Gluten quality of bread wheat is associated with activity of RabD GTPases

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Summary
In the developing endosperm of bread wheat (Triticum aestivum), seed storage proteins are produced on the rough endoplasmic reticulum (ER) and transported to protein bodies, specialized vacuoles for the storage of protein. The functionally important gluten proteins of wheat are transported by two distinct routes to the protein bodies where they are stored: vesicles that bud directly off the ER and transport through the Golgi. However, little is known about the processing of glutenin and gliadin proteins during these steps or the possible impact on their properties. In plants, the RabD GTPases mediate ER-to-Golgi vesicle transport. Available sequence information for Rab GT Pases in Arabidopsis, rice, Brachypodium and bread wheat was compiled and compared to identify wheat RabD orthologs. Partial genetic sequences were assembled using the first draft of the Chinese Spring wheat genome. A suitable candidate gene from the RabD clade (TaRabD2a) was chosen for down-regulation by RNA interference (RNAi), and an RNAi construct was used to transform wheat plants. All four available RabD genes were shown by qRT-PCR to be down-regulated in the transgenic developing endosperm. The transgenic grain was found to produce flour with significantly altered processing properties when measured by farinograph and extensograph. SE-HPLC found that a smaller proportion of HMW-GS and large proportion of LMW-GS are incorporated into the glutenin macropolymer in the transgenic dough. Lower protein content but a similar protein profile on SDS-PAGE was seen in the transgenic grain.

Introduction
Different Rab GT Pases are involved in regulation fusion of transport vesicles with their different target membranes. In plants, certain types have expanded in number compared with yeast and mammalian systems, and in some classes, members may have diverged somewhat in function (Lunn et al., 2013; Lycett, 2008; Pereira-Leaf and Seabra, 2001; Rutherford and Moore, 2002). The RabD clade is involved in ER-to-Golgi trafficking (Batoko et al., 2000; Cheung et al., 2002; Zheng et al., 2005), and in Arabidopsis, dominant negative mutant forms of RabD cause accumulation of secreted proteins and Golgi-resident proteins in an ER-like reticulate compartment (Batoko et al., 2000). However, in leaf protoplasts of other plant species, dominant negative RabD mutant proteins do not stop accumulation of storage proteins in protein bodies (Park et al., 2004), suggesting that RabD does not control all possible routes to the protein bodies.

The major wheat gluten proteins comprise two classes of prolamins, the monomeric gliadins and the glutenins that have interchain disulphide bonds that enable a complex polymeric structure to form (Kreis et al., 1985). The glutenins are classified into high molecular weight glutenin subunits (HMW-GS) and low molecular weight ones (LMW-GS), whereas the gliadins are classified by acidic polyacrylamide gel fractionation into α, β, γ and ω subunits (Woychik et al., 1961). Although the situation is complex, it seems that the elasticity of dough is conferred mainly by the glutenins and the extensibility mainly by the gliadins (Shewry et al., 1995). Good breadmaking wheat is characterized by a high protein content, but the type of protein is also important.

Gluten proteins are synthesized on the rough endoplasmic reticulum (RER) where proteins may be folded, disulphide-bonded and hydrogen-bonded. This can cause them to precipitate to form hydrated protein particles (Tosi et al., 2009). Whether different proteins are trafficked at different times and whether they go by different routes from the ER to the protein bodies is still uncertain. Rubin et al. (1992) proposed that one type of protein body, containing large insoluble HMW protein aggregates, buds directly from the ER, whereas less dense bodies form from protein trafficked in transport vesicles via the Golgi apparatus. An attempt to confirm this by the use of specific antibodies for α, β, γ and ω gliadins, and HMW and LMW glutenin subunits failed to show any difference in the protein content of storage vesicles (Loussett et al., 2008), but protein sorting into different protein bodies was shown by Tosi et al. (2009) using a very specific antibody for a LMW glutenin subunit. Golgi trafficking is favoured during the early grain-filling stage, whereas aggregation in the ER occurs at later stages and Golgi-stacked cisternae are no longer visible after 12 days postanthesis (dpa) (Levanony et al., 1992; Loussett et al., 2008; Tosi et al., 2009).

Biotechnological attempts to improve gluten quality have mainly focused on expression of specific proteins, especially the HMW glutenins (Leon et al., 2009, 2010, Rakszegi et al., 2008,
Tatham et al., 1990). However, the use of a dominant negative mutant tobacco RabD transgene improved gluten strength and increased disulphide bonding in tetraploid durum wheat (Di Luccia et al., 2005). In this study, we have used RNAi to knock down the expression of RabD GTPase genes in the hexaploid breadmaking wheat variety Cadenza and have analysed the effect on dough quality.

Results

Bioinformatics

Phenetic analyses were carried out using Rab protein and nucleotide sequence data from Arabidopsis thaliana, Brachypodium distachyon, rice, wheat and tobacco. Selected sequences were aligned, and a phenetic tree was generated to discern relationships between all RabD proteins from the four organisms (Figure 1).

A cladogram comparing all Rab proteins identified (Figure 2) proved helpful in the more specific identification of Rabs in Brachypodium and wheat. The Rab protein family is conserved within clades rather than within species, demonstrating their equivalent function in the two different plants.

Production, selection and screening of transgenic wheat lines

For the production of transgenic plants, we chose the variety Cadenza, which is a facultative winter variety with moderate breadmaking quality, rated 6 on a 1–9 scale where 9 is highest quality (Rakszegi et al., 2008). Wheat plants were transformed by biolistics with an RNAi construct cloned into the pHMW-Adh-Nos vector (Nemeth et al., 2010; Figure S1) to give endosperm-specific expression. This construct targeted a 270-nucleotide section of the Ta.54382 (RabD2a) coding sequence that included a 23nt match with Ta.54881 (RabD2b). All 29 T0 plants, designated L1 to L29, were screened for transgene presence using PCR. Based on PCR results, 16 T0 plants were selected, each of which was an independent transformation event. From these 16 selected T0 plants, 24 seeds from each plant were initially sown, of which 12 from each plant were grown on to maturity. The designation for each of these mature T1 plants consists of the name of the parent plant followed by .1 to .12. Probable zygosity of all 192 T1 plants was determined by PCR of T2 seedlings for transgene presence. T2 grains from lines that had sufficient grain weight for rheological testing were analysed for size and shape, protein content, moisture content and dough-mixing properties, along with controls. A homozygous transgenic line (L10.5) that displayed a large difference in mixing properties yet similar protein and moisture content compared with the controls was selected to be grown alongside a control line (L24.2).

Expression of RabD genes in transgenic lines

To establish that the target RabD gene (Ta.54382) had been successfully knocked down and to check whether other RabD genes with close sequence similarity were also affected, a

Figure 1 Phenogram with Rab D protein sequences of wheat, Brachypodium, rice, Arabidopsis and tobacco. Rab D2a homologs are highlighted in blue. Alignment and phenetic tree file were generated using MAFFT version 6 online (Katoh and Toh, 2010). Tree is neighbour-joining with 1000 bootstrap replicates, shown on branches as percentage confidence values.
quantitative real-time PCR was performed looking at the expression of four RabD genes in the endosperm of T3 lines L10.5 (knock-down) and L24.2 (control) at 14 and 21 dpa.

Three other genes—Ta.2291, Ta.2776 and Ta.54227—were also included as potential normalization genes based on a study identifying reference genes in wheat (Paolacci et al., 2009). However, expression of Ta.2291 appeared to be 79% higher at 14 dpa and 120% higher at 21 dpa in the transgenic line compared with the control and was therefore not used as a normalization gene in this study. qRT-PCR data of the RabD genes were normalized using the Ta.54227 gene (for primers see Tables 1 and 2). Calibration curves were set up for each primer pair using serial dilutions of a sample containing a mixture of all cDNA samples.

All four RabD genes were found to some extent to have lower transcript levels at 14 dpa in the transgenic line compared with the control (Figure 3). The RabD1 genes Ta.35418 and Ta.47209 had 49% and 22% reductions in transcript levels, respectively. The expression of RabD2a target gene Ta.54382 and its closest relative gene Ta.54881 showed a 60% and 50% reduction, respectively, compared with the control. At 21 dpa, the transcript levels of RabD2 genes Ta.54382 and Ta.54881 again showed similar reductions in the transgenic line compared with the control line of 58% and 60%, respectively. However, at 21 dpa, whereas Ta.47209 transcript levels were 51% lower in the RNAi line than the control, there was no significant difference in the Ta.35418 transcript levels in the two lines. The transgene clearly had a large effect on expression of RabD genes in the developing endosperm, with RabD2 genes down-regulated by 50%–60% at both 14 and 21 dpa.

Size and shape analysis of transgenic grain

To investigate potential effects of the transgene on grain dimensions and proportions, an analysis of the size and shape
of the T2 grain was performed. Using DigiEye apparatus, photographs of grain belonging to 41 lines were taken, including six lines from the L24 bar-only control parent. The photographs were analysed with ImageJ software to give values for area, perimeter, length, width and circularity of each grain image. The number of grains analysed for each line ranged from 76 to 703 but was mostly in the range 150–200. The mean of each of the values was determined for each line. While L05.6 apparently had the highest mean perimeter and width, it had the lowest number of grains analysed (76); therefore, its mean values are the least reliable (Figure S2). The control lines tend towards the mid range in most of the properties, with a slight leaning towards the higher range in area and the lower range in perimeter, and with a wide spread in length. This implies that the transgene has little effect on these grain properties. However, the control lines appeared to have greater circularity than most of the other lines, which would suggest that the transgene reduces grain circularity (i.e. causes a more elongated grain shape).

To determine whether differences seen in the T2 generation were also present between lines of T3 grain, a similar analysis was performed on T3 grain from the selected T2 knock-down line L10.5 and a T2 control line L24.2. The data set for L10.5 was produced through analysis of 1329 grains and that for L24.2 from 984 grains. There is a slightly greater perimeter and smaller area in the grains of the T3 knock-down line compared with the control line (Figure S3). Additionally, the knock-down grains are slightly longer and thinner. These combined small differences result in a significant reduction in circularity of the knock-down line compared with the control. This finding agrees with the results from the previous generation, which further suggests that the changes seen are caused by the transgene.

**Protein content of grain**

After milling, protein content of the T2 flour was measured to compare the total levels of protein in the 20 selected lines. The mean protein content of the control T2 lines on a dry weight basis was 16.38%, while that of the T2 lines found to contain the transgene was 14.83% (Figure S4). This drop in mean protein content of 1.55% in the transgenic lines was despite the anomalously high protein level of transgenic line L03.11.

### Table 1

<table>
<thead>
<tr>
<th>Unigene</th>
<th>Primer F name</th>
<th>Primer F sequence</th>
<th>Primer R name</th>
<th>Primer R sequence</th>
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<tr>
<td>Ta.35418</td>
<td>Ta.35418-R1-F</td>
<td>TCC GTT TCT CCG ATT CG</td>
<td>Ta.35418-R1-R</td>
<td>TCC TGC GTC CCA AAT CTG</td>
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<td>Ta.47209</td>
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<td>AGG TCG TCG ATA CAG AGG AG</td>
<td>Ta.47209-R4-R</td>
<td>GCC TCG CCA TCT TGT TCT TG</td>
</tr>
<tr>
<td>Ta.54832</td>
<td>Ta.54832-R3-F</td>
<td>ATC GGA GAC TCA GGT GTT GG</td>
<td>Ta.54832-R3-R</td>
<td>GTC CTG AAG CGT TCT TGC CC</td>
</tr>
<tr>
<td>Ta.54881</td>
<td>Ta.54881-R3-F</td>
<td>AAC TCT TGC CTG CTG AG</td>
<td>Ta.54881-R2-R</td>
<td>TCG CTC TTG TCG AGC AGT ATC</td>
</tr>
<tr>
<td>Ta.2291</td>
<td>Ta.2291-P1-F</td>
<td>GCT CTC CAA CAA CAT TGC CAA C</td>
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<td>GCT TCT GCC TGT CAC ATA CCG</td>
</tr>
<tr>
<td>Ta.2776</td>
<td>Ta.2776-P1-F</td>
<td>CGA TCT AGA GCA GCG TAT TGT TG</td>
<td>Ta.2776-P1-R</td>
<td>AGT TGG TCG GTG CTC TTC TAA ATG</td>
</tr>
<tr>
<td>Ta.54227</td>
<td>Ta.54227-P1-F</td>
<td>CAA ATA CGC CAT CAG GGA GAA CAT C</td>
<td>Ta.54227-P1-R</td>
<td>CGC TGC CGA AAC CAC GAG AC</td>
</tr>
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</table>

Sequences are 5'-3'.

### Table 2

<table>
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<tr>
<th>Unigene</th>
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<th>cDNA product/bp</th>
<th>gDNA product/bp</th>
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<tr>
<td>Ta.35418</td>
<td>RabD1a</td>
<td>122</td>
<td>&gt;122</td>
</tr>
<tr>
<td>Ta.47209</td>
<td>RabD1b</td>
<td>141</td>
<td>547/866</td>
</tr>
<tr>
<td>Ta.54832</td>
<td>RabD2a</td>
<td>176</td>
<td>1176</td>
</tr>
<tr>
<td>Ta.54881</td>
<td>RabD2b</td>
<td>147</td>
<td>1517</td>
</tr>
</tbody>
</table>

### Figure 3

Expression of RabD genes at 14 and 21 days postanthesis in the developing endosperm of T3 wheat. Blue bars: L10.5 (GM), green bars: L24.2 (control). Expression normalized with Ta.5427 gene (cell division control). Error bars indicate standard error. RabD1a = Ta.35418; RabD1b = Ta.47209; RabD2a = Ta.54382; RabD2b = Ta.54881.
There was also a 1.37% reduction in total protein in the T3 transgenic line L10.5 compared with the control line L24.2 (Figure 4a). This finding concurs with the differences in protein content of T2 transgenic lines compared with the T2 controls and specifically the difference between T2 lines L10.5 and L24.2, which indicates the difference is a result of the transgene. No significant difference in starch content was found between the T3 knock-down line and control line (Figure 4b).

**SDS-PAGE analysis of mature wheat seed protein**

To better understand protein deposition in the transgenic grain, SDS-PAGE experiments were carried out on mature seeds of T0 plants (Figure S5) and the mature seeds of 30 lines selected by PCR and grain weight (Figure S6). Controls used included grain from bar-only and gold-only T0 plants and from a bar-only T1 plant. Densitometry was performed by the Cereals and Milling Department at Campden BRI on 5 of the gels, each comparing 2–3 transgenic lines with control line L24.12 in triplicate. Only very small differences were found between the intensity of the bands in transgenic lines compared with the controls (Figure 5). This suggests that the transgene has little, if any, effect on the ratio of different seed storage proteins.

**Dough rheology**

The main factor in deciding which homozygous T2 lines to grow on in the next generation was a study of the processing properties of the dough. Twenty lines were chosen to be milled, based on transgene PCR confirmation and grain weight. Milling was carried out in a laboratory scale Quadrumat mill. Coarse semolina and bran particles were subjected to two passes through the milling process. The resulting flour samples were tested for moisture and protein content and mixed into dough using a Reomixer apparatus. The Reomixer produced traces of resistance to mixing over the course of 10 min, measured in arbitrary units of torque from 0 to 10. The important features of the trace are peak torque, dough development time (time at peak torque), breakdown amount/angle (reduction in dough strength after peak torque) and trace width.

The high protein line L25.1 showed a high peak torque of 9 but a thin trace and short development time of 130 s (Figure 6a). On the other hand, homozygous T2 lines L10.5, L12.11 and L17.12 had substantially lower peak torques of around 7 but somewhat longer development times (150–190 s, Figure 6b–d). Trace width varied between them—L17.12 had the widest trace (similar to the controls) and the trace of the other two lines appeared to gradually narrow after peak torque. The peak torque of the four control lines was in the range 8.1–8.4 and occurred after 195–225 s. Trace width was greater than most of the transgenic lines.

From the traces of the twenty lines, variables were fed into two dough quality indicators—PC1 and PC2, as defined by Anderson (2004). PC1 increases as peak torque and dough development time decrease; therefore, weak biscuit-like flours have a higher PC1 and strong bread-like flours give a lower PC1. PC2 is determined by breakdown—greater breakdown gives a higher PC2 value—so a more stable dough will have a lower PC2. The PC values were calculated for each line and plotted on a quality map (Figure 7).

Excluding the anomalous high protein line L03.11, transgenic T2 lines had higher PC1 values and lower PC2 values than the controls, and homozygous lines more so than hemizygous lines. A null segregant included in the experiment showed similar PC1 to the controls but somewhat decreased PC2, although only one hemizygous line had a PC2 value between the null segregant and the control lines. These results strongly suggest that presence of the transgene significantly altered the processing properties of the dough.

The small-scale rheology results from the T2 generation showed substantial changes in the processing characteristics of the dough of transgenic lines. To demonstrate the specific changes in gluten quality on a larger scale, farinograph and extensograph tests were carried out on T3 dough. While similar to the Reomixer test performed on T2 flour, the farinograph is a more conventional method of determining dough processing properties.

In the farinograph traces, there are clear differences between the T3 knock-down line L10.5 and the T3 control line L24.2 (Table 4, Figure 8). The dough development time (time until peak resistance) was noticeably shorter in the knock-down line and the dough started to break down more quickly after peak resistance.

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![Figure 4](image_url)  
Figure 4: Protein and starch content of flour from knockdown and control T3 lines as a percentage of dry weight. (a): Protein content of T3 lines on dry weight basis; (b): starch content of T3 lines. The blue bars represent T3 knockdown line L10.5, the green bars represent T3 bar-only control line L24.2. Error bars indicate predicted error of measuring techniques.
Additionally, the width of the trace in the knock-down line was smaller than in the control throughout the mixing, and the resistance dropped to a lower level compared with the control. The specific measurements made by the farinograph back up these observations. The knock-down line had a 43% reduction in development time, 40% reduction in stability (time during which the top edge of the trace is above the centre of the peak resistance) and 25% increase in degree of softening (difference in resistance between peak resistance and 12 min after peak resistance) compared with the control.

The extensograph traces and corresponding values show that dough made from the T3 knock-down line was weaker and less extensible than the control line (Table 4, Figure 9). This is reflected by the resistance and extensibility figures, which were, respectively, 43% lower and 32% lower in the knock-down line compared with the control.

SE-HPLC analysis of gluten macropolymer

As the difference in protein content was relatively small compared with the alteration of dough rheology in the T2 generation and the relative levels of gluten subunits were very similar (as shown by SDS-PAGE results), it was likely that other factors were contributing to the processing properties of the dough. To investigate the characteristics of the large disulphide-linked aggregates formed by polymeric subunits in the glutenin macropolymer that gives dough its elastic properties, a size exclusion high-performance liquid chromatography (SE-HPLC) experiment was carried out using T3 flour. The largest polymeric gluten proteins, high molecular weight glutenin polymers, elute as a peak between 9m and 10m10s (fraction F1); the smaller polymeric gluten proteins, low molecular weight glutenin polymers, elute between 10m11s and 13m40s (fraction F2); the monomeric gliadin proteins elute between 13m41s and 14m45s and between 14m46s and 16m50s (fractions F3 and F4, respectively); and the globulin/albumin fraction elutes between 16m51s and 19m (fraction F5). Two replicates of each T3 line were run untreated (i.e. without sonication) and with sonication during extraction with SDS solution. Sonication conditions were selected to maximize the solubilization of the polymeric proteins, while minimizing the breakdown of the polymers and disruption of the native protein structures within the flour (Morel et al., 2000).

The SE-HPLC profiles of the untreated (not sonicated) samples show that a greater proportion of polymeric high molecular weight glutenin polymer and low molecular weight glutenin polymer proteins, in fractions F1 and F2, respectively, had been extracted in the T3 knock-down line L10.5 compared with the T3 control line L24.2 (Table 5, Figure 10a). This
suggests that the modified knock-down line featured a gluten macropolymer complex that was easily solubilized and hence was weaker than the control line. Examination of the sonicated samples showed that the relative size distribution of the polymeric proteins within the total extractable gluten protein fraction was different between the knock-down lines and the control lines (Table 5, Figure 10b). Here, the knock-down line shows smaller peaks eluting in F1 and between 10m11s and 11m20s in F2 compared with the control line, indicating that it contained a lower proportion of the higher molecular weight polymeric gluten material. The presence of more, smaller low molecular weight glutenin polymer polymeric protein in the knock-down line, eluting between 11m21 and 13m40s in F2, suggests that it had been only weakly bound within the gluten macropolymer matrix, compared with the control line.

**Discussion**

Transcript levels of all four wheat RabD genes studied were reduced in the transgenic line at 14 dpa, and all but Ta.35418 (RabD1) were knocked down at 21 dpa. The transgenic effect is a result of a pHMW-Adh-Nos RNAi vector containing a 270-bp sequence taken from a single Rab gene, Ta.54382 (RabD2a) with

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>--site primer F</td>
<td>GTTGGCAAGTCATGCCCCTTC</td>
</tr>
<tr>
<td>--site primer R</td>
<td>GCGATCAATCTCGTTCAACCC</td>
</tr>
<tr>
<td>+site primer F</td>
<td>CTAAA GGATCC GTTGGCAAGTCATGCCC</td>
</tr>
<tr>
<td>+site primer R</td>
<td>GCCCA AGATCT GCGATCAATCTCGTT</td>
</tr>
</tbody>
</table>

The parts of the +site primers that match the gene (underlined) are preceded by a restriction site (bold), plus extra nucleotides to ensure efficient restriction (italics). +site primer F contains a BamHI site, and +site primer R contains a BglII site.

**Table 3** Primers designed to anneal to the target sequence in PCR

<table>
<thead>
<tr>
<th>Primer name</th>
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</tr>
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<tbody>
<tr>
<td>--site primer F</td>
<td>GTTGGCAAGTCATGCCCCTTC</td>
</tr>
<tr>
<td>--site primer R</td>
<td>GCGATCAATCTCGTTCAACCC</td>
</tr>
<tr>
<td>+site primer F</td>
<td>CTAAA GGATCC GTTGGCAAGTCATGCCC</td>
</tr>
<tr>
<td>+site primer R</td>
<td>GCCCA AGATCT GCGATCAATCTCGTT</td>
</tr>
</tbody>
</table>

The parts of the +site primers that match the gene (underlined) are preceded by a restriction site (bold), plus extra nucleotides to ensure efficient restriction (italics). +site primer F contains a BamHI site, and +site primer R contains a BglII site.

**Table 4** Farinograph and extensograph results

<table>
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<th>Control line L24.2</th>
<th>Transgenic line L10.5</th>
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<td></td>
<td>Stability (min)†</td>
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<tr>
<td></td>
<td>Degree of softening (BU)‡</td>
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<td>Extensograph features</td>
<td>Resistance (BU)§</td>
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</tr>
<tr>
<td></td>
<td>Extensibility (cm)¶</td>
<td>23.5</td>
</tr>
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*Development time refers to time until peak resistance.
†Stability is the time during which the top edge of the farinograph trace is above the centre of the peak resistance.
‡Degree of softening is the difference in resistance between peak resistance and 12 min after peak resistance in Brabender units (BU).
§The extensograph resistance value refers to maximum resistance in Brabender units (BU).
¶Extensibility refers to the distance stretched before breaking.
a 21nt match with Ta.54881 (RabD2b). A recent study by Wang et al. (2012) found that overexpression of a YFP-associated mutant RabD2b gene in Arabidopsis led to co-suppression of the native RabD2b gene along with down-regulation of RabD1 and RabD2c genes, although RabD2a appeared unaffected. A similar down-regulation mechanism might help account for the expression pattern seen in the developing T3 seeds, where aside from the target gene, three other genes of the same subfamily showed reduced transcript levels. Alternatively, the fact that this strategy appeared to affect a number of similar genes regardless of whether or not there was a perfect 21nt match between them could be due to cleavage of mRNA molecules with sequences that are highly conserved with the siRNA incorporated into RISC, in addition to those that are fully identical as suggested by a number of recent studies (Birmingham et al., 2006; Fedorov et al., 2006; Haley and Zamore, 2004; Sigoillot et al., 2012).

A potential reference gene included in the qRT-PCR experiment, Ta.2291 (Arf2), which had previously been described as a suitable reference gene for qPCR in wheat (Paolacci et al., 2009), seemed to be unsuitable as a reference gene and in fact appeared to be significantly up-regulated in the transgenic line compared with the control at both 14 dpa and 21 dpa. This is an interesting result as Arf (ADP ribosylation factor) proteins are small GTPases involved in vesicle trafficking and are part of the same Ras superfamily as Rab GTPases (Wennnerberg et al., 2005). It would appear that reduced RabD levels affected some equilibrium or feedback mechanism in the vesicle transport system, leading to increased expression of at least one Arf gene, perhaps to compensate for reduced ER-to-Golgi vesicle traffic.

The T3 rheology results clearly indicate a reduction in dough strength and extensibility in the knock-down line compared with the control. It is likely that altered intermolecular bonding in the glutenin macropolymer contributed to this effect, combined with a drop in total protein content. As it has been confirmed that the RabD genes are knocked down in the developing endosperm in the T3 transgenic line relative to the control line, it is reasonable to conclude that the expression of one or more of these genes is instrumental in gluten quality.

The SE-HPLC findings provide evidence that the RabD knock-down transgene has markedly affected interactions or bonding between glutenin subunits in the gluten macropolymer of dough. Bonding strength appears most reduced in high molecular weight glutenin polymer and some of the larger low molecular weight glutenin polymers. As these subunits are probably the most important in giving gluten its elastic properties (Anjum et al., 2007), the transgenic dough would be expected to have weaker gluten strength when processed. This correlates with the results of the rheology experiments, in which the knock-down lines exhibited considerable reductions in gluten strength and extensibility compared with controls. These changes are within the
found that gliadins and glutenins were both glycosylated, and aggregated LMW-GS showed N-glycans with xylose, thought to indicate processing in the Golgi (Lauriere et al., 1996). Meanwhile, many other studies describe finding little or no glycosylation of HMW-GS and α-gliadins using mostly mass spectrometric approaches such as GC–MS, RP-HPLC/ESI-MS and MALDI-TOF-MS (Bollecker et al., 1998; Cunsolo et al., 2002, 2003, 2004; Foti et al., 2000; Roels and Delcour, 1996; Turner et al., 2002, Zhang et al., 2008). Additionally, some of these studies also discovered false-positive results when using other techniques including periodate-digoxigenin glycan detection assay and acid PAGE (Bollecker et al., 1998; Roels and Delcour, 1996). While evidence has built up that HMW-GS are probably not commonly glycosylated, LMW-GS and gliadins are less well studied, and there is still uncertainty whether they undergo glycosylation or not (Li et al., 2008). If any LMW-GS or gliadins are glycosylated, then it is likely that reducing ER-to-Golgi vesicle traffic would have a profound effect on this process. It is possible that a change in prolamin glycosylation could be at least partly responsible for differences seen in the transgenic wheat grain, although it is unclear whether or not this is the case.

SE-HPLC and rheology results show a tangible difference in dough quality between the transgenic and control lines. However, SDS-PAGE results suggest that the relative proportions of the different gluten subunits have not been affected. A significant contributing factor to this may be the likelihood that fewer gluten proteins in the transgenic lines than in the control lines go through important post-translational modifications that occur in the Golgi, due to inhibited ER-to-Golgi trafficking. It is likely that these transgenic modifications could be in the form of disulphide bonding, hydrogen bonding, glycosylation or folding into a structure able to produce the loop that is dependent on processing in the Golgi (Lauriere et al., 1996). Protein disulphide isomerase (PDI) is located in the ER and protein bodies, and although this glycoprotein is thought to catalyse intermolecular covalent bonding between gluten subunits, it is not known whether it has a role in folding of the subunits (Shimoni et al., 1995).

There is a general consensus that wheat prolamins are transported to the protein storage vacuole by two different pathways: Golgi dependent and Golgi independent (Tosi, 2012). However, there is disagreement as to whether or not different prolamins are transported through the two routes differentially, and if so, what causes the difference (Levanony et al., 1992; Loussert et al., 2008; Rubin et al., 1992; Tosi et al., 2009). Based on SE-HPLC results of this study, the reduction of RabD expression in wheat endosperm appears to reduce the bonding ability of gluten proteins in the dough.
HMW-GS and larger LMW-GS while smaller LMW-GS are less affected. This may point towards different glutenin subunits favouring different trafficking routes or alternatively that smaller glutenins are not as dependent on processing in the Golgi to retain their ability to bond in the glutenin macropolymer. However, if there is a difference in the ratio of proteins trafficked via the Golgi compared with ER aggregation, it is not apparent in the SDS-PAGE results, which do not show any differences in subunit proportions present in the mature seed between the knock-down and control lines. It is possible that proteins blocked by one route could, subsequently, be trafficked by the other route.

The knowledge gained from this study regarding the sequence and function of Rab GTPases in the endosperm of hexaploid wheat could be further exploited to better understand, and possibly improve, the technological properties of flour. Based on the results of this work, it would appear that RabD expression is linked to dough processing quality in bread wheat, at least in that gluten quality appears reduced when RabD expression is reduced. Conversely, it may therefore be inferred that an increased level of RabD expression might improve gluten quality. However, overexpression of native RabD genes has met with a lack of phenotype in studies on durum wheat and Arabidopsis (Di Luccia et al., 2005; Wang et al., 2012). When no phenotype was found in transgenic Arabidopsis overexpressing RAC/ROP small GTPases, the authors of the study suggested that interactors including GAPs and GDIs could be responsible for keeping GTPase activity stable despite a greater quantity of protein present (Brembu et al., 2005). It is reasonable to conclude that the wide range of Rab interaction partners could prove problematic to direct efforts to up-regulate vesicle trafficking from ER to Golgi by genetic modification.

Most attempts to alter breadmaking quality in bread wheat using biotechnology have targeted the gluten proteins themselves, particularly the HMW glutenin subunits. This study represents a novel approach to this area of work by altering the transport of these important proteins in the endosperm. The resulting effects on grain shape, protein content, bonding in the glutenin macropolymer and dough rheology reported here expand the understanding of factors that contribute towards gluten quality in bread wheat and could be of interest to breeders looking for loci-controlling quality.

Experimental procedures

Laboratory chemicals and reagents
Chemicals and reagents were supplied by Fisher Scientific (Fisher Scientific UK Ltd, Loughborough, UK) or Sigma-Aldrich (Sigma-Aldrich Corporation, St. Louis, MO) unless otherwise stated.

Primers and DNA
Primers were obtained from Eurofins MWG Operon (Ebersberg, Germany). The dNTPs used in PCR were obtained from Promega (Promega UK Ltd, Southampton, UK). Primers and dNTPs were diluted as in the manufacturer’s instructions and stored at −20 °C.

Polymerase chain reaction (PCR)
PCR was originally described by Saiki et al. (1988). PCRs were performed using an Eppendorf Mastercycler PCR machine. Each reaction tube contained a total reaction mixture of 20 µL composed of 12.8 µL H2O, 2 µL 10× PCR buffer, 0.6 µL 50 mM MgCl2, 1.6 µL 2.5 mM dNTPs, 0.4 µL forwards primer,
l reverse primer, 2 L DNA template and 0.2 L Taq polymerase. PCR buffer and MgCl2 solutions were supplied by Bioline (Bioline Reagents Ltd., London, UK). Unless otherwise stated, the DNA template used in PCR was from a sample of wheat DNA extracted from leaf tissue of Cadenza variety wheat. The general PCR programme used was 94 °C for 5 min, followed by a denaturing, annealing and amplification cycle (repeated 35 times) of 94, 60 (for nongradient PCR) and 72 °C. Once complete, this repeated cycle was followed by 72 °C for 10 min prior to removal of tubes from machine and storage at 4 °C.

Agarose gel

Electrophoresis gels were 2% molecular biology grade agarose (Bio-Rad Laboratories Inc., Hercules, CA), made up with 0.5× TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA). 0.01% w/v 10 mg/mL ethidium bromide was mixed into the buffer once the agarose had been dissolved by heating in a microwave oven and the liquid had begun to cool. Gels were left to solidify in a microgel former with appropriate combs before being placed in a microgel bath and submerged in 0.5× TBE buffer. Next, 2 μL of loading buffer (0.25% w/v xylene cyanol, 0.25% w/v bromophenol blue, 30% w/v glycerol) was added to 10 μL of PCR product in each of the experiment wells (mixed before loading), and 5 μL of DNA ladder was added to each marker well. Once PCR products and marker had been loaded, gels were run at 10 V/cm and then photographed under ultraviolet light.

Plasmid miniprep

Plasmid was recovered from transformed Escherichia coli culture using a Fermentas GeneJET™ plasmid miniprep kit (Fermentas GmbH, St. Leon-Rot, Germany) following the manufacturer’s instructions.

E. coli transformation and culture

Competent DH5-α E. coli cells were transformed with pHMW-Adh-Nos vector using the method described by Sambrook et al. (1989).

Spectrophotometric analysis of nucleic acids

A NanoDrop 1000 machine (NanoDrop products, Wilmington, DE) was used to quantify DNA and RNA samples using the manufacturer’s instructions.
DNA sequencing

DNA samples were sequenced by Eurofins MWG Operon. DNA samples were prepared for sequencing by following the instructions provided.

Total RNA extraction from developing wheat endosperm

Total RNA was extracted from wheat endosperm tissue using the method described by Chang et al. (1993).

cDNA synthesis using random hexamer primers

Random hexamer primers were used to synthesize cDNA from total RNA using the method described by Parr et al. (1992). Random hexamer primers, RT enzyme, buffer, RNasin RNase inhibitor and dNTPs were supplied by Promega.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Real-time quantitative PCR was first described by Heid et al. (1996). qPCR was carried out using 384-well plates in a Roche LightCycler 480 qPCR machine. SYBR Green I master mix was used, and manufacturer’s instructions followed for plate preparation. Programme was 95 °C for 5 min; 45 cycles of 95 °C for 10 s, 60 °C for 15 s and 72 °C for 15 s; 95 °C for 5 s, 65 °C for 1 min, slow ramp to 97 and 40 °C for 10 s.

Phenetic analysis

Phenetic analyses were carried out using Rab coding sequence (CDS) data that had been obtained by BLAST or string searches of the NCBI nucleotide and EST databases, as well as Rab protein sequences that had been obtained by following links from corresponding entries in the NCBI nucleotide database or by translating from a CDS that had been assembled from ESTs. The plant species included were Arabidopsis thaliana, Oryza sativa, Brachypodium distachyon, Triticum aestivum and Nicotiana tabacum. Alignments and phenetic tree files for rectangular grids were generated using MAFFT version 6 online (Katoh and Toh, 2010). Alignments and phenograms for circular trees were produced in MEGA 5 (Tamura et al., 2011). Alignments and phenograms for circular trees were produced in MEGA 5 (Tamura et al., 2011). All trees were neighbour-joining with 1000 bootstrap replicates, shown on branches as percentage confidence values (Felsenstein, 1985; Saitou and Nei, 1987). Branches corresponding to partitions reproduced in <50% bootstrap replicates were collapsed. The trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site.

Cloning of RNAi construct

Bovine serum albumin, calf intestinal alkaline phosphatase, T4 DNA ligase enzyme and BamHI and BglII restriction enzymes were acquired from Promega. A 270-bp section of the CDS of wheat RabD2a gene Ta.54382 was obtained by PCR using primers with BamHI and BglII restriction sites added to their 5’ ends (Table 3, Figure S7). The PCR product was purified and cloned into the vector pHMV-Adh-Nos (Nemeth et al., 2010; Figure S1). Digestion and ligation of insert and vector were performed using the supplier’s instructions. Negative controls used were no enzyme digest (for both insert and vector digests), no enzyme ligation, no vector ligation and no insert ligation. Competent DH5a E. coli cells were used for transformation. E. coli cells were grown in LB-ampicillin medium (Per plate: 10 mL sterile LB medium mixed with 10 µL 100 mg/mL ampicillin).

Wheat transformation and plant growth conditions

Biologic transformation of wheat (Triticum aestivum var. Cadenza) embryos and regeneration of T0 plants were carried out as in Jones and Sparks (2009). Transgenic lines and control plants were grown in the same glasshouse compartments, which were certified for contained-use GMOs. For production of plants, T0 seedlings or seeds were potted in soil and grown in the glasshouse with 18–20 °C day and 14–16 °C night temperatures with a 16-h photoperiod provided by natural light supplemented with banks of Son-T 400 W sodium lamps (Osram Limited, Langley, UK) giving 400–1,000 µmol/m2s PAR. Soil composition contained 75% fine-grade peat, 12% screened sterilized loam, 10% 6-mm screened lime-free grit, 3% medium vermiculite, 2 kg osmocote plus/m3 (slow-release fertilizer, 15N/11P/13K plus micronutrients) and 0.5 kg PG mix/m3 (14N/16P/18K granular fertilizer plus micronutrients; Petersfield Products, Leicestershire, UK).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of T2 seeds

Three mature seeds from each line to be tested were placed in separate folded pieces of paper and crushed using a pestle. One line per gel was a control line. A 10 mg sample from each crushed seed was weighed out into a 1.5-mL microcentrifuge tube, and a modified version of the gel manufacturer’s instructions was followed. Gels were preformed 15-well Bis-Tris 10% acrylamide gels (Invitrogen, Life Technologies Ltd, Paisley, UK), buffer was MOPS SDS running buffer (Invitrogen), gel equipment was a mini dual vertical electrophoresis system (Sigma-Aldrich), and stain was colloidal Coomassie blue stain (Sigma-Aldrich). Phoretix 1D software by Totalab was used for densitometry analysis at Campden BRI.

Grain size and shape analysis

Grain size and shape analyses were performed on the grain size and shape using DigEye apparatus (VeriVide Limited, Enderby, Leicester, LE19 4SG, UK).

Measurement of flour protein content

Protein content was determined by Dumas combustion in an oxygen-rich environment at high temperatures. Protein is calculated using a factor of 5.7 from total nitrogen determination. Combustion was carried out in a Leco FP528 at Campden BRI.

Size exclusion high-performance light chromatography (SE-HPLC)

SE-HPLC was carried out on flour samples using the Profiblé methodology developed jointly by ARVALIS—Institut du vegetal (France) and l’Institut National de la Recherche Agronomique (INRA; Morel et al., 2000).

Dough rheology

Brabender farinograph, extensograph and Reomixer tests were carried out using the manufacturer’s instructions. Reomixer traces were converted to PCA components according to the method of Anderson (2004). This uses equations determined at Campden BRI. The smoothed standard-dough traces are converted into two
numbers (principal component scores) that are used to plot the position on a quality map, which summarizes the trace characteristics of varying dough development, peak time, peak height and differing breakdown.

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References


Supporting information

Additional Supporting information may be found in the online version of this article:

Figure 51 Vector map of pHMW-Adh-Nos (Nemeth et al., 2010). Figure 52 Size and shape analysis of grain from T2 lines. Figure 53 Mean size and shape values of grain from T2 lines showing selected target region. Figure 54 Protein content of flour from 20 shortlisted T2 lines as a percentage of dry weight. Figure 55 SDS-PAGE of mature T1 seeds. Figure 56 SDS-PAGE of mature T2 seeds. Figure 57 The Ta.54382 (wheat RabD2a) coding sequence showing selected target region.