RNA interference targeting rye secalins alters flour protein composition in a wheat variety carrying a 1BL.1RS translocation

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Abbreviations: 1-D, 1-dimensional; 2-DE 2-dimensional gel electrophoresis; AATI, alpha-amylase trypsin inhibitor; HMW, high-molecular-weight; LMW, low-molecular-weight; MS/MS, tandem mass spectrometry; RNAi, RNA interference

20

21 **Abstract**

22 Wheat varieties carrying chromosome translocations from rye are part of the international
23 wheat breeding pool despite being associated with defects in dough processing quality.
24 One cause proposed for the quality defects of wheat carrying 1Bl.1RS translocations is
25 the presence of the secalins encoded by the *Sec1* locus on the short arm of rye
26 chromosome 1. In this report, we decrease the levels of these and related wheat proteins
27 by RNA interference (RNAi). A plasmid designed to down-regulate secalin was
28 introduced by biolistics into 'Bobwhite', which carries a 1BL.1RS translocation. Flour
29 proteins of two independent transformants were analyzed in detail by 2-dimensional gel
30 electrophoresis and tandem mass spectrometry. Compared to the parent, the
31 transformants exhibited up to 5.5-fold decreases in the levels of individual secalins, up to
32 7-fold decreases in the closely related omega gliadins, and lesser decreases in many
33 gamma gliadins, low-molecular-weight (LMW)-glutenins, and farinins. Increased levels
34 were found for nearly all identified alpha gliadins, triticins, and y-type high-molecular-
35 weight (HMW)-glutenins. Flour from the transgenic lines exhibited changes in dough
36 mixing behaviour in the 2-gram mixograph, including longer development times and
37 greater tolerance to mixing. We conclude that changes in protein composition engineered
38 by this RNAi construct improved dough handling characteristics.

1. Introduction

Many wheat lines used in breeding and commercial production contain translocations from rye chromosomes (reviewed and referenced in Graybosch, 2001). The most common of these translocations are between the short arm of rye chromosome 1 and the short arm of wheat chromosome 1B (1BL.1RS) or 1A (1AL.1RS). The rye segments carry genes for fungal resistance, improved grain yield potential (Graybosch, 2001 and references therein) and drought tolerance (Ehdaie et al., 2012; Howell et al., 2014). The presence of the 1BL.1RS translocation has also often been associated with defects in end-use quality, including poor mixing tolerance, dough stickiness, and low loaf volumes (Graybosch, 2001).

Several hypotheses have been put forward to explain the effects of the 1RS rye translocation on end-use properties (Barbeau et al., 2003). Prominent among these are substitution of the omega secalins and 40 kDa gamma secalins encoded at the *Sec-1* locus on the short arm of chromosome 1R (Tatham and Shewry, 2012) for the wheat low-molecular-weight (LMW)-glutenins and gamma and omega gliadins encoded by the *Gli-B1/Glu-B3* or *Gli-A1/Glu-A3* loci in varieties carrying the 1BL.1RS or 1AL.1RS translocation, respectively (Singh and Shepherd, 1988). In particular, the lack of LMW-glutenins is a plausible hypothesis for explaining weaknesses in dough formation because these proteins participate via disulfide bonds in the gluten polymer that forms the backbone of dough (Wieser, 2007). Since the gain of the *Sec-1* locus coincides with the loss of the *Gli-1* and *Glu-3* loci, it is difficult to distinguish whether the absence of the

wheat LMW-glutenins, gamma and omega gliadins, the presence of the monomeric rye secalins, or a combination of both factors affect wheat dough processing properties.

The origin of the dough stickiness property of 1BL.1RS lines has been investigated by reconstitution experiments, which have shown it to be attributed to the water soluble components of flours from translocation lines (Dhaliwal and MacRitchie, 1990). The omega secalins are highly water soluble, but so are other flour components including pentosans that are abundant in rye flour and ferulic acid-linked polysaccharides (reviewed in Graybosch, 2001 and Barbeau et al., 2003).

One way to assess the contribution of the secalins to wheat end-use properties would be to suppress their accumulation in 1BL.1RS lines via RNA interference (RNAi). RNAi is a strategy that utilizes the natural defences of plant cells to degrade double-stranded RNA structures (Fu et al., 2007). In this approach, genetic transformation is used to introduce a construction that will be transcribed into dsRNA that will trigger its own degradation and that of any RNAs with high degrees of sequence homology. RNAi was first used in wheat to validate candidate sequences for the *Vrn2* gene (Yan et al., 2004). It has since been used to decrease the levels of various groups of wheat seed storage proteins, including some that are known or potential allergens (Altenbach and Allen, 2011; Barro et al., 2015; Becker et al., 2012; Gil-Humanes et al., 2008, 2010; Yue et al., 2007).

The purpose of this study was to use RNA interference to reduce the levels of rye seed storage proteins in a wheat variety carrying a 1BL.1RS translocation and to measure the effects of the flour protein changes in small-scale quality tests.

2. Materials and Methods

The hard white spring wheat ‘Bobwhite’ (BW) utilized for genetic transformation experiments in our laboratory is one of 129 derivatives of a CIMMYT cross with the pedigree ‘Aurora’//‘Kalyan’/‘Bluebird 3’/Woodpecker’ and carries the 1BL.1RS translocation from the “Aurora” parent. The rye chromosome segment was originally from ‘Petkus’ rye (Warburton et al., 2002). A secalin gene was cloned from genomic DNA of BW and used in construction of RNAi vector pSHP1 (Supplementary Fig. 1). The pSHP1 plasmid consists of 481 bp of the secalin coding region in the sense orientation, and 498 bp of the same coding region in the antisense orientation, separated by an 189 bp intron from the potato *ST-LSI* gene (coordinates 2652 -2832 of emb[X04753.1]) that was embedded within a 390 bp fragment of the coding region from the *E. coli uidA* gene (coordinates 1675629 – 1676019 of gb|CP012127.1). The latter serves only as a single-stranded spacer region between hairpin arms in the double-stranded RNA that results from splicing out the *ST-LSI* intron. These sequences were transcriptionally fused for endosperm-specific expression to a 2933 bp fragment of the *Idy10* gene promoter [beginning at the *EcoRI* site in GenBank accession X12929.2]. The transcription terminator from the CaMV 35S gene (coordinates 351-124 of

gb|HQ593861.1) was added 3' to the RNAi expression cassette, which was then cloned as an *EcoRI* fragment into pUC18.

Immature embryos of 'Bobwhite' were co-transformed by the biolistic method using the Ubi:BAR selectable marker gene and a 3:1 molar excess of the pSHP1 RNAi construct. The transformation method and subsequent regeneration under bialaphos selection was as described previously (Blechl et al., 2007).

To detect the transgene, total DNA was isolated from young leaves of primary transformants and their progeny using the XNA-R Plant PCR kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. The presence of the secalin RNAi construct was detected by polymerase chain reaction (PCR), using the primers (Supplementary Fig. 1) Sec3630F 5'GGTATCAGCGCGAAGTC3' from the GUS spacer region and Sec4138R 5'CCCAGAGACCAGAGCAAC3' from the secalin coding region. Amplification was for 40 cycles, utilizing an annealing temperature of 55°C and the buffer from the PCR kit with addition of 0.3mM EDTA to improve specificity of the amplification. The expected amplified fragment is 526 bp.

Twenty-two independent lines were identified that transmitted the pSHP1 PCR product to the first seed generation. Selfed progeny homozygous for the RNAi transgene were identified by PCR in the 2nd or subsequent generations of 15 of these lines. Two independent homozygous transgenic lines, L25-121 (L25) and L26-20 (L26) were chosen for further analyses.

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129 To obtain the flour samples used for both proteomics and small-scale quality analyses,
130 sixteen pots containing 8 homozygous T₃ or T₂ plants from line L25 or L26, respectively,
131 were grown on a single bench in the greenhouse with continuous fertilizer
132 (20N:20P:20K). T₄ seeds for L25 and T₃ seeds for L26 were harvested, pooled and milled
133 as described previously (Blechl et al., 2007). The resultant flours were assayed for protein
134 content, SDS-sedimentation rates and in 2-g mixograph tests, as previously described
135 (Blechl et al., 2007).

136

137 Soluble proteins were prepared from ground dry seeds by two extractions with 50%
138 propanol. The extracts were pooled, dried, suspended in SDS-PAGE buffer, and
139 subjected to 1-dimensional (1-D) SDS-PAGE (Blechl et al., 2007). Total flour proteins
140 were prepared for 2-dimensional gel electrophoresis (2-DE) as described previously
141 (Dupont et al., 2011; Hurkman and Tanaka, 2004). Three independent 2-D gels were run
142 and analyzed each for non-transformed BW and for transgenic events L25 and L26.
143 Normalized spot volumes were determined for each gel and compared between the non-
144 transgenic and the transgenic samples by ANOVA using Same Spot Software (TotalLab,
145 Ltd., Newcastle upon Tyne, UK). Differences in spot volumes were considered
146 significant if the ANOVA value was ≤ 0.02 and the change was greater than 20%.

147

148 Protein spots were excised from the 2-D gels and placed into individual wells of reaction
149 plates of a DigestPro (Itavis, Koeln, DE), where they were destained, reduced, alkylated,
150 and digested with trypsin, chymotrypsin or thermolysin. The resulting peptides were

automatically eluted into a 96-well plate in preparation for liquid chromatography followed by tandem mass spectrometry (MS/MS) (Dupont et al., 2011).

Identification of protein-containing bands was carried out as previously described (Blechl and Vensel, 2011; Vensel et al., 2005). The 96-well collection plate from the DigestPro was inserted into the autosampler of the Reverse Phase-HPLC system that was interfaced to a QSTAR PULSAR *i* quadrupole time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, CA). Conditions for operation of the instrument were as previously described (Vensel et al., 2005). The data were analyzed by Scaffold against the SuperWheat Database built with all Triticae sequences in NCBI as of September 1, 2014 and added sequences for the beta-lactamase and BAR genes included in the transformation constructs.

3. Results

3.1 Identification of transgenic plants containing pSHP1

An accession of the wheat variety ‘Bobwhite’ (BW) that carries the 1BL.1RS translocation was transformed with an RNAi plasmid (Supplementary Fig. 1) designed to suppress synthesis of the rye secalins. Putative transgenic plants were identified by their resistance to bialaphos, and the presence of the RNAi construction was confirmed in the primary regenerants and their progeny by PCR (Supplementary Fig. 2A), using primers that spanned the junction between the secalin coding region and the *uidA* spacer fragment

(Supplementary Fig.1). Several amplification products were obtained (Supplemental Fig. 2A), but the presence of the predicted fragment of 526 bp was correlated with decreases in one protein band in SDS-PAGE (Supplemental Fig. 2B). The inheritance of the pSHP1-specific PCR product was followed in subsequent generations and used to identify homozygous lines (not shown).

3.2 Analyses of flour proteins from pSHP1 transgenic plants

To better visualize the effects of the pSHP1 transgene on seed proteins, crushed whole seeds from homozygous plants were extracted with 50% propanol and the soluble proteins were separated by 1-D SDS-PAGE. At least four prominent propanol-soluble protein bands in the LMW-glutenin/gliadin region of the gel were reduced in the transgenic seeds (Fig. 1A). Further resolution was obtained by 2-DE of flour proteins, which showed differences in the levels of many spots between the transgenics (Fig. 1B, 1D) and their non-transgenic parent (Fig. 1C). Two independent transgenic lines, L25 and L26, were chosen for more detailed analyses.

The gel region from 30-60 kDa most affected by the RNAi construct is the most complex, even after 2-DE, and contains most of the LMW-glutenin and gliadin storage proteins from wheat (Supplemental Fig. 3). In addition, this is the region of the gel expected to contain the omega (MW 40,000 – 51,000) and gamma secalins (36,000 to 40,000) encoded by the *Sec1* locus.

Supplemental Table 1 lists all the flour protein spots whose normalized volumes were determined in three separate gels each for L25, L26, and their non-transformed parent. In this table, the averages of spot volumes are grouped into worksheets by decreases in one or both transgenics, increases in one or both transgenics, or no significant changes. Also shown in Supplemental Table 1 are the identifications of spots based on MS/MS of peptides after digestion with the proteolytic enzymes trypsin, chymotrypsin or thermolysin. Best matches and database accession numbers (where available) are shown. The assignment of individual protein spots to the exact genes that encode them cannot be made because the proteome of BW has not been mapped in detail and most of the seed transcriptome has not been sequenced. Nevertheless, the proteins can be assigned to groups, based on their identification by MS/MS. Tentative assignments can also be made by their migrations in 2-D gels in comparison to those of the well-characterized proteome of 'Butte 86' (Dupont et al., 2011).

There were 66 spots that showed significant decreases in both transgenic flours compared to the non-transformed flour. Generally, spot volume decreases in L25 were greater than those in L26 (Supplemental Table 1; Table 1). Of 46 spots (Supplemental Table 1) excised from the gels and subjected to MS/MS, 33 were identified with overall probabilities of 100% and at least two peptide identifications with probabilities greater than 90%. Thirty-one spots that are decreased in both transgenic flours and that fall between 60 kDa and 30 kDa are labelled on the 2-D gel image of BW flour in Figure 2A. All spots shown to contain secalins (6 spots) or omega gliadins (7 spots) were decreased in both transgenic flours. Eleven spots containing gamma gliadins and four spots

identified as LMW-glutenins also were decreased in both transgenic flours. A minor spot (647) identified as HMW-glutenin 1Bx7 (not shown) was decreased in both transgenic flours as well as four spots containing farinin. Ten unidentified spots also were decreased. Two of them (351, 356) migrate at about 50 kDa within the row of other spots identified as secalins (Figure 2A), while three others (425, 431, 489) are in the region populated by gamma gliadins. One (288) is located on the acidic side of the region populated by omega gliadins and beta-amylase. Unidentified spots 378 and 82 (not shown) are in the gel regions populated by LMW-glutenins/secalins and HMW-glutenins, respectively. There are two unidentified spots (508 and 518) from the region of the gels containing the farinins and one unidentified spot (541) located just below that region. The location of unidentified spot 579 is shown in Figure 1C.

In addition, there were 33 and 35 spots that were significantly reduced compared to the control in only one of the two transgenic lines, L25 and L26, respectively. Of these, 17 and 14, respectively, could be assigned identities by MS/MS. As observed in both transgenics, decreases in the L25 transgenic were in spots that contained gamma gliadins, LMW-glutenins, HMW-glutenins and farinins. In addition, there were small decreases (1.2-fold) in a spot (433) identified as an alpha gliadin and in one of the major spots (78) that contains HMW-glutenin 1Bx7. In fact, all but one of the HMW-glutenin spots that were decreased in one or both transgenic flours were identified as 1Bx7. For L26, a different pattern was evident in that several prominent members of the alpha amylase/trypsin inhibitor (AATI) gene family (635, 631, 637, 612, 629 and 624) were decreased. The locations of these spots except 635 are shown in Figure 1C.

243

244 There were also small but significant increases in the levels of a number of spots,

245 including some that were shown to contain alpha gliadins, triticins or y-type HMW-

246 glutenins (Table 2). Forty-nine spots were significantly increased in both transgenic

247 flours relative to that of their non-transformed parent (Supplemental Table 1). Of these,

248 20 were identified (Table 2); twelve are labelled in Fig. 2B and two (594, 600) in Fig. 1C.

249 Several of these identifications were made in spots excised from L25 gels (d superscripts

250 in Table 2 because they were better isolated in regions where nearby proteins were

251 decreased in the transgenic flours. Four spots (456, 417, 721, 441) contained alpha

252 gliadins; two (739, 139) contained HMW-glutenin By9; one (154) contained HMW-

253 glutenin Dy10; six (383, 395, 594, 384, 600, 744) contained triticins; one contained serpin

254 (439); one contained sucrose synthase (137); and one (205) contained a protein with 5

255 peptides identical to those in barley 70 kDa heat shock protein. Several spots that

256 contained alpha gliadins also contained other proteins. Three with increased levels in

257 both transgenic flours were mixed with gamma gliadins (438, 447, 494) and one (733)

258 was a mixture with triticein.

259

260 Forty-one more spots showed significant increases only in L25 flour compared to the

261 non-transformed flour (Supplemental Table 1). Of the 18 that were subjected to MS/MS,

262 eight were identified. Two contained alpha gliadins (529 496), one contained a mixture of

263 alpha gliadins and LMW-glutenin (361), two contained HMW-glutenins [Ax2* (731) and

264 Dy10 (235)], one farinin (503), triosephosphate isomerase (565), and 1-cys peroxiredoxin

265 (574) (Table 2). Forty-seven different spots showed significant increases only in L26

(Supplemental Table 1). Of the 24 subjected to MS/MS, seventeen were identified. Nine contained alpha gliadins (487, 440, 474, 435, 460, 464, 780, 495, 436), one gamma gliadin (461), major spots for HMW-glutenins By9 (142), Dy10 (150), a medium-sized spot for HMW-glutenin Ax2* (95), one serpin (404), protein disulfide isomerase (269), beta amylase (302), and an embryo globulin (335) (Table 2).

There were 304 spots whose levels did not differ significantly between either transgenic and the non-transformed flour (“No Change” tab in Supplemental Table 1). The seventy-one of these spots that were identified by MS/MS or with average BW values greater than 20 are listed from highest spot volume to lowest in Supplemental Table 2. Among them are representatives of each seed storage protein family and nearly every protein class that showed significant increases or decreases in one or both transgenic flours. Major spots identified as HMW-glutenins Dx5 (42) and Ax2* (72) did not differ significantly in volume between the non-transformed and transgenic flours. Several lesser spots containing HMW-glutenins (318, 163, 185, 167, 134, 248, 173, 97, 644, 135, 113) did not differ in volume among the three flours. Other spots with no significant volume differences were identified as LMW-glutenins (448, 450, 449, 403, 391, 443), serpins (459, 434), farinins (592, 505), alpha gliadins (492, 683), tritin (522), triticin (380, 575, 599, 736), gamma gliadin (468), beta-amylase (306), triosephosphate isomerase (566), globulin 3 (473), rye gamma prolamin (458) and members of the AATI family (627, 626, 632, 633, 587, 614, 630, 622, 636). Proteins found only in the spots that did not differ significantly between the transgenics and their non-transformed parent were puroindoline

(609), lactoglutathione lyase (687), dehydroascorbate reductase (586), rye seed chitinase (561), and superoxide dismutase (613).

3.3 Small scale quality analyses

The flour protein content of non-transformed BW (20.4%) was higher than those of transgenic flours L25 (18.1%) and L26 (17.3%) (Table 3). No significant differences in SDS-sedimentation were detected among the flours (Table 3). The mixing properties of the transgenic flours were measured in the 2-gram mixograph (Figure 3). The trace of resistance over time of mixing of non-transformed BW flour exhibits characteristics of doughs with low mixing strength and tolerance. The time to peak resistance (Mix Time) is short (4.5 min.) and the decline in resistance after peak is very steep (slope of -6.7 and trace width of 12.5 AU eight minutes after peak) (Fig. 3; Table 3). Flours from the transgenic plants show greater tolerance to mixing, with less steep declines (slopes of -2.1 and -2.5) and nearly twice the thickness in traces eight minutes after attaining peak resistance (24.3 and 23.4). Transgenic flours from L25 and L26 also have longer dough development times of 11.0 and 8.7 minutes, respectively (Figure 3; Table 3). Dough stickiness and loaf volumes were not evaluated in these small-scale analyses.

4. Discussion

Genetic engineering allows us to assess the contributions of individual seed proteins and protein families to wheat dough properties by comparing flours prepared from the seeds of closely related genotypes that differ only or mainly in the presence/absence of those

proteins. In the present study, introduction of an RNAi construct containing 481 bp of the coding region of a rye omega secalin substantially decreased the levels of not only the secalins, but also of the highly homologous wheat omega gliadins. In addition, the levels of several other types of wheat seed storage proteins were reduced, including gamma gliadins, and to a lesser extent LMW-glutenins, farinins, HMW-glutenin subunit 1Bx7, and in one transgenic, some AATI. The widespread effects of the RNAi construct could be due to the large size of its trigger region, which included codons that specify the repetitive regions of the protein. Indeed, a BLASTn search of Triticae sequences in GenBank identifies numerous omega and gamma gliadins as well as gamma and omega secalins with perfect matches of 24 nucleotides or more to the trigger sequence. A more selective suppression might be achieved by using a smaller region more specific to the secalins.

At the same time, many wheat flour proteins increased in relative abundance in the transgenic flours, including alpha gliadins, triticins and some y-type HMW-glutenins. The increases tend to be small; only 4 spots had increases of more than 2-fold. These increases may be due to proteome rebalancing that redistributes nitrogen reserves from the suppressed set of storage proteins to others in the seed (Schmidt et al., 2011). Increases in other seed storage proteins have been reported in every case where RNAi has been used to suppress families or sub-families of gliadins (Altenbach et al, 2014a; Barro et al., 2015; Becker et al., 2012; Gil-Humanes et al., 2010; Piston et al., 2011).

Changes for members of some seed storage protein families were consistent between the two independent transgenic events. Spots identified as containing secalins or omega gliadins all showed significant decreases in both transgenics compared to the non-transgenic flour (Table 1). This was true even when the spots also contained other proteins (363, 274, 284, 781, 283). Spots only containing triticins were never significantly lower in the transgenics. Spots containing gamma gliadins, LMW-glutenins, farinins and AATI tended to be at lower levels in the transgenic flours, while spots containing alpha gliadins or serpins tended to be higher in the transgenics. There were exceptions to these trends. The spot (433) that contained an alpha gliadin and was 1.2-fold lower in L25 included a partial identification of a gamma gliadin whereas the spot (461) that contained a gamma gliadin and was 1.4-fold higher in L26 included a partial identification of an alpha gliadin (Supplemental Table 1). This is not surprising given the considerable overlap among alpha gliadins, gamma gliadins and LMW-GS in 2-D gels (Dupont et al., 2011). A couple of mixed spots (478, 467) whose levels did not differ between non-transformed and transformed flours may represent offsets of presumptive decreases in gamma gliadins with increases in alpha gliadins or triticins.

Other experiments that have used RNAi strategies to decrease the levels of families and sub-families of wheat seed storage proteins have had similarly mixed and event-specific results. Yue et al. (2008) transformed a 'Bobwhite' with an RNAi construct that included 210 bp from the 5' coding region of the *1Dx5* gene. They achieved reductions of nearly 100% in levels of HMW-glutenin 1Dx5, but also lesser reductions of subunit 1Bx7. Total HMW-glutenin levels were decreased 17% and 30% in two different lines. They did not

report effects on other proteins or protein fractions. Gil-Humanes et al. (2008) transformed two sister lines from the ‘Bobwhite’ cross. One of their lines ‘BW2003’ contains the 1BL.1RS translocation and is likely the same or very closely related to the ‘Bobwhite’ used in this report. Their construct consisted of 169 bp from a gamma gliadin coding region. They identified 9 lines with reductions in the gamma gliadin fractions that averaged 78% in the 1BL.1RS translocation background (Piston et al., 2011). They also measured increases in the alpha gliadin and omega gliadin fractions that offset the gamma gliadin decreases, resulting in the same total gliadin levels in the transgenics as in their untransformed parents.

Altenbach and Allen (2011) designed a very specific RNAi trigger of 153 bp from the 3’ coding region of an omega-5 gliadin gene and transformed it into the spring wheat ‘Butte 86’. They used quantitative 2-DE to characterize the effects of suppression on individual proteins in four different transgenic events (Altenbach et al., 2014a) The availability of a comprehensive proteomic map of Butte 86 flour (Dupont et al., 2011) made it possible to consider changes in individual protein types in addition to individual 2-DE spots. They reported that the same RNAi plasmid had differential effects on the wheat grain proteome. In one line with an 80% reduction in omega-5 gliadin levels, few changes in other proteins were detected. In another line the omega-5 gliadins were virtually absent, but the omega-1,2 gliadins were also partially suppressed. Two other lines showed alterations in a variety of proteins.

Becker et al. (2012) used RNAi with a trigger of 313 bp from the 5' coding region to reduce the levels of alpha gliadins in winter wheat 'Florida'. They measured changes in individual protein spots from 2-D gels of one line that had 39% the levels of alpha gliadins as wild type. In addition to 2-14-fold reductions in 20 spots identified as alpha gliadins, they also observed reductions of about 2-fold in two spots identified as HMW-glutenins.

The widespread changes in the levels of different protein classes in secalin RNAi transgenic flours did not result in changes in SDS-sedimentation volume. However, mixing behavior was improved, in particular mixing tolerance. We tentatively conclude that in this respect, the flours of the RNAi secalins transgenics have improved properties for bread-making. It will be interesting to measure the end-use properties of the RNAi transgenic lines, including stickiness, in larger scale quality analyses when large quantities of field-grown seed are available.

Altenbach et al. (2014b) also found improved mix times and mixing tolerance in two transgenic lines in which the omega-5 gliadins were suppressed and concluded that the omega gliadins have a negative effect on mixing properties. In comparison, reductions in the contents of other classes of gliadins by RNAi have not had large impacts on dough processing properties. Becker et al. (2012) detected no differences in micro-Kiefer rig maximum resistance and extensibility in doughs made from wild type compared to a composite of transgenic flours with 63% reductions in alpha gliadins. They did detect difference in the properties of glutens prepared from these flours and hypothesize that

increases in other seed protein fractions, particularly of the non-gluten albumins and globulins, compensate for the lack of alpha gliadins in their transgenic flours. The RNAi gamma transgenic lines analyzed by Piston et al. (2011) exhibited higher SDS sedimentation values in six of the nine lines that also contained the 1BL.1RS translocation. Ten-gram mixograph parameters did not differ much among the transgenics and their non-transformed parent.

Major changes in gliadin levels that result in changes to the ratio of glutenin/gliadin do have effects on dough mixing properties. Gil-Humanes et al. (2014) analyzed doughs from 20 transgenic ‘Bobwhite’ lines with reductions in multiple gliadin families in a Mixolab and a 35-g mixograph. Whether or not they contained the 1BL.1RS translocation, most of the transgenics exhibited shorter dough development times and improved stability in the Mixolab, but were too strong to be mixed in the mixograph. In contrast, reductions in 1Dx5 and 1Bx7 and total HMW-glutenins in RNAi Dx5 transgenic lines resulted in decreases in SDS-sedimentation and farinograph dough development time and stability, consistent with the role of HMW-glutenins in dough strength (Yue et al., 2008).

Wheat lines containing the 1BL.1RS translocation continue to be employed in wheat breeding because they contain some effective fungal resistances and confer yield advantages in some environments. Recent research has shown that the rye *Pm3* gene confers resistance to powdery mildew in backgrounds that lack the orthologous wheat *Pm8* gene (Hurni et al., 2014) and that rye translocation lines have a yield advantage

under drought conditions (Ehdaie et al., 2012; Howell et al., 2014). Not all wheats that contain rye translocations have weak doughs; it appears that at least some quality defects can be overcome by the presence of HMW- and LMW-glutenin alleles from the A and D genomes that are favorable for dough strength (Barbeau et al., 2003). In the work presented here, we have shown that use of RNAi to shift the protein composition of wheat endosperm is an alternative and effective strategy for mitigating the effects of rye translocations on wheat dough mixing and processing quality.

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Appendices A-D. Supplementary data

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547 **Table 1. Individual spots that were less abundant in flours from transgenic events L25 and L26, compared to their non-**
548 **transformed parent.** Normalized spot volumes are averages for three separate 2-D gels. This table lists spots that were identified
549 and/or with average BW volumes of at least 20. (Values for all spots are listed in Supplemental Table 1). The spots are grouped by
550 gene families, based on their identifications and/or gel positions.

551

Gel Spot number ^a	Identification ^b	Ave. BW spot volume	Ave. L 25 spot volume	Ave. L26 spot volume	Fold change in L25	Fold change in L26
351	No ID	132.1	20.1	43.7	-6.6	-3.0
385	Omega secalin	44.7	8.1	15.7	-5.5	-2.8
360	Omega secalin	105.2	20.8	35.0	-5.1	-3.0
366	Omega secalin	93.2	24.3	51.3	-3.8	-1.8
359	Secalin	78.1	21.0	26.3	-3.7	-3.0
363	Secalin / tritacin ^c	126.9	34.6	48.8	-3.7	-2.6
356	No ID	233.1	67.3	106.2	-3.5	-2.2
354	Secalin / enolase ^c	139.2	47.8	73.0	-2.9	-1.9

365	Omega gliadin	119.6	17.1	34.4	-7.0	-3.5
341	Omega gliadin (D genome)	110.5	19.5	31.3	-5.7	-3.5
274	Omega gliadin + Beta amylase	28.9	11.9	7.0	-2.4	-4.1
284	Omega gliadin + Beta amylase	119.5	50.7	76.5	-2.4	-1.6
782	Omega gliadin + Beta amylase	100.6	44.0	52.5	-2.3	-1.9
283	Omega gliadin + Beta amylase	251.3	116.9	119.9	-2.2	-2.1
288	No ID	84.5	41.1	50.4	-2.1	-1.7
282	Omega gliadin + Beta amylase	44.4	23.9	22.3	-1.9	-2.0
425	No ID	27.0	4.3	8.0	-6.3	-3.4
479	Gamma gliadin	257.6	47.4	112.0	-5.4	-2.3
428	Gamma gliadin / gamma gliadin ^c	297.2	59.9	148.6	-5.0	-2.0
455	Gamma gliadin	67.3	15.2	31.7	-4.4	-2.1
402	Gamma gliadin	279.2	66.1	114.0	-4.2	-2.4
489	No ID	43.2	10.4	18.8	-4.1	-2.3
490	Gamma gliadin	257.4	73.2	142.2	-3.5	-1.8

431	No ID	32.1	10.5	9.4	-3.1	-3.4
442	Gamma gliadin	91.3	30.6	46.7	-3.0	-2.0
374	Gamma gliadin + LMW-GS	136.0	61.1	98.9	-2.2	-1.4
427	Gamma gliadin	57.9	41.1	50.4	-2.1	-1.9
406	Gamma gliadin	41.2	21.0	20.7	-2.0	-2.0
399	Gamma gliadin + tritacin	38.2	21.1	15.5	-1.8	-2.5
420	Gamma gliadin	21.2	8.7	20.9	-2.3	n.s.
423	Gamma gliadin + barley protein (Acc. BAK04052) / tritacin + barley protein ^c	66.1	45.6	52.3	-1.5	n.s.
463	Gamma gliadin	115.1	84.0	97.4	-1.4	n.s.
421	Gamma gliadin + Alpha gliadin	16.8	11.8	19.4	-1.4	n.s.
433	Alpha gliadin	240.1	201.9	222.3	-1.2	n.s.
407	LMW-GS + Gamma gliadin	33.0	11.3	14.6	-2.9	-2.3
643	LMW-GS	82.8	15.6	33.3	-5.3	-2.5
390	LMW-GS	254.2	153.1	205.4	-1.7	-1.2

351	LMW-GS	411.4	252.3	354.4	-1.6	-1.2
346	LMW-GS	161.9	108.6	141.3	-1.5	-1.1
388	LMW-GS	14.9	9.0	11.1	-1.7	n.s.
453	LMW-GS + peroxidase	100.3	70.3	89.1	-1.4	n.s.
348	LMW-GS	51.7	36.7	49.8	-1.4	n.s.
400	LMW-GS	152.4	109.0	137.9	-1.4	n.s.
378	No ID	75.2	52.1	74.8	-1.4	n.s.
372	LMW-GS	250.4	214.2	249.4	-1.2	n.s.
387	LMW-GS	25.0	17.5	14.7	n.s.	-1.7
647	HMW-GS Bx7	40.8	13.8	23.8	-2.9	-1.7
191	HMW-GS Bx7	11.9	5.5	4.0	n.s.	-3.0
204	HMW-GS Dx5 + Beta amylase	13.4	15.9	11.3	n.s.	-1.2
90	HMW-GS Bx7	46.2	35.2	38.2	n.s.	-1.2
78	HMW-GS Bx7	237.5	195.1	252.6	-1.2	n.s.
685	HMW-GS Bx7	21.5	17.9	16.0	-1.2	n.s.

122	LOS1 copper-binding Elongation Factor	3.23	0.7	3.6	-4.3	n.s.
507	Farinin	105.1	38.9	50.1	-2.7	-2.1
508	No ID	21.2	9.4	9.9	-2.7	-2.1
510	Farinin + Tritin	52.3	23.6	33.4	-2.2	-1.6
518	No ID	20.2	10.7	10.0	-1.9	-2.0
519	Farinin + Alpha gliadin ^d	36.8	22.0	22.7	-1.7	-1.6
516	Farinin	53.0	35.8	35.2	-1.5	-1.5
471	Farinin	13.7	4.9	6.4	n.s.	-2.2
509	Farinin	51.6	39.4	40.8	-1.3	n.s.
635	Monomeric alpha-amylase inhibitor	41.5	33.0	25.3	n.s.	-1.6
631	Tetrameric alpha-amylase inhibitor	183.5	159.1	112.7	n.s.	-1.6
637	Monomeric alpha-amylase inhibitor	159.7	144.6	99.9	n.s.	-1.6
612	Tetrameric alpha-amylase inhibitor	285.3	291.1	188.5	n.s.	-1.5
629	Tetrameric alpha-amylase inhibitor	314	333.8	225.0	n.s.	-1.4
624	Tetrameric alpha-amylase inhibitor	277.5	259.8	218.9	n.s.	-1.3

541	No ID	33.8	19.4	18.2	-1.7	-1.9
579	No ID	56.6	45.2	33.5	-1.3	-1.7
584	No ID	88.6	88.7	71.9	n.s.	-1.2

552 ^aMost spots with decreases in both L25 and L26 are shown in Figure 3.

553 ^bIdentified spot from BW flour with at least 2 peptides, each with at least 90% probability

554 ^cIdentified spot from both BW and transgenic L25. Both ID's are shown separated by /.

555 ^dIdentified spot from L25 only.

556 n.s. not significant at $p = 0.02$.

557 No ID: No identification of at least 2 peptides, each with at least 90% probability. The positions of most of the unidentified spots in

558 the table were chosen based on partial ID's (Supplemental Table 1) and/or the identity of nearby spots in 2-DE (Figures 2C and 3A).

559

560 **Table 2. Individual spots that were more abundant in flours from transgenic events L25 and L26 than in their non-**
561 **transformed parent BW.** Normalized spot volumes are averages for three separate 2-D gels. This table lists spots that were identified
562 and/or with average BW volumes of at least 20. (Values for all spots are listed in Supplemental Table 1). The spots are grouped by
563 gene families, based on their identifications and/or gel positions.
564

Gel Spot number ^a	Identification ^b	Ave. BW spot volume	Ave. L 25 spot volume	Ave. L26 spot volume	Fold change in L25	Fold change in L26
438	Alpha gliadin + Gamma gliadin	25.8	86.7	72.9	3.4	2.8
447	Alpha gliadin + Gamma gliadin / Alpha gliadin ^c	32.8	92.0	86.5	2.8	2.6
456	Alpha gliadin	20.4	45.2	36.8	2.2	1.8
417	No ID / Alpha gliadin ^c	20.8	37.1	39.0	1.8	1.9
728	Alpha gliadin ^d	21.0	32.2	29.7	1.5	1.4
494	Alpha gliadin + Gamma gliadin / Alpha gliadin ^c	24.0	35.1	43.5	1.5	1.8

441	Alpha gliadin	89.1	115.8	114.7	1.3	1.3
733	Alpha gliadin + Triticin ^d	29.8	38.4	39.6	1.3	1.3
529	Alpha gliadin / Alpha gliadin ^c	67.5	96.8	78.7	1.4	n.s.
496	Alpha gliadin	66.1	89.4	86.6	1.4	n.s.
361	Alpha gliadin + LMW-GS / No ID	72.1	84.5	84.4	1.2	n.s.
466	No ID	24.9	30.3	29.6	1.2	n.s.
487	Alpha gliadin	48.7	59.2	73.4	n.s.	1.5
440	Alpha gliadin	41.8	52.4	60.1	n.s.	1.4
474	Alpha gliadin	172.7	204.7	240.0	n.s.	1.4
435	Alpha gliadin	115.8	139.5	147.2	n.s.	1.3
780	Alpha gliadin	162.6	186.3	211.4	n.s.	1.3
464	Alpha gliadin	209.0	230.4	264.1	n.s.	1.3
734	Alpha gliadin ^d	11.5	12.7	14.8	n.s.	1.3
495	Alpha gliadin ^d	19.8	21.2	25.4	n.s.	1.3
436	Alpha gliadin	196.3	190.5	219.8	n.s.	1.1

461	Gamma gliadin	51.0	61.4	70.4	n.s.	1.4
739	HMW-GS By9 ^d	7.2	22.7	18.6	3.1	2.6
139	HMW-GS By9	20.3	32.0	39.3	1.6	1.9
154	HMW-GS Dy10	31.6	44.8	35.2	1.4	1.1
731	HMW-GS Ax2* ^d	3.0	23.6	5.5	7.8	n.s.
96	No ID / No ID ^c	30.0	48.6	31.6	1.6	n.s.
235	HMW-GS Dy10	13.8	18.5	14.5	1.3	n.s.
150	HMW-GS Dy10	214.6	253.8	268.3	n.s.	1.3
95	HMW-GS Ax2*	38.9	38.4	47.4	n.s.	1.2
142	HMW-GS By9	174.9	173.6	202.7	n.s.	1.2
744	Triticin	44.1	83.2	81.6	1.9	1.8
383	Triticin ^c	71.2	119.5	122.8	1.7	1.7
395	Triticin / Triticin	61.1	93.4	99.9	1.5	1.6
594	Triticin	56.1	85.1	83.7	1.5	1.5
384	Triticin	46.0	64.4	71.0	1.4	1.5

600	Triticin	102.4	134.7	139.9	1.3	1.4
503	Farinin	23.5	43.7	34.5	1.9	n.s.
439	Serpin	14.2	20.1	22.6	1.4	1.6
429	No ID	25.2	33.1	36.9	n.s.	1.5
426	No ID	76.9	95.0	113.0	n.s.	1.5
419	No ID	24.5	32.6	34.0	n.s.	1.4
404	Serpin	37.7	47.8	50.3	n.s.	1.3
137	Sucrose synthase	24.6	31.6	29.5	1.3	1.2
205	Barley heat shock 70	10.1	17.6	17.3	1.7	1.7
269	Protein disulfide isomerase ^d	29.6	36.9	62.7	n.s.	2.1
290	No ID	61.6	71.6	82.1	n.s.	1.3
302	Beta amylase	32.6	41.6	44.3	n.s.	1.4
315	No ID	21.5	21.5	25.5	n.s.	1.2
316	No ID	46.0	49.1	65.4	n.s.	1.4
335	Globulin 3	10.3	12.5	17.6	n.s.	1.7

491	Cytosolic malate dehydrogenase	40.1	58.0	58.2	1.4	1.5
565	Triose phosphate isomerase	21.6	28.5	23.9	1.3	n.s.
573	No ID	116.4	153.6	111.7	1.3	n.s.
574	1-cys peroxiredoxin	51.7	59.6	45.9	1.2	n.s.
691	No ID	44.5	63.1	74.1	n.s.	1.7

565 ^aSome spots with changes in both transgenics are shown in Figure 3B.

566 ^bIdentified spot from BW flour with at least 2 peptides, each with at least 90% probability

567 ^cIdentified spot from both BW and transgenic L25. Both ID's are shown separated by /.

568 ^dIdentified spot from L25 only.

569 n.s. not significant at $p = 0.02$.

570 No ID: No identification of at least 2 peptides, each with at least 90% probability. The positions of most of the unidentified spots in
571 the table were chosen based on partial ID's (Supplemental Table 1) and/or the identity of nearby spots in 2-DE (Figures 2C and 3A).

572

573 **Table 3. Total protein, SDS sedimentation values and two-gram mixograph parameters for transgenic flours L25 and L26**
574 **containing pSHP1 and their non-transformed parent BW.** Except where noted, the averages and standard deviations of two
575 determinations for a one flour sample are shown.
576

Parameter	Bobwhite	L25	L26
Flour Protein (% based on 14% moisture)	20.4* (0.02)	17.3* (0.18)	19.1 (0.00)
SDS Sedimentation (mls.)	5.0 (0.28)	5.05 (0.14)	5.0 (0.07)
Mixograph Mix Time (min.)	5.7 (0.44)	11.6 (0.47)	8.7 (0.13)
Mixograph Peak Height (AU)	63.0 (4.88)	47.5 (1.41)	45.9 (0.35)
Mixograph Width at Peak (AU)	27.2 (1.84)	23.2 (1.56)	22.1 (0.57)
Mixograph Slope after Peak	-6.7 (2.05)	-2.1 (0.62)	-2.5 (0.30)
Mixograph Width at 8 min. after Peak (AU)	12.5 (0.49)	24.3 (0.28)	23.4 (0.42)

577 AU: Arbitrary units of resistance.

578 *Three independent technical determinations for one flour

579

580 **Fig. 1.** Effects of pSHP1 on the propanol-soluble (**A**) and total flour (**B**) proteins of BW,
581 L25 and L26. **A.** SDS-PAGE of seed proteins soluble in 50% 1-propanol from BW (N)
582 and homozygous transgenic events L25 and L26. The regions of the gel containing
583 HMW-glutenin subunits (HMW-GS), LMW-glutenin subunits (LMW-GS) and gliadins
584 are indicated by brackets. Red dots mark the bands most obviously reduced in the
585 transgenic seeds. **B-D.** 2-DE of flour proteins from non-transformed BW (**C**) and
586 transgenics L25 (**B**) and L26 (**D**). Proteins are separated by charge in the horizontal
587 direction and by size in the vertical direction. The isoelectric point (pI) gradient and the
588 molecular weights of the standards are shown to the top and right, respectively, in panel
589 **B.** In **C**, numbers mark the locations of protein spots discussed in the text. One major
590 spot for each HMW-glutenin is labeled with the subunit name.

591

592 **Fig. 2.** Close-up of regions of the 2-DE gels that contain most of the flour proteins that
593 differ between non-transformed BW (upper panel from Fig. 1C) and transgenic L25
594 (lower panel from Fig. 1B). The locations of 31 spots significantly decreased in both L25
595 and L26 are labeled in the BW image and of 12 spots significantly increased in both
596 transgenic lines are labeled in the L25 image. Labels are colored by seed storage protein
597 family: omega secalins (brighter red), omega gliadins (blue), gamma gliadins (lighter
598 purple), LMW-glutenins (green), farinin (gray), alpha gliadins (black), triticins (orange),
599 cytosolic malate dehydrogenase (darker red, lower panel) and HMW-glutenin (darker
600 purple, lower panel). Spot 374 contains both gamma gliadin and LMW-glutenin.

601

602 **Fig. 3.** Two-gram mixograph traces of flours from transgenic wheats L25 and L26
603 containing the secalin RNAi construct and from their non-transformed parent BW.
604 Arbitrary units (AU) of resistance are plotted against time in minutes.