



## Association analysis reveals effects of wheat glutenin alleles and rye translocations on dough-mixing properties

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### ABSTRACT

The glutenin loci of wheat (*Triticum aestivum* L.) are important determinants of bread-making quality, although the effects of alleles at those loci are incompletely understood. We applied an association analysis method to assess the effects of glutenin alleles and 1RS wheat-rye (*Secale cereale* L.) translocations on dough-mixing properties in 96 wheat cultivars and advanced lines grown at three Colorado locations while accounting for population structure and relatedness of individuals in the population. The results indicated that (1) in the majority of cases, controlling relatedness of individuals reduced the significance of associations between glutenin loci and Mixograph traits; (2) the *Glu-D1* and *Glu-B3* loci and 1RS translocations had greater impacts on dough-mixing properties compared to other glutenin loci; (3) *Glu-B1w*, *Glu-D1d*, and *Glu-B3b* were consistently associated with greater (more favorable) Mixograph peak time (MPT) than other alleles at the respective loci, whereas *Glu-B1e*, *Glu-D1a*, and *Glu-B3c* were associated with reduced MPT; (4) the 1BL.1RS translocation was associated with a decrease in Mixograph properties. Our results indicate that taking multiple-level relatedness of individuals into account can improve the results of association analysis for wheat-quality traits.

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### 1. Introduction

Wheat (*Triticum aestivum* L.) bread-making quality is a complex collection of traits influenced by a number of environmental, genetic, and biochemical factors. Among them, grain protein concentration and its composition are of primary importance (reviewed by Singh and Khatkar, 2005). Glutenin, a major component of wheat protein, is formed of polymers linked by disulphide bonds. Glutenin proteins are further classified into two groups, the

high-molecular-weight glutenin subunits (HMW-GS) and low-molecular-weight glutenin subunits (LMW-GS), based on their mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reduced conditions (Singh and Khatkar, 2005). The HMW-GS have been studied in detail because of their important roles in determining bread-making quality, as well as their ease of separation on SDS-PAGE. The LMW-GS have not been studied as thoroughly because they are relatively difficult to separate in gels (Singh and Khatkar, 2005).

A number of genes of agronomic importance, including disease and pest resistance genes, have been transferred from rye (*Secale cereale* L.) to wheat in the form of 1AL.1RS, 1BL.1RS, and 1DL.1RS wheat-rye chromosomal translocations (1AL, 1BL, and 1DL are the long arms of group 1 wheat chromosomes; 1RS signifies the short arm of rye chromosome 1) (Graybosch, 2001). Among these, the 1AL.1RS and 1BL.1RS translocations are most common in wheat cultivars. Unfortunately, the 1RS translocation has often resulted in significant reduction in wheat end-use quality (Graybosch, 2001). Severe quality problems are recognized when 1BL.1RS is present in hard wheat backgrounds, including low SDS sedimentation volumes, the production of 'sticky' doughs, a lack of

**Abbreviations:** HMW-GS, high-molecular-weight glutenin subunits; LMW-GS, low-molecular-weight glutenin subunits; MPT, Mixograph midline peak time; MPV, Mixograph midline peak value (height); MPW, Mixograph band width at peak; MRS, Mixograph midline right slope, calculated as (MRV-MPV)/2; MRV, Mixograph midline right value (height) at two minutes after peak; MRW, Mixograph band width at two minutes after peak; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SSR, simple-sequence repeat; 1RS, the short arm of rye chromosome.

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tolerance of dough to overmixing, and low loaf volumes (Graybosch, 2001). Wheats cultivars with the 1AL.1RS translocation have better end-product qualities than those with the 1BL.1RS translocation (Graybosch et al., 1993; Kumlay et al., 2003), although 1AL.1RS reduces mixing strength and SDS sedimentation volumes compared to non-1RS sibling lines (Espitia-Rangel et al., 1999; Graybosch et al., 1993).

Both irrigated and rainfed (dryland) environments are important for wheat production in semi-arid areas of the west central Great Plains of the US. For example, irrigated wheat accounted for one-sixth of the total wheat production in Colorado in 2007, with rainfed wheat making up the rest ([www.nass.usda.gov/Statistics\\_by\\_State/Colorado/Publications/County\\_Estimates/alwhitce07.pdf](http://www.nass.usda.gov/Statistics_by_State/Colorado/Publications/County_Estimates/alwhitce07.pdf), verified on Dec. 15, 2008). Breeders would benefit from knowing whether the same genetic factors affect bread-making quality under both sets of moisture conditions.

The Mixograph has traditionally been used to determine the rheological characteristics and bread-making potential of dough systems by the wheat milling and baking industry (Kunerth and D'Appolonia, 1985). US breeding programs typically prefer the Mixograph over other methods for assessing the functional properties of flour because the method is fast (typically no more than 8 min), can accommodate a relatively small amount of flour (10 g), and imparts a comparatively large amount of work input to the dough that provides greater discrimination of the end-use performance of breeding lines.

The quality effects of glutenin alleles and 1RS have been studied in two types of populations: structured populations, typically derived from biparental crosses and investigated via quantitative trait locus (QTL) analysis, and non-structured populations, usually collections of cultivars and breeding lines that are analyzed by association analysis methods (Yu and Buckler, 2006). Non-structured populations have several advantages, including the ability to simultaneously evaluate many alleles at each locus, assess allele function in genetic backgrounds of current breeding materials, and use multi-location and multi-year quality data at no additional costs because these data are collected routinely in many wheat-breeding programs (Brescghello and Sorrells, 2006; Cane et al., 2008; Eagles et al., 2002). Due to these advantages, the largest number of associations between glutenin alleles and quality characteristics have been established in collections of wheat cultivars and advanced lines (Bekes et al., 2006; Branlard et al., 2001; Cane et al., 2008; Eagles et al., 2002; He et al., 2005).

Nevertheless, there are certain precautions that must be heeded with association analysis of non-structured populations to avoid reaching incorrect conclusions. Due to geographic isolation, natural selection, or artificial selection, population stratification is common for plant germplasm (Yu et al., 2006), and has been observed in collections of wheat germplasm (Brescghello and Sorrells, 2006; Ravel et al., 2006). Population stratification is the presence of a systematic difference in allele frequencies between subpopulations within a population (Yu and Buckler, 2006). This population stratification and an unequal distribution of alleles within a population can result in spurious associations (Knowler et al., 1988). Therefore, genome-wide distributed molecular markers have been used to estimate population structure and relatedness of individuals, and incorporating descriptions of these relationship into the statistical model has helped to reduce false associations (Pritchard et al., 2000; Yu and Buckler, 2006). A unified mixed model (Q+K) was proposed to account for both population structure and relatedness among individuals (Yu et al., 2006).

Therefore, the objective of this study was to evaluate the effects of alleles at the *Glu-1* and *Glu-3* loci and the presence of the 1RS translocation on dough-mixing properties in a collection of 96

wheat cultivars and lines in irrigated and rainfed Colorado environments while accounting for multiple-level relatedness of individuals.

## 2. Experimental

### 2.1. Plant materials

A population of 96 hard red and hard white winter wheat cultivars and advanced lines was evaluated in this study. These included 87 entries chosen from a larger set of wheat germplasm released or developed since 1991 in the Hard Winter Wheat Region of the US Great Plains and which were mostly included in the glutenin survey by Shan et al. (2007). Based on pedigree information, we attempted to avoid including sister lines or other closely related materials. In addition, eight entries from Eastern Europe and Central Asia were included (Supplementary Table S1).

### 2.2. Field experiments

The 96 genotypes were grown at three locations in the north-east quadrant of Colorado in the 2005–2006 growing season: the Agricultural Research, Development and Education Center of Colorado State University (CSU), Fort Collins; the Central Great Plains Research Station, USDA Agricultural Research Service, Akron; and a farmer's field near Dailey. At all locations, the fields were arranged in an alpha-lattice incomplete block design (Patterson et al., 1978) with two replications, and each plot consisted of two rows with 25 cm spacing between rows. Plots at Fort Collins were 3.4 m long, and plots at Akron and Dailey were 3.7 m long. Ten grams of seed per plot were planted in each trial. The trial at Fort Collins was irrigated with a linear overhead sprinkler system as needed to maintain optimum moisture conditions until three weeks before harvest. Akron and Dailey were rainfed locations with no additional water applied to those trials. All plots were hand harvested and threshed separately. There was very little difference in growing season temperature among the three locations according to the CoAgMet weather database (<http://ccc.atmos.colostate.edu/~coagmet/>, verified on June 25, 2009). Of the rainfed locations, Dailey received twice as much precipitation as Akron in May (10.5 and 4.5 cm, respectively), coinciding with anthesis and early grain filling, a critical time for wheat grain development.

### 2.3. Phenotypic evaluation

#### 2.3.1. Agronomic traits

Phenotypic data were recorded or calculated for each plot for grain yield, kernel weight, days to heading, and grain fill duration. The single kernel weight was estimated using a 200-kernel subsample from each plot. Days to heading was the number of days from January 1, 2006 to the day on which 50% of the spikes were fully visible above the flag leaf collar. Grain fill duration was determined by subtracting days to heading from the number of days from January 1, 2006 to the date when 50% of peduncles showed complete loss of green color.

#### 2.3.2. Mixograph properties

Fifty grams of seed from each plot were milled to flour with a Brabender Quadrumat<sup>®</sup> Jr. Mill. (C. W. Brabender Instruments, Inc., South Hackensack, NJ) following AACC Method 26-50 (AACC, 2004). Dough-mixing properties were analyzed for each plot with a 10 g-Mixograph at room temperature, according to the approved AACC method 54-40A (AACC, 2004). Mixograph characteristics were determined with the software program Mixsmart<sup>®</sup> version

3.80 (AEW Consulting, Lincoln, NE, commercially available through National Manufacturing Division of TMCO, Lincoln NE, USA). An explanation of the Mixogram output was presented by Bordes et al. (2008). Briefly, all mixing characteristics were measured from the center line (Mixograph midline) of the mixogram, and all characteristics except mixing time were reported on a 100Mixograph unit scale (% height). Mixograph midline peak time (MPT) was visually determined. Other characteristics were automatically estimated by Mixsmart<sup>®</sup>, including midline peak (height) value (MPV), midline peak width (MPW), midline right value (height) at two minutes after peak (MRV), right width at two minutes after peak (MRW), and midline right slope (MRS) calculated as  $(MRV-MPV)/2$ , as shown in the mixogram (Fig. 1, Bordes et al., 2008). The optimum MPT for hard red winter wheat is 3–6 min, and the target for mixing tolerance is at least 3 on a 7-point scale (0–6), as suggested in the report by Chen and Seabourn (2006). For this population, higher values of MPT, MPW, MRW, and MRS and lower value of MPV are considered desirable for bread-making quality.

## 2.4. Genotypic evaluation

### 2.4.1. Glutenin analysis

The glutenin proteins from each entry (cultivar or advanced line) were extracted and analyzed by SDS-PAGE according to the protocols described by Shan et al. (2007). Ten random seeds from each genotype were bulked for each extraction. For each genotype, loci with protein bands corresponding to more than one allele were scored as the allele with the higher intensity. The only exception was for *Glu-A1a* and *b* whose intensities were very similar; these were scored as having both alleles present and denoted as *Glu-A1a/b*. Assignment of alleles at *Glu-A3* and *Glu-B3* was sometimes problematic in cases where the cultivar contained a 1AL.1RS or 1BL.1RS translocation, respectively. If a translocation replaces the whole short arm of the respective wheat chromosome, then no *Glu-A3* or *Glu-B3* locus would be present. However, we detected bands for *Glu-B3* alleles in 13 1BL.1RS cultivars, indicating most likely that these lines were heterogeneous for the 1RS translocation. For *Glu-A3*, only seven of 17 lines scored as having the *Glu-A3e* (null) allele also carried the 1AL.1RS translocation and we were unable to resolve that potential discrepancy.

### 2.4.2. Simple-sequence repeats (SSR) marker analysis

DNA was extracted from bulked leaves of 10 seedlings of each entry using the method described by J.A. Anderson, Department of Agronomy and Plant Genetics, University of Minnesota (<http://maswheat.ucdavis.edu/PDF/DNA0002.pdf>, verified on June 25, 2009). SSR primers were selected based on the map of Somers et al. (2004). For each chromosome, we chose at least three SSR markers (one in each telomere region and one in the centromere region) for testing, and had the primers synthesized according to information available in the GrainGenes database (<http://wheat.pw.usda.gov/GG2/index.shtml>, verified on June 25, 2009). A total of 63 SSR markers were applied to the 96 entries. The presence of the 1AL.1RS and 1BL.1RS translocations was determined with the rye-specific SSR marker 'SCM9' (Saal and Wricke, 1999).

All SSR genotyping was carried out in the USDA Small Grain Genotyping Laboratory in Manhattan, KS, following the method of Chen et al. (2008) with some modifications. In brief, a 13  $\mu$ l polymerase chain reaction (PCR) mixture contained 50–150 ng/ $\mu$ l DNA template, 200 mM of dNTP, 1  $\times$  ASB buffer (Bioline USA Inc. Taunton, MA), 2.5 mM MgCl<sub>2</sub>, 1 U *Taq* polymerase, 100 nM forward primer, 300 nM of reverse primer, and 200 nM fluorescence dye-labeled M13 primer compatible with an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). The PCR were conducted in an MJ-PTC200 Cycler (Biorad, Hercules, CA) and detected by an ABI

3730 DNA analyzer. The marker data were scored using GeneMarker<sup>®</sup> version 1.5 (SoftGenetics<sup>®</sup>, LLC, State College, PA). Markers producing a single band were preferred. If markers produced two well-separated bands, those markers were scored as independent loci. A total of 69 SSR loci were scored from these SSR markers, including one locus each from 57 markers and two loci each from the other six markers.

## 2.5. Data analysis

### 2.5.1. Multiple levels of relatedness of 96 entries

To minimize spurious associations, the mixed model (Q+K) (Yu et al., 2006) was used to account for population structure and cousin-level relatedness of individuals among 96 entries. The genetic structure among the 96 entries was inferred with the 60 SSR loci that had a maximum of 16 missing values, using the admixture and correlated allele frequency options in the program STRUCTURE version 2.2 (Pritchard et al., 2000). All the alleles of these SSR markers were used to estimate the population structure. The *a priori* subpopulation number (*k*) was estimated with the program StructuRAMA (Huelsenbeck and Andolfatto, 2007) with the same set of 60 SSR loci used for the STRUCTURE analysis. The estimated *k* was eight. The Q-matrix was estimated in STRUCTURE with 60 SSR markers, eight subpopulations, and one million 'burn-in' periods, followed by two million Markov Chain Monte Carlo iterations. The Q-matrix was visualized with the program Distruct (Rosenberg, 2004). The relative kinship (*K*) matrix (*K* being distinct from the *a priori* subpopulation number *k*) was calculated on the basis of 69 SSR loci using the method proposed by Ritland (1996), which is built into the program SPAGeDi (Hardy and Vekemans, 2002).

### 2.5.2. Least squares means, correlation, and association analysis

Data were analyzed with SAS software version 9.2 (SAS Institute, Cary, NC). First, exploratory association analysis was performed on Mixograph properties combining data from the three locations. This analysis revealed highly significant ( $P < 0.0001$ ) genotype  $\times$  environment interactions between most of the glutenin loci and growing locations (data not shown). Thus, our data were analyzed and are presented separately by location. Second, least squares means of all traits at each location were estimated with the MIXED procedure of SAS. In the model, 'entry' was treated as a fixed effect and 'incomplete block' as a random effect. Third, homogeneity of error variance of Mixograph properties was evaluated by the Kolmogorov–Smirnov test and visually with residual plots. The expected and residual values used in residual plots were obtained from the MIXED procedure for calculating least square means. MPW and MRW were not consistent with an acceptable level of homogeneity of error variance across the three locations, so they were  $\log_e(\ln)$  transformed to achieve homogeneous variance in all the three environments. The results were back transformed to original units for presentation purposes. Fourth, the association of glutenin loci and 1RS translocations with Mixograph properties was tested with the Q+K model (Yu et al., 2006). The SAS code for the Q+K model was adapted from E. Buckler (USDA-ARS, Plant, Soil and Nutrition Research Unit, Ithaca, NY, <http://www2.maizegenetics.net/images/stories/interests/qk.txt>, verified on June 25, 2009). The subpopulations together with one glutenin locus or 1RS translocation were treated as fixed effects, 'incomplete block' as a random effect, and 'entry' as a random effect with the *K*-matrix defining the degree of genetic covariance between a pair of individuals. The LSMEAN statement in the MIXED procedure was used to estimate the least squares means for each allele of the glutenin loci and 1RS status. Henceforth, we will use "mean" to refer to the least squares mean. The means for different allelic classes at

glutenin loci or presence–absence of 1RS were compared with Tukey–Kramer multiple-range tests.

### 3. Results

#### 3.1. Trait means

Wheat from the irrigated location, Fort Collins, had higher values ( $P < 0.05$ ) of most agronomic traits (grain yield, single kernel weight, and grain fill duration) compared to those from the rainfed locations, Dailey and Akron (Table 1). Wheat from Akron had the highest grain protein concentration (170 g/kg), followed by those from Fort Collins (165 g/kg), and Dailey (142.7 g/kg). The MPT for the three locations were in the order of Dailey > Akron > Fort Collins ( $P < 0.001$ ). Wheat from Fort Collins had wider MPW than those from Dailey and Akron ( $P < 0.001$ ), whereas wheat from Dailey had a wider MRW than those from Fort Collins and Akron ( $P < 0.001$ ). Wheat from Dailey had lower MPV and higher MRS than Fort Collins and Akron ( $P < 0.001$ ), indicating that flours from wheat grown at Dailey were more tolerant to mixing. Significant ( $P < 0.05$ ) differences among the entries were found for each trait at each location (data not shown) except single kernel weight at Fort Collins and grain fill duration at Akron.

#### 3.2. Glutenin allelic and 1RS diversity

A high level of diversity was observed for most glutenin loci in this set of materials (Tables 2–4). The most common alleles at different glutenin loci were *Glu-A1b*, *Glu-B1b* and *c*, *Glu-D1d*, *Glu-A3c*, *Glu-B3g*, and *Glu-D3a*, *b*, and *c*. Some alleles were present in less than three entries, including *Glu-B1a* (one entry), *Glu-D1e* (one entry), *Glu-A3b* (two entries), and *Glu-B3d* (one entry). These rare alleles were not included in the analysis to reduce the level of multiple comparisons. There were four classes of entries with regard to the presence of 1RS translocations (Tables 2–4). Sixty-five entries had no rye translocation (non-1RS), 17 entries had a 1AL1RS translocation, 13 entries had a 1BL1RS translocation, and one entry (Infinity) was found to have both 1AL1RS and 1BL1RS translocations. Because Infinity was the only entry in this class, it was excluded from the analysis for 1RS effects. When the genotypic data were analyzed with the program STRUCTURE, population stratification of the 96 wheat cultivars was obvious (Supplementary Fig. S1). The entries formed eight hypothetical ancestral groups. Most entries were not assigned to a single group, but to different proportions of multiple groups.

**Table 1**  
Least squares means and ranges of Mixograph properties and agronomic traits of 96 wheat genotypes grown in three Colorado locations in the 2005–2006 growing season ( $n = 96$  except KW and SPH from Dailey, for which  $n = 95$ ).

Variable <sup>a</sup>	Fort Collins		Akron		Dailey		LSD <sub>0.05</sub> <sup>b</sup>
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	
Grain yield (kg/ha)	2867 ± 655	1151–4365	1313 ± 308	726–2046	1440 ± 270	379–2082	127
Kernel weight (mg)	28.5 ± 2.4	20.2–34.1	22.7 ± 2.5	16.9–28.9	25.0 ± 2.5	19–31.3	0.7
Days to heading (days)	141.2 ± 1.5	138.5–145.4	142.7 ± 1.5	140.1–146.8	142.3 ± 1.7	138.7–147.4	0.4
Grain filling duration (days)	26.3 ± 1.4	23.2–30.2	23.2 ± 1.1	20.6–26.4	23.4 ± 1.2	20.4–26.2	0.3
Grain protein (g/kg)	165.1 ± 8.9	135.4–183.6	170.1 ± 5.7	157.5–187.2	142.7 ± 8	118.0–159.4	2.2
MPT (min)	2.9 ± 0.6	1.5–4.7	3.3 ± 0.8	1.8–5.1	4.6 ± 0.9	2.6–7.1	0.2
MPW (% height <sup>c</sup> )	21.6 ± 3.7	13.3–33.8	19.1 ± 3.6	12.6–30.7	19.1 ± 3.1	10.5–27.5	1.0
MRW (% height)	10.8 ± 3.4	5.3–22.5	10.5 ± 3.4	5.1–22.2	15 ± 3.5	5.4–23.7	1.0
MPV (% height)	50.6 ± 4.2	41.3–60.7	48.6 ± 4.1	38.1–61.6	43.5 ± 3.9	34.5–57.1	1.2
MRS (% height/min)	−3.8 ± 1.1	−6.1 to −1.3	−3.2 ± 1.1	−5.6 to −1	−1.4 ± 0.8	−4.1 to −0.5	0.3

<sup>a</sup> SD, standard deviation; MPT, Mixograph peak time; MPW, Mixograph band width at MPT; MRW, Mixograph band width at two minutes after MPT; MPV, Mixograph midline peak value; MRS, Mixograph midline right slope, calculated by (Mixograph right value at two minutes after MPT-MPV)/2.

<sup>b</sup> LSD<sub>0.05</sub>, Fisher's least significant difference among environments at  $P < 0.05$ , calculated based on the SAS PROC MIXED analysis of variance table.

<sup>c</sup> % height, Mixograph units in 100-unit scale.

**Table 2**

Least squares means of Mixograph properties for glutenin allelic classes and 1RS translocations of 96 wheat genotypes at Akron, CO, in the 2005–2006 growing season.

Loci	Allele <sup>A</sup>	<i>n</i>	Mixograph properties <sup>B</sup>				
			MPW <sup>C</sup>	MRW	MPT	MPV	MRS
<i>Glu-A1</i>	<i>a</i>	14	19.1a	10.3a	3.2b	48.3ab	−3.0a
	<i>a/b</i>	8	18.8a	11.0a	3.9ab	50.2a	−3.1a
	<i>b</i>	71	18.6a	9.8a	3.4ab	48.7a	−3.2a
	<i>c</i>	3	16.0a	9.3a	4.0a	43.9b	−2.4a
<i>Glu-B1</i>	<i>b</i>	33	19.0a	10.2a	3.5a	48.2a	−2.9b
	<i>c</i>	38	18.4a	10.2a	3.6a	48.8a	−3.1b
	<i>e</i>	10	18.9a	8.7a	2.7b	51.0a	−4.1a
	<i>i</i>	7	19.6a	10.1a	3.1ab	48.8a	−3.5ab
	<i>w</i>	7	17.3a	10.1a	3.7a	48.2a	−3.0ab
<i>Glu-D1</i>	<i>a</i>	10	19.5ab	11.6a	2.7b	50.9a	−3.6a
	<i>b</i>	6	22.4a	11.5a	3.2ab	50.2a	−3.0a
	<i>d</i>	79	18.2b	10.0a	3.4a	48.2a	−3.1a
<i>Glu-A3</i>	<i>a</i>	14	18.2a	9.7a	3.2ab	47.8a	−3.6a
	<i>c</i>	51	19.1a	9.7a	3.2b	49.3a	−3.3a
	<i>d</i>	7	19.2a	9.9a	3.8ab	49.0a	−3.1ab
	<i>e</i>	18	18.2a	11.1a	3.9a	47.7a	−2.5b
	<i>g</i>	4	17.6a	9.0a	2.9ab	47.0a	−3.1ab
<i>Glu-B3</i>	<i>a</i>	3	15.8a	13.1a	3.8ab	43.3bc	−1.6b
	<i>b</i>	14	17.8a	11.1a	3.9a	48.8ab	−2.8ab
	<i>c</i>	5	16.9a	5.7b	2.8b	41.9c	−3.4ab
	<i>e</i>	8	19.4a	10.9a	3.1ab	49.3ab	−3.4ab
	<i>f</i>	11	20.4a	10.3a	3.2ab	51.2a	−3.3ab
	<i>g</i>	40	18.9a	9.9a	3.3ab	48.8ab	−3.3ab
	<i>h</i>	4	18.6a	10.0a	2.8b	51.0a	−4.3a
	<i>i</i>	10	18.4a	9.5a	3.0b	48.2ab	−3.2ab
	<i>g</i>	40	18.9a	9.9a	3.3ab	48.8ab	−3.3ab
<i>Glu-D3</i>	<i>a</i>	30	18.6a	9.5a	3.4ab	48.2a	−3.3a
	<i>b</i>	27	18.2a	10.6a	3.7a	48.3a	−3.0a
	<i>c</i>	28	18.5a	10.3a	3.3ab	49.2a	−3.1a
	<i>d</i>	6	18.6a	8.8a	2.8b	50.3a	−3.6a
	<i>e</i>	5	21.9a	10.9a	3.3ab	50.0a	−3.0a
1RS	1AL1RS	17	18.0ab	9.1b	3.2a	48.4a	−3.3a
	1BL1RS	13	16.8b	7.6c	3.1a	43.2b	−3.0a
	Non-1RS	65	19.2a	10.6a	3.4a	49.8a	−3.3a

<sup>A</sup> Subunit designations for the HMW-GS loci are *Glu-A1*: *a*, 1; *b*, 2\*; *c*, null; *Glu-B1*: *b*, 7+8; *b*, 7+9; *e*, 20x+20y; *i*, 17+18; *w*, 6\*+8\*; *Glu-D1*: *a*, 2+12; *b*, 3+12; *d*, 5+10.

<sup>B</sup> Means followed by different letters indicate a significant difference at the  $P < 0.05$  level.

<sup>C</sup> MPT, Mixograph peak time; MPW, Mixograph band width at MPT; MRW, Mixograph band width at two minutes after MPT; MPV, Mixograph midline peak value; MRS, Mixograph midline right slope, calculated by (Mixograph right value at two minutes after peak-MPV)/2.

**Table 3**

Least squares means of Mixograph properties for glutenin allelic classes and 1RS translocations of 96 wheat genotypes at Dailey, CO, in the 2005–2006 growing season.

Loci	Allele	n	Mixograph properties <sup>A</sup>				
			MPW <sup>B</sup>	MRW	MPT	MPV	MRS
<i>Glu-A1</i>	<i>a</i>	14	18.6a	13.4a	4.8a	42.4a	−1.4a
	<i>a/b</i>	8	18.7a	15.3a	4.5a	41.7a	−0.7a
	<i>b</i>	71	19.0a	14.7a	4.5a	44.1a	−1.6a
	<i>c</i>	3	16.0a	14.0a	5.0a	40.9a	−0.7a
<i>Glu-B1</i>	<i>b</i>	33	19.1a	15.5a	4.8a	43.4a	−1.2a
	<i>c</i>	38	18.8a	14.7a	4.6a	43.7a	−1.3a
	<i>e</i>	10	19.1a	13.2a	3.9a	44.8a	−1.8a
	<i>i</i>	7	18.4a	13.4a	4.2a	44.6a	−1.8a
	<i>w</i>	7	17.7a	13.9a	5.0a	41.1a	−0.8a
	<i>d</i>	79	18.4b	14.4a	4.7a	42.9b	−1.2b
<i>Glu-D1</i>	<i>a</i>	10	21.0a	14.8a	3.7b	46.2a	−2.1a
	<i>b</i>	6	21.4a	14.0a	3.8b	46.2a	−1.9ab
	<i>d</i>	79	18.4b	14.4a	4.7a	42.9b	−1.2b
	<i>a</i>	14	18.8a	13.5a	4.3a	44.2a	−1.6a
	<i>c</i>	51	19.0a	14.4a	4.5a	44.0a	−1.4a
	<i>d</i>	7	20.3a	14.8a	4.6a	42.6a	−1.2a
<i>Glu-A3</i>	<i>e</i>	18	18.2a	15.5a	5.0a	42.7a	−0.9a
	<i>g</i>	4	18.1a	13.7a	4.7a	43.4a	−2.0a
	<i>a</i>	3	16.5a	16.3a	5.2a	38.1b	−0.6a
	<i>b</i>	14	20.1a	16.2a	5.0a	43.8ab	−1.2a
	<i>c</i>	5	18.0a	8.7b	4.1a	41.4ab	−2.0a
	<i>e</i>	8	19.0a	14.2a	4.6a	43.6ab	−1.5a
<i>Glu-B3</i>	<i>f</i>	11	19.3a	15.3a	4.5a	45.7a	−1.6a
	<i>g</i>	40	18.8a	14.6a	4.5a	43.5ab	−1.3a
	<i>h</i>	4	18.2a	12.6ab	4.1a	46.7a	−2.0a
	<i>i</i>	10	18.8a	13.6a	4.1a	43.8ab	−1.6a
	<i>a</i>	30	18.6a	14.1a	4.7a	43.5a	−1.6ab
	<i>b</i>	27	19.2a	14.9a	4.7a	43.6a	−1.3a
	<i>c</i>	28	18.6a	15.4a	4.4a	44.3a	−1.3a
	<i>d</i>	6	18.7a	12.3a	4.3a	41.7a	−3.0b
<i>e</i>	5	21.2a	15.2a	4.8a	44.4a	−1.3ab	
1RS	1AL1RS	17	18.3a	14.0a	4.3a	44.3a	−1.3a
	1BL1RS	13	16.4b	10.6b	4.3a	40.5b	−1.5a
	Non-1RS	65	19.5a	15.3a	4.6a	44.1a	−1.4a

<sup>A</sup> Means followed by different letters indicate a significant difference at the  $P < 0.05$  level.

<sup>B</sup> Trait abbreviations are the same as for Table 2.

### 3.3. Relationship of glutenin loci and 1RS translocations with dough-mixing properties

We tested the association of the six glutenin loci and the 1RS translocations with Mixograph properties by using the Q+K model (Yu et al., 2006) and a basic model (not accounting for Q+K). The Q+K model gave better model fit as indicated by lower Akaike Information Criterion (AIC) values (data not shown). The Q+K model reduced the significance levels of most associations in the basic association model (Table 5). For example, *Glu-D3* was significantly ( $P < 0.05$ ) associated with MPW, MRW, and MPV at Fort Collins in the basic model, whereas none of these associations was significant in the Q+K model. On the other hand, the Q+K model detected additional significant ( $P < 0.05$ ) associations among glutenin loci and Mixograph properties which were not significant with the basic model. For example, in the Q+K model, there were significant ( $P < 0.05$ ) associations between *Glu-D1* and MPW and *Glu-A3* and MPT at Dailey and between *Glu-A1*, *Glu-A3*, and *Glu-D3* and MPT at Akron. These associations were not significant in the basic model. Hereafter, only the results from the Q+K model are presented.

All six glutenin loci and translocation status were associated with Mixograph properties, with *Glu-D1*, *Glu-B3*, and 1RS status

**Table 4**

Least squares means of Mixograph properties for glutenin allelic classes of 96 wheat genotypes at Fort Collins, CO, in the 2005–2006 growing season.

Loci	Allele	n	Mixograph properties <sup>A</sup>				
			MPW <sup>B</sup>	MRW	MPT	MPV	MRS
<i>Glu-A1</i>	<i>a</i>	14	21.3a	9.7a	3.0a	50.5a	−3.5a
	<i>a/b</i>	8	20.2a	12.4a	3.5a	50.4a	−2.9a
	<i>b</i>	71	21.2a	10.2a	2.9a	50.8a	−3.8a
	<i>c</i>	3	20.6a	9.3a	3.2a	48.6a	−3.5a
<i>Glu-B1</i>	<i>b</i>	33	21.6a	11.0a	3.0a	50.1a	−3.4b
	<i>c</i>	38	20.8a	10.0a	3.0a	51.1a	−3.8ab
	<i>e</i>	10	19.5a	8.6a	2.2b	50.7a	−4.8a
	<i>i</i>	7	21.5a	10.6a	2.8ab	49.5a	−3.9ab
	<i>w</i>	7	21.9a	11.0a	3.4a	52.2a	−3.0ab
	<i>d</i>	79	21.0a	10.5a	3.0a	50.1a	−3.5b
<i>Glu-D1</i>	<i>a</i>	10	21.1a	9.3a	2.4b	52.1a	−4.6a
	<i>b</i>	6	22.3a	9.6a	2.6ab	51.6a	−4.3ab
	<i>d</i>	79	21.0a	10.5a	3.0a	50.1a	−3.5b
<i>Glu-A3</i>	<i>a</i>	14	21.0a	9.7a	2.8a	50.3a	−4.0a
	<i>c</i>	51	21.1a	9.9a	2.9a	51.6a	−3.9a
	<i>d</i>	7	21.8a	11.0a	3.0a	48.8a	−3.3a
	<i>e</i>	18	20.8a	11.3a	3.2a	49.7a	−3.3a
	<i>g</i>	4	22.4a	10.0a	2.4a	48.0a	−3.9a
	<i>d</i>	79	21.0a	10.5a	3.0a	50.1a	−3.5b
<i>Glu-B3</i>	<i>a</i>	3	21.7a	14.0a	3.3ab	47.8ab	−2.1b
	<i>b</i>	14	22.3ab	11.7a	3.2a	50.2a	−3.2ab
	<i>c</i>	5	15.9c	6.7b	2.3b	42.8b	−4.1ab
	<i>e</i>	8	22.1ab	11.5a	2.9ab	52.4a	−3.9ab
	<i>f</i>	11	23.3a	10.7a	2.9ab	53.8a	−4.1a
	<i>g</i>	40	20.8ab	9.9a	2.9ab	50.5a	−3.7ab
	<i>h</i>	4	24.7a	9.8ab	2.8ab	53.3a	−4.6a
	<i>i</i>	10	19.3bc	9.1ab	2.5b	50.8a	−4.3a
	<i>a</i>	30	21.0a	10.0a	3.0a	50.8a	−3.9a
	<i>b</i>	27	21.0a	10.7a	3.1a	50.1a	−3.4a
<i>Glu-D3</i>	<i>c</i>	28	21.8a	10.7a	2.9a	51.3a	−3.7a
	<i>d</i>	6	20.7a	9.3a	2.4a	49.9a	−4.0a
	<i>e</i>	5	19.2a	9.3a	2.8a	50.7a	−4.1a
	1AL1RS	17	19.8b	8.9b	2.8a	49.5b	−3.9a
	1BL1RS	13	17.6c	8.2b	2.8a	45.8c	−3.6a
Non-1RS	65	22.1a	10.9a	2.9a	51.8a	−3.8a	

<sup>A</sup> Means followed by different letters indicate a significant difference at the  $P < 0.05$  level.

<sup>B</sup> Trait abbreviations are the same as for Table 2.

affecting most traits at all the three locations (Table 5). The associations among genetic factors and Mixograph properties were highly influenced by wheat-growing environment (Table 5). The associations detected at the irrigated location, Fort Collins, were very similar to those of the rainfed location Akron, but different from the rainfed location Dailey. Results for specific loci are described in the following paragraphs.

#### 3.3.1. *Glu-A1*

*Glu-A1c* was associated with higher ( $P < 0.05$ ) MPT than *Glu-A1a*, and lower ( $P < 0.05$ ) MPV than *Glu-A1b* and *a/b* at Akron, but not at other locations (Tables 2–4). *Glu-A1a*, *b*, and *a/b* were not significantly different from each other for any of the Mixograph properties.

#### 3.3.2. *Glu-B1*

Variation at the *Glu-B1* locus was significantly associated with MPT ( $P < 0.01$ ) at Fort Collins and Akron (Tables 2 and 4). This was mainly because *Glu-B1e* produced lower MPT than *Glu-B1b*, *c*, and *w*. In addition, *Glu-B1e* was associated with a more negative MRS than *Glu-B1b* at Akron and Fort Collins, and therefore is an undesirable allele at the *Glu-B1* locus. In this first study of the effects of *Glu-B1w*, we observed that *Glu-B1w* had similar values for

**Table 5**  
The effects of glutenin loci and the presence of 1RS translocations on Mixograph properties of 96 wheat genotypes at Fort Collins, Dailey, and Akron, CO, in the 2005–2006 growing season.

Location	Loci	Basic model (not adjusted for Q+K)					Q+K model				
		MPW <sup>a</sup>	MRW	MPT	MPV	MRS	MPW	MRW	MPT	MPV	MRS
Akron	<i>Glu-A1</i>				*,b				**	*	
	<i>Glu-B1</i>			***		***		***		*	
	<i>Glu-D1</i>	*		***	*	*	*	*			
	<i>Glu-A3</i>				**	***			**		*
	<i>Glu-B3</i>	*	***	***	***	**		***	**	***	*
	<i>Glu-D3</i>				*	*			*		
	1RS	**	***		***		*	***		***	
Dailey	<i>Glu-A1</i>				*						
	<i>Glu-B1</i>		**	**	*						
	<i>Glu-D1</i>			***	*		**		**	**	**
	<i>Glu-A3</i>				*			*		*	*
	<i>Glu-B3</i>		***	**	*	*		***		*	
	<i>Glu-D3</i>		*			*					*
	1RS	***	***	*	***	***	***	***		***	
Fort Collins	<i>Glu-A1</i>		** <sup>b</sup>	*		*					
	<i>Glu-B1</i>			*					***		
	<i>Glu-D1</i>		**	***		***		**		**	**
	<i>Glu-A3</i>				**	**					
	<i>Glu-B3</i>	***	***	***	***	*	***	***	*	***	**
	<i>Glu-D3</i>	*	**	*	*	*					
	1RS	***	***		***		***	***		***	

Results from the basic model are to the left and those from the Q+K model are to the right.

\*, \*\*, \*\*\*, *F* test significant at 0.05, 0.01, and 0.001 levels, respectively; empty cells indicate not significant ( $P > 0.05$ ) in the *F* test.

<sup>a</sup> Trait abbreviations are the same as for Table 2.

<sup>b</sup> Cells shaded gray in the basic model indicate that the strength of associations was reduced in the Q+K model. Cells shaded black in the Q+K model indicate the strength of associations was increased in the Q+K model.

all Mixograph traits compared to other *Glu-B1* alleles, except for MPT, which tended to be higher. Other alleles were not significantly different from each other for any of the Mixograph properties.

### 3.3.3. *Glu-D1*

*Glu-D1d* was associated with a higher MPT ( $P < 0.01$ ) than *Glu-D1a* at all the three locations and higher ( $P < 0.01$ ) MPT than *Glu-D1b* at Dailey. *Glu-D1d* was associated with lower MPW ( $P < 0.05$ ) than *Glu-D1b* at Akron and Dailey, and higher MRS than *Glu-D1a* at Fort Collins and Dailey (Tables 2–4).

### 3.3.4. *Glu-A3*

Variation at *Glu-A3* was significantly ( $P < 0.05$ ) associated with MPT and MRS at Akron (Tables 2 and 5). The allele *e* was associated with higher ( $P < 0.05$ ) MPT and MRS than allele *a* or *c* at Akron. The means of allelic classes for MPT were in the order of  $e > d > a > c > g$  at Akron (Tables 2–4).

### 3.3.5. *Glu-B3*

Variation at *Glu-B3* was significantly ( $P < 0.05$ ) associated with MRW, MPT, MPV, and MRS at Akron, MRW and MPV at Dailey, and all the properties at Fort Collins (Table 5). *Glu-B3c* was associated with lower ( $P < 0.05$ ) MRW than *Glu-B3a*, *b*, *e*, *f*, and *g* at all the three locations, lower MPV ( $P < 0.01$ ) than most other *Glu-B3* alleles at Akron and Fort Collins, and lower MPW ( $P < 0.01$ ) than most other *Glu-B3* alleles at Fort Collins except *Glu-B3i* (Tables 2–4). *Glu-B3i* produced lower MPT ( $P < 0.05$ ) than *Glu-B3b* at Akron and Fort Collins (Tables 2 and 4). *Glu-B3h* was associated with the most negative MRS among *Glu-B3* alleles at Akron and Fort Collins (Tables 2 and 4).

### 3.3.6. *Glu-D3*

The *Glu-D3d* allelic class was significantly ( $P < 0.05$ ) associated with shorter MPT than *Glu-D3b* at Akron and more negative

( $P < 0.05$ ) MRS than *Glu-D3b* and *c* at Dailey (Tables 2–4). Other alleles were associated with similar values for Mixograph properties across the three locations (Tables 2–4).

### 3.3.7. 1RS translocation

1BL.1RS was associated with the lowest values of MPW, MRW, and MPV at all the three locations (Tables 2–4). Non-1RS was associated with higher MPW, MRW, and MPV than 1AL.1RS at Fort Collins and higher MRW than 1AL.1RS at Akron.

## 4. Discussion

We used a candidate gene association analysis to study the HMW-GS, LMW-GS, and 1RS translocation effects on wheat quality as measured by the Mixograph. The analysis accounted for multiple-level relatedness with the unified mixed model suggested by Yu et al. (2006). Of 64 trait–locus associations that were significant ( $P < 0.05$ ) in the basic model, 52 showed a reduced strength of association when the Q+K model was applied (Table 5). Many of these were presumably spurious associations or showed inflated significance levels due to the relatedness among some of the entries. In a smaller number of cases (11), the significance of the association was increased with the Q+K model compared to the basic model, suggesting that false negative as well as false positive associations can be detected in the absence of adjustment for population structure (Table 5).

Population stratification in wheat has been observed in the previous studies (Brescghello and Sorrells, 2006; Ravel et al., 2006). Cane et al. (2008) were aware that the relatedness of cultivars might influence the association between alleles and quality traits, so they included a relationship matrix generated from pedigree information in the analysis to minimize bias. However, when they compared the effects of glutenin allelic classes with the results published earlier on a similar dataset (Eagles et al., 2006), the

results were nearly the same. They concluded that a large dataset ( $n = 899$ ) minimized the influence of relationship among entries for the association between glutenin alleles and quality traits. In our smaller study, the 96 wheats were from eight hypothetical ancestral groups, with most entries showing a high level of admixture (Supplementary Fig. S1). This study demonstrated the importance of accounting for multiple-level relatedness of individuals when studying the quality effects of the glutenin loci in collections of breeding lines and cultivars.

Our results confirmed that the HMW-GS, LMW-GS, and 1RS translocations play important roles in determining dough-mixing properties. Among them, 1RS translocations, and the *Glu-B3* and *Glu-D1* loci were of major importance in this set of genotypes, as discussed in detail below.

#### 4.1. *Glu-A1*

Variation at *Glu-A1* played a minor role in accounting for variation in dough-mixing properties. In this population, most entries carried *Glu-A1a*, *b*, or both, and those alleles had equivalent effects. This is in agreement with the previous studies on materials from Australia (Bekes et al., 2001; Eagles et al., 2002; Ma et al., 2005) and France (Branlard et al., 2001). However, Moonen et al. (1983) found that *Glu-A1b* was associated with higher loaf volume and SDS sedimentation volumes than *Glu-A1a* in wheat grown in the Netherlands. There was some indication that *Glu-A1c*, the null allele, was inferior in dough strength compared to *Glu-A1a* and *Glu-A1b* as shown by lower MPW and MRW. However, only three of the 96 entries in our study carried *Glu-A1c*.

#### 4.2. *Glu-B1*

To the best of our knowledge, the quality effects of *Glu-B1w* have not been previously reported. In our study, the seven entries with *Glu-B1w* also carried *Glu-D1d*. To minimize the influence of *Glu-D1d* on the results for *Glu-B1w*, we compared *Glu-B1w* to other *Glu-B1* alleles within the set of 79 lines carrying *Glu-D1d*. The results showed that *Glu-B1w* was associated with higher MPV, and lower MRW, MRS, and MPT than *Glu-B1b* and *c* at all the three locations (Supplementary Table S2). Compared to *Glu-B1e*, *Glu-B1w* was associated with higher MPW, MRW, MPT, and MRS at all the three locations (Supplementary Table S2). However, none of these associations was significant at  $\alpha = 0.05$ . In general, *Glu-B1w* could be considered slightly inferior to *Glu-B1b* and *c*, but better than *Glu-B1e* for dough-mixing properties. The frequency of *Glu-B1w* in US wheat is low, only 5% in 111 US wheat cultivars released after 1991 in the US Great Plains (Shan et al., 2007).

*Glu-B1e* appeared to be an inferior allele compared to other alleles at the *Glu-B1* locus for Mixograph properties, especially for MPT (Tables 2–4). This is in agreement with the score of Payne et al. (1987) and previous reports for bread wheat (Cane et al., 2008; Eagles et al., 2004; Ma et al., 2005) and durum wheat (Brites and Carrillo, 2001; Martinez et al., 2005).

#### 4.3. *Glu-D1*

We confirmed the beneficial effects of *Glu-D1d* over *Glu-D1a* (Tables 2–4). This agreed with the gluten-quality score (Payne et al., 1987) and many other studies (Bekes et al., 2001; Eagles et al., 2002; He et al., 2005; Huang et al., 2006). Selection for improved bread-making quality in breeding programs might explain why the ratio between *Glu-D1d* and *Glu-D1a* changed from 3:2 in US hard winter wheat cultivars released between 1970 and 1990 compared to 4:1 in those released from 1991 to 1996 (Graybosch, 1992; Shan et al., 2007).

#### 4.4. *Glu-A3*

*Glu-A3e*, the null allele, was a favorable allele for bread-making quality because there was a trend for *Glu-A3e* to be associated with higher MRS, MRW, and MPT than other *Glu-A3* alleles at all the three locations, though not all differences reached a significance level of  $P < 0.05$  (Tables 2–4). However, this differs from a previous report in which *Glu-A3e* was associated with a lower extensibility and Rmax than *Glu-A3d*, *b*, and *c* (Cane et al., 2008; Eagles et al., 2002).

#### 4.5. *Glu-B3*

Our study confirmed the important role of the *Glu-B3* locus for dough-mixing properties. *Glu-B3c* was associated with low Mixograph properties at all the locations. However, *Glu-B3c* was present in only five of the 96 entries (Wendy, SD97538, Cougar, Rowdy, and Postrock) and four of these entries carried 1BL1RS. To minimize the influence of 1BL1RS, we compared lines with *Glu-B3c* to lines with other alleles at the *Glu-B3* locus within a subgroup of 13 lines having 1BL1RS. We observed that *Glu-B3c* was consistently associated with low values of Mixograph properties in all the three environments; however, the *t*-tests were not all significant at  $\alpha = 0.05$  due to the limited number of entries (Supplementary Table S3). Inferior quality effects of *Glu-B3c* are in agreement with many previous studies (Bekes et al., 2001; Branlard et al., 2001; Cane et al., 2008; Eagles et al., 2002; Ma et al., 2005).

#### 4.6. *Glu-D3*

The five alleles at the *Glu-D3* locus produced similar values for Mixograph properties in our study. The only significant differences observed were for higher MPT for *Glu-D3b* compared to *Glu-D3d* at Akron, and more negative MRS for *Glu-D3d* compared to *Glu-D3b* and *c* at Dailey. Other studies have shown that the *Glu-D3* alleles had similar Rmax and extensibility in a large collection of wheats (Branlard et al., 2001; Cane et al., 2008; Eagles et al., 2002). These results indicate that *Glu-D3* would have the lowest priority among glutenin loci in which to invest efforts to improve wheat quality because of its minor role in determining quality.

Lack of independence between the six glutenin loci has been observed in other studies (Eagles et al., 2002; He et al., 2005). However, due to the low number of entries in our study, we were not comfortable in inferring any interaction effects, although it is likely there will be significant interactions among glutenin loci and among glutenin loci and other non-glutenin loci as previously reported (Eagles et al., 2002; Huang et al., 2006).

#### 4.7. 1RS translocation

In this study, 1BL1RS was clearly the more detrimental form of the 1RS translocation in all the three locations. Wheat with 1AL1RS was comparable to non-1RS wheat in most cases, but was inferior for three Mixograph properties at Fort Collins and one Mixograph property in Akron (Tables 2–4). The quality defects associated with the 1RS translocation may be the consequence of a change in the protein composition of the recipient wheats because incoming rye genes, such as secalin genes, are detrimental to wheat end-use quality. In addition, 1RS replaces the short arm of at least one wheat chromosome pair, leading to a permanent loss of some potentially important wheat genes, such as those encoding the LMW glutenins (Graybosch, 2001; Kumlay et al., 2003).

In conclusion, we have completed one of the few association analysis studies in wheat that has taken multiple-level relatedness into account. Controlling for population structure did indeed

reduce the number of significant associations, presumably including many that were false positives. Due to small sample size and unequal distribution of alleles in the population, we lacked the statistical power to distinguish alleles with similar quality effects. However, when comparisons were possible, our results were remarkably similar to those of the previous studies, indicating that candidate gene association analysis can be efficient in estimating the effects of glutenin alleles. Our results indicate that taking kinship into account can improve the results of association analysis for wheat-quality traits.

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## Appendix. Supplementary information

Supplementary information associated with this article can be found, in the online version, at doi:10.1016/j.jcs.2009.06.008.

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