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

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ABSTRACT

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

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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 *Sec1* 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 *Sec1* 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

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.

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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., 2008).

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-LS1* gene (coordinates 2652–2832 of emb|X04753.1) that was embedded within a 390 bp fragment of the coding region from the *Escherichia 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-LS1* intron. These sequences were transcriptionally fused for endosperm-specific expression to a 2933 bp fragment of the *1Dy10* gene promoter [beginning at the *Eco*RI site in GenBank accession X12929.2]. The transcription terminator from the CaMV 35*S* gene (coordinates 351-124 of gb|HQ593861.1) was added 3′ to the RNAi expression cassette, which was then cloned as an *Eco*RI 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 M 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 0.3 mM 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.

To obtain the flour samples used for both proteomics and small-scale quality analyses, sixteen pots containing 8 homozygous T_3 or T_2 plants from line L25 or L26, respectively, were grown on a single bench in the greenhouse with continuous fertilizer (20N:20P:20K). T_4 seeds for L25 and T_3 seeds for L26 were harvested, pooled and milled as described previously (Blechl et al., 2007). The resultant flours were assayed for protein content, SDS-sedimentation rates and in 2-g mixograph tests, as previously described (Blechl et al., 2007).

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

Protein spots were excised from the 2-D gels and placed into individual wells of reaction plates of a DigestPro (Itavis, Koeln, DE), where they were destained, reduced, alkylated, 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