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Variant high-molecular-weight glutenin subunits arising from biolistic transformation of wheat

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ABSTRACT

Genetic transformation *via* the biolistic method has been used to introduce genes encoding natural and novel high-molecular-weight glutenin subunits (HMW-GS) into wheat. The appearance of new seed proteins of sizes not predicted by the transgene coding sequences was noted in some experiments. In this report, the identities of thirteen of these novel proteins were determined by tandem mass spectrometry (MS/MS). Seven different proteins larger than and two proteins smaller than the native protein were shown to contain peptides from 1Dx5. A novel protein found in some progeny of crosses between a transgenic plant and Great Plains winter wheats was larger than but contained several peptides from 1Dy10. In one line, a protein larger than and a protein smaller than HMW-GS each contained peptides from the N- and C-terminus of 1Dx5 and from the repeat region of 1Dy10. In a sixth transgenic line, the native *Bx7* gene was apparently replaced by a gene that encodes a larger version of 1Bx7. The variant proteins accumulate in the polymeric protein fraction, indicating that they can form inter-molecular disulfide bonds. These results show that novel proteins found in some transformants are encoded by altered versions of either the transforming or endogenous HMW-GS genes.

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1. Introduction

Genetic transformation is one of several approaches used to understand the contributions of individual genes and seed proteins to wheat (*Triticum aestivum*) end-use functionality. The most-studied wheat quality genes are those encoding high-molecular-weight glutenin subunits (HMW-GS), which typically constitute 5–10% of seed storage proteins and are important determinants of dough strength and elasticity (reviewed and referenced in Shewry et al., 2003). Addition of native or modified HMW-GS genes to wheat generally results in accumulation of proteins of the expected sizes in addition to those encoded by the native genes (Altpeter et al., 1996; Alvarez et al., 2000; Barro et al., 1997; Blechl and Anderson, 1996; He et al., 1999, 2005; León et al., 2009). Transformants carrying expressed transgenes encoding native HMW-GS produce flours that make doughs with increased mixing times and tolerances compared to their non-transformed parent lines (Alvarez et al., 2001; Barro et al., 1997, 2003; Blechl et al., 2007; Darlington et al., 2003; He et al., 1999; León et al., 2009; Popineau et al., 2001; Rakszegi et al., 2005; Rooke et al., 1999; Vasil et al., 2001). However, some HMW-GS gene transformation events contain proteins of unpredicted sizes in addition to or instead of those encoded by the introduced transgenes (Altpeter et al., 1996; Alvarez et al., 2000; Barro et al., 1997; Blechl and Lin, 2007; He et al., 2005). In this report, we characterize thirteen of these proteins by tandem mass spectrometry (MS/MS) and show that they are size variants of HMW-GS.

2. Materials and methods

2.1. Derivation of transformed lines

The transformation events characterized here were produced in the same experiments as those described previously (Blechl et al., 2007). Briefly, cultivar 'Bobwhite' was co-transformed with the Ubi::BAR selection plasmid and separate DNA plasmids carrying the wheat genomic *Eco*R1 fragments from cultivar 'Cheyenne' that encode either 1Dx5 or 1Dy10 (Anderson et al., 1989). After selection with bialaphos, putative transformants were identified by changes in the levels of 1Dx5 and/or Dy10 in T_1 seeds. Homozygous progeny from each event were identified by SDS-PAGE of seed proteins and used to establish true-breeding lines (Blechl et al., 2007). Lines were named by the introduced HMW-GS gene(s), e.g., Dx5 + Dy10, followed by a unique event letter.

Abbreviations: HMW-GS, high-molecular-weight glutenin subunits; MS/MS, tandem mass spectrometry; RP-HPLC, reverse-phase liquid chromatography.

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An additional event "B52a-6" was characterized after the transgene had been crossed into hard winter wheat lines. The derivation and characterization of those lines are described in Graybosch et al. (2011). The original 'Bobwhite' transformant was co-transformed by Ubi::BAR and a plasmid carrying the native gene encoding 1Dy10.

2.2. Protein characterization

2.2.1. Mass spectrometry

To prepare individual protein bands for mass spectrometry, SDS-PAGE was performed as described previously (Blechl and Anderson, 1996 for Fig. 1C; Blechl et al., 2007 for Figs. 1A, B, D and 3). In some experiments, proteins were reduced and then alkylated by incubation for 30 min at room temperature in 4% (w/v) iodoactamide, 50 mM Tris–HCl (pH 8.8), 2% (w/v) SDS before loading onto the gels (method adapted from Görg et al., 2004). Bands corresponding to novel proteins were excised from the gels and placed into the reaction plate of a DigestPro (Itavis, Koeln, DE), where the protein-containing spots were destained, reduced, alkylated, and digested with trypsin. The resulting tryptic peptides were automatically eluted into a 96-well plate in preparation for LC-MS/MS.

Identification of protein-containing bands was carried out as previously described (Vensel et al., 2005). The 96-well collection plate from the DigestPro was inserted into the autosampler of the Reverse-Phase (RP)-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). A minimum of four independent samples for each protein variant were excised from gels and analyzed.

2.2.2. Protein identification

Extraction of peak lists from the QSTAR Analyst QS wiff files and their conversion to text files was carried out using Mascot Daemon (http://www.matrixscience.com/). MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.1.04) and X! Tandem (www.thegpm.org; version 2006.04.01.2). Mascot was set up to search a specially constructed database with all Triticae sequences (as of January 2009) and the protein sequences of Phosphinothricin Acetyl Transferase (Accession No. P16426 encoded by the BAR gene), beta lactamase (Accession No. AAB59737.1 encoded by the *bla1* gene for ampicillin resistance), and β-galactosidase (Accession No. P00722 encoded by the *lacZ* gene). The specified digestion enzyme was trypsin. Using the post-analysis software package Mascot and X! Tandem (Craig and Beavis, 2004), results were searched with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 1.00 Da. Iodoacetamide derivative of cysteine was specified in Mascot and X! Tandem as a fixed modification while oxidation of methionine and deamidation at N and Q were specified as variable modifications.

Validations of tandem MS-based peptide and protein identifications were performed using Scaffold (Scaffold version 2.06.01, Proteome Software Inc., Portland, OR). Protein probabilities were assigned by the Protein Prophet algorithm (Keller et al., 2002; Nesvizhskii et al., 2003). The protein identifications reported in Table 1 were established at 100.0% probability and contain at least 2 peptides identified with greater than 90% probability.

2.2.3. Protein solubility

Whole seeds homozygous for the transgenes were ground and extracted three times with 50% propanol. The residue was dissolved in SDS-PAGE sample buffer containing 2-mercaptoethanol and loaded onto 4–12% gradient gels for electrophoresis (Blechl et al., 2007).

3. Results

Previously we reported the generation of multiple independent transformation events obtained by particle bombardment with the Ubi::BAR selection gene and plasmids containing the native genes encoding HMW-GS 1Dx5 and/or 1Dy10 (Blechl et al., 2007). Analyses by SDS-PAGE of seed proteins showed that 23 of 30 transformants had an increase in the protein band(s) corresponding to HMW-GS 1Dx5 and/or 1Dy10, as expected (open circles in Fig. 1), while the other seven exhibited transgene-mediated co-suppression (Blechl and Lin, 2007; lane 2 of Fig. 1B). However, seven of the events with increased expression of 1Dx5 and/or 1Dy10 also contained one or more novel protein bands (arrowheads in lanes 1, 3–7, 9, 11, and 12 of Fig. 1). Most of the novel proteins were of higher molecular weight than native HMW-GS, although lower molecular-weight bands were also apparent in three transformants (lanes 1, 3 and 4, Fig. 1). Among 13 transformants with the *1Dx5* gene, we observed three lines with variant proteins. Among 10 transformants with both *1Dx5* and *1Dy10* genes, we observed four such lines (Blechl and Lin, 2007). Among 7 lines transformed with

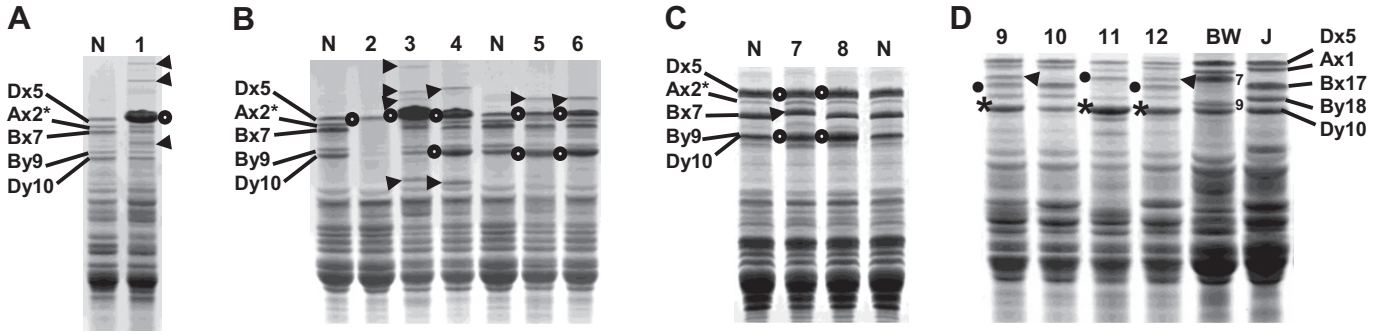

Fig. 1. SDS-PAGE of seed proteins from transgenic (numbered) and non-transformed 'Bobwhite' (N, BW) and 'Jagger' (J) wheat plants. Arrowheads to the left or right of the lanes indicate the positions of the variant proteins analyzed in this paper. Open circles indicate the subunit(s) with increased accumulation due to transgene expression. The names and positions of HMW-GS native to 'Bobwhite' are shown to the left of the first three panels. The names and positions of HMW-GS native to 'Jagger' are shown to the right of panel D. A. Transgenic line Dx-M (1) containing three variants. B. Transgenic lines are as follows: a segregant of transgenic event Dx5-F that showed transgene-mediated suppression (2); a different segregant of transgenic event Dx5-F, called Dx5-F* (3), that contained four proteins not found in (2) or in non-transformed Bobwhite extracts; Dx5 + Dy10-H (4) containing two variants; Dx5 + Dy10-I (5); Dx5 + Dy10-C (6). C. Transgenic line Dx5 + Dy10-A: a segregant called Dx5 + Dy10-A* (7), that contained a variant protein that migrated more slowly than native 1Bx7 (arrowhead), and a different segregant (8) that contained unaltered 1Bx7. D. Individual seeds (9–12) from a segregating population derived from crosses between transgenic event B52a-6 and Nebraska hard winter wheat lines (Graybosch et al., 2011). Asterisks indicate over-expressed 1Dy10 encoded by the transgene. Closed circles indicate other HMW-GS identified by tandem MS. The positions of subunits 1Bx7 (7) and 1By9 (9) are indicated to the right of the BW lane.