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Celiac-safe wheat genotypes: *A dietary solution to the gluten-induced disorders.*

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Wheat and derived products are elicitors of a number of frequent diet-induced health issues, including gluten intolerance, sensitivity, and allergy, collectively known as the 'gluten syndrome'. These disorders cumulatively affect more than 7.5% of the U.S. population (Rustgi 2013; Rosella et al. 2013). In particular, gluten intolerance or celiac disease alone affects more than 71 million individuals around the globe (i.e., ~1% of the world population), which makes it one of the most devastating disorders of the gastrointestinal tract (Bai et al. 2012). The seed storage proteins of wheat, in particular prolamins (i.e., gliadins and glutenins), are known to trigger this autoimmune condition. So far, 190 celiac-causing epitopes were identified from wheat prolamins, where origin of the 180 epitopes were tracked back to α/β-, γ-, and ω-gliadins and the remaining 10 to low- and high-molecular-weight glutenin subunits. Interestingly, out of these 10 epitopes from glutenins, the high molecular weight glutenins (HMWgs) contribute to only two epitopes, which have shown to elicit immune responses in relatively fewer cases (Comino et al. 2013). The HMW glutenins also are vital for the baking properties of common wheat. Furthermore, the low molecular weight glutenins (LMWgs) and gliadins have imbalanced amino acid profiles, with 15% proline and 35% glutamine, and a reduced content of the essential amino acids lysine, threonine, methionine, and histidine (Koehler and Wieser 2013). Parallel research also has demonstrated that gliadins and LMWgs are inessential for baking, because the flours derived from wheat deletion lines and transformants lacking one or more families of the gluten proteins baked into a normal bread loaf with characteristic organoleptic properties (van den Broeck et al. 2011; Gil-Humanes et al. 2014). Similarly, *in vitro* experiments with washed-out wheat flour residues mixed with recombinant HMWgs HMWDx5 and HMWDy10 baked into normal-looking bread loaves, which further supported the observations made with the wheat transformants and deletion lines (Wen et al. 2012 and references cited therein). Moreover, reduced-gluten, transgenic wheat lines exhibited improved nutritional properties, because their lysine content was significantly higher than that of normal flour due to the compensatory increase in the amount of lysine-rich proteins (Gil-Humanes et al. 2014).

Epigenetic elimination of immunogenic prolamins. Because HMWgs largely contribute to the baking properties of wheat, and are primarily non-immunogenic, we undertook a strategy to specifically eliminate LMWgs and gliadins from grains by endosperm-specific silencing of wheat *DEMETER* (*DME*) homoeologues. DME enzymes regulate transcriptional activation of the prolamin genes (except HMW glutenin genes) during endosperm development by demethylation of their promoters (Osorio et al. 2012; Wen et al. 2012). Under the auspices of NIH (National Institutes of Health) and

LSDF (Life Sciences Discovery Fund) funded research projects, we undertook cloning of wheat *DME* homoeologues, established connections between temporal expression of *DME* homoeologues and accumulation of specific prolamins, and transformed wheat cultivar Brundage 96 to express DME-targeting hairpin (hp) and artificial micro (ami) RNAs in the endosperm. Using this RNA interference-based approach, 401 candidate transformants were obtained (Rustgi et al. 2014). Of these 401 transformants, 333 were obtained through particle bombardment and 68 via microspore electroporation. Using protein gel electrophoresis and liquid chromatography, 19 viable wheat transformants showing the elimination of 45.2–76.4% immunogenic prolamins were identified. Protein profiling of these transformants exhibited elimination of specific prolamins and/or prolamin groups (Wen et al. 2012; Rustgi et al. 2014; Mejias et al. 2014). Differential silencing of three *DME* homoeologues in individual transformants due to variation in number and site of transgene integration(s), the *DME* site targeted by hp- and amiRNAs, and the level of conservation among *DME* homoeologues at the small interfering RNA targeted sites, explain the observed incomplete elimination of gluten proteins. This partial elimination of prolamins has motivated us to pyramid the effects of different transformants into a single plant to obtain genotypes completely devoid of celiac-causing prolamins. To achieve the desired objective, crossing of selected transformants after doubled haploidization is currently underway.

Determining the end-use quality of the gluten-deficient wheat transformants. In order to get the preliminary idea about the end-use quality of the selected wheat transformants, T_4 grains of these genotypes were used for detailed mixing and baking experiments at the Western Wheat Quality Laboratory in Pullman. In view of the importance of the physical properties of grain in determining end-use quality, a number of single-kernel parameters, such as grain hardness, grain weight, and grain size, were studied using the Single-Kernel Characterization System (SKCS). However, no major difference in the physical properties of the selected transformants and the untransformed control was observed. In order to get a deeper insight into the end-use quality of wheat transformants, other physical parameters, such as flour yield, break flour yield, flour ash content, and milling score, were recorded. For these parameters, the transformants exhibited subtle differences among themselves and with the control. Because most of the above-mentioned parameters are reflective of kernel hardness, and the literature suggests that it is not a sole determinative characteristic for bread-making properties, various other parameters that represent flour protein content and gluten strength were studied. Specifically, the SDS (sodium dodecyl sulfate)-sedimentation test and mixograph analyses, which are considered good indicators of bread-making quality, were studied in the selected transformants. The analyses suggested significant gluten strength in transformants P42G4, P32F2, P31D12, P48F6, P78E7, and P48F5 compared with that of the wild-type control, Brundage 96. Interestingly, different transformants exhibited higher scores for different mixograph parameters. Specifically, wheat transformant P31D12, which exhibited a 76.4% reduction in the amount of immunogenic gluten proteins (Wen et al. 2012), also showed the highest gluten strength. In addition to the mixing assay, a baking experiment also was performed with these transformants. In this experiment, the loaf volume of breads baked from the selected wheat transformants ranged from 775 cubic centimeter (CC) for P22H3 and P48F6 to 930 CC for P42G4, whereas the loaf volume of the untransformed control was 765 CC. Four transformants, P42G5, P42G4, P32F2, and P31D12, exhibited significantly high loaf volumes compared with that of the control. Collectively, these biochemical and baking experiments unambiguously suggested that these transformants exhibit physical properties similar to soft wheat genotypes, however, they posses the potential to be baked into breads somewhat similar to hard wheat genotypes.

Background effect of silencing wheat *DEMETER* homoeologues. To obtain detailed understanding of the global genomic changes taking place due to the silencing of the homoeologous wheat *DME* genes, a bi-partite approach was adapted, which involves phenotype charcterizing and transcript profiling of the selected wheat transformants. Due to limited availability of the T_2 grains, 3×12 ft. plots with 10 rows each were planted per genotype and four agronomical traits, grain number, grain weight, heading date, and anthesis date, were recorded. Because phenotypic data alone were inadequate for predicting the agronomical potential of the selected wheat transformants, we undertook transcript profiling of these transformants by RNA sequencing (RNA-seq). In this particular case, RNA-seq is expected to provide the information necessary for 'genomic selection', where prediction about a genotype's breeding value is made on the basis of understanding of its genomic constitution. In genomic selection, predictions about the breeding value of an uncharacterized genotype rely on a training set of plants, which is a collection of densely genotyped and precisely phenotyped individuals. To make it financially feasible, we modified this approach by undertaking sequencing of the grain transcriptome instead of the whole genome of the two selected wheat transformants, P37A3, which showed elimination of specific prolamins, and P48F6, which showed an over all reduction in the prolamin content, and compared their transcript profiles with that of the untransformed control and a genotype (12B-Bar) exclusively transformed with selectable marker gene (*Bar*) construct (Fig. 1). In this case, the two controls, i.e., the untransformed Brundage 96 and 12B-Bar, will serve as the training set, because great body of information about the phenotypic characteristics of Brundage 96 is already available. Transcript profile of 12B-Bar will help in separating out the effect of silencing wheat *DME* genes from

that of the genetic transformation. The comparisons based on the transcriptomic data and magnitude of expression level differences between the selected wheat transformants and controls will give a fair idea about the performance of the two gluten-deficient transformants. Other advantages associated with this analysis are the precision and depth of information one receives in comparison to recording a few phenotypic traits. Because many genomic changes do not result in a visible phenotype, due to the masking effect of homoeologous and paralogous gene actions and/or genotype–environment interactions, their effects can be missed by the traditional phenotypic screens. Despite the fact that the methylation status of a gene and its transcriptional pattern are not perfectly correlated, we expect that this analysis will provide sufficient information about the epigenetic regulation of genes involved in endosperm development, which significantly contributes to grain yield. In order to study transcript profiles of the selected transformants and controls, RNA was extracted from 50–70%-filled grains and used for RNA-seq. For this purpose, cDNA libraries were prepared for each genotype and sequenced in-depth using Ion Torrent sequencing procedure. After removal of transcripts derived from the chloroplast and mitochondrial genes, the two controls, Brundage 96 and 12B-Bar, respectively, yielded 107.28 and 84.92 Mb of clean sequence. Similarly, for the two selected transformants, P48F6, which exhibited a 77.8% reduction in immunogenic prolamins, and P37A3, which showed a 40.2% reduction in toxic gluten proteins, 93.95 and 74.75 Mb sequences, respectively, were obtained (Fig. 1).

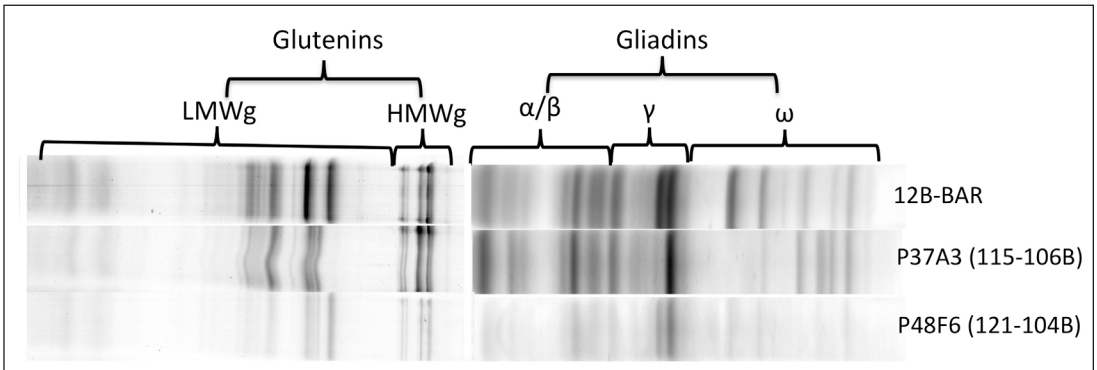

Fig. 1. Prolamin profiles of gluten-deficient transformants selected for RNA sequencing and compared with the wheat genotype (12B-Bar) exclusively transformed with the *Bar* gene expression vector.

The analysis revealed at least 262 and 327 genes showing perturbed expression in P48F6 and P37A3 when respectively compared with 12B-Bar. However, the phenotypic screen performed earlier on these genotypes suggested close similarity among them. Plotting these differentially expressed (DE) genes using a homology search on the wheat chromosome assembles revealed that, in several cases, the number of DE genes does not correspond well with the total number of genes predicted for that chromosome or group of homoeologous chromosomes.

This biased distribution of differentially expressed genes in the wheat genome has prompted us to investigate if there is a location effect within each chromosome by virtually mapping differentially expressed genes onto the chromosome assemblies. In several instances, plotting the observed number of differentially expressed genes onto the chromosome axis with the expected number of differentially expressed genes revealed biased distribution. This analysis clearly showed that the DME targeted sites are nonrandomly distributed in the wheat genome. The preliminary data from *Arabidopsis* exhibited localized demethylation at >9,000 loci in the euchromatic regions (Ibarra et al. 2012). Whereas, the preliminary data presented here do not suggest that the magnitude of changes is similar in two plant species, this aspect warrants further investigation.

In order to demonstrate correspondence between gene methylation/demethylation and its transcription, 10 up and 10 down regulated genes showing maximum expression level differences were listed in each combination. Interestingly, the genes showing maximum expression level differences overlapped among different combinations, which further supported the idea that these observations are non-random. These highly up/down regulated genes currently are being tested for the endosperm-specific changes in DNA methylation pattern using bisulphite sequencing in two wheat transformants, P48F6 and P37A3. This analysis will throw further light on the role of DME during the grain development in small grain cereals.

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