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Engineering wheat for celiac patients.

Wheat is one of the elementary nutritional elements of human civilization. Wheat has accompanied human beings since the dawn of civilization, and so has celiac disease. Removing wheat from the daily diet has severe consequences not only for human diet but also for social life. Celiac disease is a complex autoimmune disorder of humans and apes and is represented by a large variety of phenotypic manifestations in different patients. In celiac sprue, genetically predisposed individuals develop intolerance to wheat and wheat products. After decades of research, the complex mixture of seed storage proteins designated as glutens were determined to be responsible for celiac disease. The glutens comprise a huge family of proteins including gliadins (alpha/beta, omega, and gamma) and high- and low-molecular-weight glutenins. Of these proteins, gliadins and low-molecular-weight glutenins (LMWgs) account for most of the celiac-causing epitopes (a peptide that can serve as an antigen) and need to be eliminated or detoxified for making celiac safe wheat. According to the Codex definition, only about 1 mg of total gluten in 50 g of food is labeled or considered to be gluten free. With the advent of better diagnostic methods, more and more celiac cases are registered ever year,.resulting in an excess of 3 millon registered cases alone in the United States, making it a serious concern for human health.

In the genetically predisposed individuals, initial presentation of gluten peptides by antigen presenting cells expressing human leukocyte antigen (HLA)-DQ2 or -DQ8 to $CD4^+$T cells results in production of interferon-gamma (IFN-γ) that causes higher expression of HLA-DQ molecules and results in increased presentation of gluten peptides. The epitopes from gluten proteins on activation to a highly immunotoxic form via enzymatic deamidation, exert both innate and immunogenic effects in susceptible individuals, triggering the onset of disease.

Gliadins and LMW glutenins are not only pathogenic in nature they also are deficient in lysine content. Eliminating these lysine-deficient proteins from the grain can improve lysine availability in wheat grains. Lysine is an essential amino acid not produced by primates, thus the need to be acquired through dietary sources. Deficiency of lysine in the diet can lead to the creation of kidney stones and other health-related troubles including fatigue, slow growth, anemia, nausea, dizziness, loss of appetite, agitation, bloodshot eyes, and reproductive disorders. Because lysine helps in the absorption of calcium, a deficiency also can lead to defective bone growth. In view of producing celiac-safe wheat lines and to improve the nutritional quality of wheat grains in general, we employ three different strategies: i) epigenetic elimination of gliadins and LMWgs by silencing DEMETER gene(s) involved in their transcriptional de-repression, ii) post-transcriptional elimination by targeting transcripts of gliadins and LMWgs using a chimeric RNAi construct, and iii) post-translational detoxification of 'gluten' proteins by ectopic expression of an endoprotease in combination with an endopeptidase.

Epigenetic elimination of gliadins and low-molecular-weight glutenins. The concept behind targeting wheat DEMETER (5-methyl cytosine DNA glycosylase/lyase) homoeologues is inspired from studies conducted in barley and wheat, where it has been shown that promoters of gliadins and LMWgs need to be demethylated in seeds for their accumulation. DME regulates activation of LMWgs and gliadins by demethylation of their promoters, thus knocking down DME genes in principle will epigenetically eliminate most of the immunogenic prolamins (~150 in total) from wheat grains. These proteins also have been shown to be superfluous for baking.

i) Cloning and sequencing of DEMETER (DME) homoeologues. A pair of degenerate DME-specific primers (potentially amplifying homoeologous copies of DME from wheat genome covering nucleotide pos. 13263-13618 on FM164415.1) was designed and used to amplify fragment(s) from wheat genomic DNA. The PCR fragment was used to screen the hexaploid wheat Chinese Spring BAC library containing 1.3 million clones. Macroarray hybridizations led to the identification of three unique BAC clones. The three BAC clones (1946D08, 2106P11, and 2159B03) were sequenced at >60-fold coverage by a 454 sequencing method at the DNA Sequencing Core, WSU, Pullman. A total of 38.9 Mb of good-quality sequences were obtained. Analysis of sequences obtained from the above three BAC clones revealed that each harbors a full-length DME sequence (accession numbers JF683316-JF683318). The three DME sequences differ in length, ranging from 12.27 kb for 2106P11 to 12.63 kb for 1946D08. The observed differences in the length of DME homoeologues are mostly due to insertions and deletions (InDels) in the introns. A large number of point mutations and small InDels between DME homoeologous also exist in exons and contribute to the observed diversity in the protein sequences. A total of 135 homoeologous sequence variants (HSVs) giving a frequency of 22.7 HSVs/kb in exons and 584 HSVs giving a frequency of 90 HSVs/kb in introns. Of the 135 HSVs, 60 (44.44%) contain amino acid substitutions in at least one of the three DME homoeologues. Comparison of DME homoeologues with mapped wheat ESTs showed high levels of homology with BE471039 and allowed their assignment to the long arm of wheat group-5 chromosomes (5AL, 5BL, and 5DL). Assignment of DME homoeologues to specific subgenomes of bread wheat was confirmed using homoeologues-specific primers derived by tagging their 3'-ends at HSVs. The homoeologue-specific primers allowed unambiguous assignment of 2159B03 to chromosome 5A, 1946D08 to chromosome 5B, and 2106P11 to chromosome 5D. Subgenome assignment of DEMETER homoeologues TaDEM-5B was validated further by the use of wheat group-5-specific nulli-tetrasomic lines, whereas, the subchromosomal location of TaDEM-5B to a subcentromeric-bin encompassing 98.78 Mb of genomic DNA on 5BL, was determined using 5B-specific, terminal and interstitial deletion lines. Comparison of the full-length DEMETER sequences with wheat ESTs available in the public domain suggested that all three copies of DEMETER are transcriptionally active.

ii) TILLING of DEMETER homoeologues in tetraploid and hexaploid wheats. In cooperation with Arcadia Biosciences, we screened for DEMETER mutations in tetraploid Kronos and hexaploid Express wheat M_2 populations by Targeting Induced Local Lesions in Genome (TILLING). The average mutation density in *ethyl methanesulfonate* (EMS) mutagenized M_2 population of Kronos was 1 mutation per 40 kb DNA, and the mutation density for Express was 1 mutation per 24 kb DNA. The mutations are mostly single nucleotide polymorphisms or small deletions. Two runs, one each, with a set of subgenome specific primers were executed on Kronos and Express M_2 DNA-bulks. The subgenome-specific DEMETER primers amplified 1,050 bp from the A subgenome and 1,044 bp from the B subgenome of tetraploid and hexaploid wheats. In total, 39 mutations in the A homoeologue and 35 mutations in the B homoeologue of TdDEM were detected in tetraploid wheat. Similarly, 42 mutations in the A homoeologue and 53 mutations in the B homoeologue of TaDEM were detected in hexaploid wheat. Heterozygous and homozygous M_3 mutants were propagated in glasshouse to obtain M_4 seeds. These were analyzed for the effect of mutations on DME transcription using qRT-PCR and accumulation of gliadin and low molecular glutenins using SDS-PAGE gene analyses, followed by RP-HPLC.

Single mutations in the A and B subgenome DEMETER homoelogues of bread and durum wheat identified as above were recently crossed in combinations to obtain DEMETER double mutants. All crosses were made reciprocally and in duplicates. The F_1 and resulting F_2 seeds were obtained for eight different mutant combinations. The F_2 grains obtained from the aforementioned crosses were propagated in 48 well flats and are currently being tested for homo-/heterozygous double mutations by PCR followed by sequencing. The preliminary analysis allowed identification of seven double mutations in the Kronos background and 14 double mutants in the Express background. These selected double mutants were transferred to larger pots and are currently being cultivated in glasshouse to obtain F_3 grains to study effect of mutations on transcriptional and translational profiles of DEMETER homoeologues.

Recently, a subgenome-specific primer pair amplifying a 1,008 bp fragment from the active site of a D subgenome DEMETER homoeologue of hexaploid wheat was used to screen the Express TILLING library. In total, 25 mutations in the DEMETER D subgenome homoeologue were detected. Some of these selected D-subgenome mutations will be crossed with the DEMETER double mutations identified from the analysis to obtain DEMETER triple mutations.

iii) Silencing wheat DEMETER genes using artificial microRNAs (amiRNAs) and hairpin constructs. A total of 342 putative transformants were obtained by four rounds of biolistic transformations using five different constructs, where three express artificial microRNAs (pRB104, pRB105, and pRB106) and two express hairpin RNA (p728 and pDRB6). All of the above single-cassette vectors were co-transformed in 2:1 proportion with another single cassette vector (pDPG165) expressing the *Bar* gene cloned under the control of 35S promoter and Nos terminator using biolistic transformation of

scutellar calli derived from soft white winter wheat varieties (Brundage 96 and Simon) adapted to Pacific Northwest United States. The co-transformation provides the option for elimination of false positives by regenerating calli on selective media with increasing quantities of herbicide (bialaphos; up to 5 µg/ml). And in principle has greater opportunity for independent integration(s) of the two cassettes at different loci, thus allowing removal of the undesirable marker gene(s) by random assortment. The plants recovered from the tissue culture were transferred to soil and vernalized for 8 weeks. After vernalization the plants were transplanted to 6-inch pots, and their leaves were painted with a 2% Ignite solution (active ingredient biolaphos) and data were recorded for injury on 0–5 scale, where 0 represents no injury and 5 represents dead tissue.

Two weeks after transplanting, leaf tissue was collected from 153 putative transformants (T_0) of the first round of transformations, and DNA was extracted to study integration of ami/hpRNA expressing cassettes in the wheat genome. Clear integrations were observed in the genomic DNA of twenty (13.07%) of the above 153 T_0 plants. The integrations were confirmed by sequencing of PCR products obtained using construct-specific primers in 11 (55%) cases. However, good quality sequencing reads could not be obtained in nine cases (plants transformed with p728), even after multiple attempts. The failure to obtain good a quality sequence can be attributed to the nature (hairpin) of construct used to transform these plants.

In the T_0 generation, transgene integrations are in hemizygous state, and plants obtained from transformed calli mostly represent chimeric plants, which also is apparent in our case by the number of tillers produced per plant. Thus, we have not discarded any plants at this stage on the basis of PCR results. T_0 spikes were collected from all 153 plants to extract RNA from immature grains to study transcriptional suppression of wheat DEMETER homoeologues.

RNA was extracted from developing T_1 grains harvested 17 (±3 days) days post anthesis (DPA) from the T_0 spikes of 153 putative transformants. The spikes were collected in liquid nitrogen. Between 0.15 to 0.3 g of the developing grains were pulverized to isolate RNA using TRIZOL reagent (Invitrogen Corp., Carlsbad, CA, USA) following the manufacturer's recommendations. The qRT-PCR analysis of TaDME transcripts was performed using the DNA Master SYBR Green 1 chemistry on the *LightCycler® 480 Real-Time PCR* System (Roche Diagnostics, Indianapolis, IN, USA) using degenerate wheat DEMETER (amplifying three DEMETER homoeologues) and Actin specific primers (used as internal control). TaDME mRNA level was normalized to Actin using the DDC_T method (Livak and Schmittgen 2001 Methods 25:402). Transcript levels were expressed as a ratio of TaDME transcripts (normalized to Actin) in control (Brundage 96) and other putative transformants (co-transformed with pDPG165 and vectors expressing hairpin and artificial micro RNAs; see above).

Of the 153 putative transformants obtained from the first round of transformations, suppression of the DMETER transcript abundance was observed in 37 cases (24.18%), where the suppression levels range from ~30% to 65%. Five of the above 37 plants also showed integration of DEMETER silencing cassettes in the wheat genome using PCR, however, integration(s) cannot be confirmed in the remaining 32 cases due to a high level of chimerism in plants and possibly due to a low copy number of integrated cassettes. The low level of DEMETER suppression observed in the transformants can also be attributed to high level of chimarism in plants.

On the basis of the results of PCR and qRT-PCR analysis, a set of 52 (33.98%) transformants was selected for further analysis. Of the above 52 plants, 50 gave seeds (T_1) and were used for protein extraction following the protocol described in Wieser et al. (1998) with minor modifications. The three different fractions albumins/globulins (salt soluble fraction), gliadins (aqueous alcohol) and glutenins (aqueous alcohol with reducing agents) were extracted from the T_1 grains obtained from each transformant, and were analyzed by loading on appropriate (SDS- or A-PAGE) gels.

The extracted proteins were first quantified using Bradford colorimetric assay followed by quantitative (by loading equal volume of extracted proteins obtained from equal amount of starting seed material) and qualitative analysis (by loading equimolar amounts of proteins) on gel and/or HPLC. Preliminary results of PAGE gel analysis and RP-HPLC revealed elimination of specific gliadins (in gamma and alpha/beta fractions) and glutenins (in LMW glutenin fraction) instead of mass eliminations. These observations have been attributed to the bulk harvest of all T_1 grains from individual T_0 plants that are chimeric in nature, thus diluting the effect in the protein gels as well as RP-HPLC profiles. In order to deal with the problem of chimerism we are currently propagating ~2,700 T_1 progeny plants in the glasshouse and equal number of plants at Cook Agronomy Farm, Washington State University, Pullman. To determine the identity of the eliminated proteins and the novel peaks that appeared in the HPLC profiles we are currently working on establishing a standard procedure for the analysis of intact proteins on MALDI-TOF MS and LC-ESI MS.

Post-transcriptional elimination of gliadins and LMWgs. In order to achieve the transcriptional silencing, we designed a novel hairpin construct which contain a chimeric stem derived from a number of miRNAs. Each are designed from a conserved region indentified by individually aligning different kind of gliadins and LMWgs. A truncated version of wheat TAK14 intron was used as the loop. The construct was cloned in gamma sub-genome of barley streak mosaic virus (BSMV) to be used in virus induced gene silencing (VIGS) and also with 1Dy HMWg promoter and Nos terminator for RNAi. The VIGS results showed significant reduction in the amount of gliadins and LMWgs. The putative transformants obtained using this construct are currently in glasshouse and will be analyzed for elimination of celiac causing gliadins and glutenins.

Post-translational detoxification of gluten proteins. Post-translational detoxification of gluten proteins by a combination of glutamine and proline specific endo-peptidase/proteases is currently considered as a therapeutic alternative for celiac patients. In view of the therapeutic potential of this approach we decided to express thermostable and codon optimized forms of these enzymes in the wheat endosperm. This anticipates obtaining transgenic grains expressing large amounts of these enzymes, which in turn can be mixed with the dough of nontransgenic wheat and baked into whole/cracked grain breads to be consumed by the celiac patients with nutritional benefits of whole grains and gluten detoxifying enzymes. The grains with these enzymes are also beneficial for the healthy individuals as it expedites and improves digestion of gluten proteins.

i) Virtual digestion of prolamins with endopeptidases and endoprotease under simulated gastro-intestinal conditions. A total of 1,336 prolamin sequences including wheat α/β- (151), γ- (272), and ω- (13) gliadins; LMW- (457) and HMW- (318) glutenins; barley B- (26), C- (22), γ- (30), and D- hordeins (4); and rye γ/ω- (26) and HMW- (17) secalins were virtually digested under simulated gastric conditions with barley EP-B2 or a mixture of wheat endopeptidases followed by *Flavobacterium meningosepticum* prolyl endopeptidase (FM-PEP) or *Aspergillus niger* prolyl endopeptidase (AN-PEP) treatment. Virtual digestion with pepsin, trypsin and chymotrypsin left a significantly large number of peptides with ≥10 residues undigested. The length of peptides left undigested ranged from 2–144 residues for γ-gliadins followed in order by ω-gliadins (2–132 residues), HMW-glutenins (2–122 residues), α/β-gliadins (2–119 residues), and LMW-glutenins (1–70 residues). The length of proteolytically resistant hordein peptides fall within the range of undigested peptides reported for wheat prolamins. For instance, the length of proteolytically resistant hordein peptides ranged from 2–130 residues for D-hordeins, 2–118 residues for C-hordeins, and 2–74 residues for B- and γ-hordeins. However, γ/ω-secalins were among the least properly digested prolamins leaving large peptides up to 339 residues undigested, in comparison with HMW-secalins (2–99 residues) and other prolamins from wheat and barley.

The optimal length of peptides stimulating T-cell response is 10–15 residues has been documented in the literature. Thus, any peptide ≥10 residues in length would potentially elicit immune response. The proteolytically resistant peptides when compared with 44 immunogenic peptides documented in the literature, encountered 413 cases showing similarity in γ-gliadins, 179 cases in α/β-gliadins, and two cases in ω-gliadins. Similarly, 17 cases were encountered in γ- and ω-secalins. However, the number of immunogenic-peptides detected in each group is biased as most of the studies conducted so far were based on wheat α/β-, γ- and ω-gliadins, thus listing only the immunogenic peptides underlying these gliadins. We consider it likely that a systematic study conducted for immunogenic-peptides underlying other prolamins will significantly add to the repertoire of immunogenic peptides.

Prolamins digested with barley EP-B2 or a mixture of wheat endoproteases significantly reduce the size of proteolytically resistant peptides leading to a greater reduction in the number of immunogenic peptides, leaving only 76 out of 179 immunogenic peptides detected in α/β-gliadins and 1 out of 2 immunogenic peptides detected in ω-gliadins. Although wheat endoproteases work better on γ and ω-gliadins, EP-B2 works better for the rest. In view of the results of the virtual digestion, and considering the fact that EP-B2 is one of the best-characterized endoproteases from Triticeae, it has been proposed as a component of combined therapy. Both FM-PEP and AN-PEP were equally active against the peptides digested with EP-B2 or wheat endoproteases under simulated gastro-intestinal conditions, except for ω-gliadins where FM-PEP performed better than AN-PEP. In view of the results of in silico analysis we undertook a nutraceutical approach to express barley EP-B2 and FM-PEP in large quantities in the wheat endosperm to detoxify immunogenic gluten proteins.

ii) Transformation of wheat scutellar calli. Biolistic bombardment of wheat scutellar calli resulted in a total of 91 putative transformants that survived bialaphos treatment. Of the 91 putative transformants, 54 were transformed with pDPG165: pHMWg+Fmen+nos: pHMWg+EP-B2+nos used in a molar ratio of 1:2:2, and 37 were transformed with pDPG165: pBSK^{+}(HMWg+Fmen+ nos/HMWg+EP-B2+nos) used in a molar ratio of 1:2. When screened us-

ing gene specific primers to confirm transgene integration(s), 20 putative transformants showed integration(s) only for pHMWg+Fmen+nos, four showed integration(s) only for pHMWg+EP-B2+nos, and six plants showed integrations for both. Of the six plants showing integrations for both genes, two plants were obtained using the double cassette linear construct and four were obtained using co-transformation of single cassette circular constructs. Results of the PCR analyses were validated by sequencing of the PCR products obtained from the positive transformants, all of the products showed perfect sequence identity with the genes used for transformation, further confirming the transgene integration(s). The results showed that the minimal gene cassettes (which were linear DNA fragments lacking vector sequences), excised from the plasmids, function as efficiently as whole plasmids containing the suitable gene constructs for wheat transformation. The linear constructs are totally devoid of the vector backbone thus allowing isolation of marker and vector free transformants, and serving as a perfect example of 'clean' DNA technology for the production of plants expressing agronomically important traits.

The isolated transformants will be raised to maturity and expression of desired enzymes will be verified at transcript level using qRT-PCR. Their activity will be determined using activity assays: FM-PEP activity will be determined as described in Yoshimoto et al. (1980; J Biological Chem 255:4786) and Chevallier et al. (1992; J Biol Chem 267:8192), for EP-B2, as described in Marti et al. (2005; J Pharmacol Exp Ther 312:19) and Bethune et al. (2006; Chem biol 13:637). The grains expressing high amounts of the two enzymes will be tested in whole grain breads prepared by the addition of whole/cracked transgenic grains to the dough (prepared from normal wheat flour) just prior to baking process to avoid hydration and thermal denaturation of the enzymes.

Establishment of a novel transformation procedure based on microspore culture and electroporation. Similar to barley, time-lapse tracking of wheat microspores clearly showed three developmental pathways for microspore development, and the fate of the developing microspore depends upon the choice of the developmental pathway. All of these developmental pathways have their hallmarks, which allow identification of the fate of microspore cultures in advance. For instance, the ultra-structural study of microspores using transmission electron microscopy revealed three cell types, where the first type displayed a thin intine layer and an undifferentiated cytoplasm, the second type showed a thick intine layer and a starch-rich cytoplasm (similar to developing pollen grains) and the third type showed an intermediate phenotype. Accumulation of starch in the pollen amyloplasts marks the commitment to the pollen developmental pathway. Our observations indicate the enlarged cells that evolved to pollen morphology after treatment with specific conditions were still committed to the gametophytic pathway, probably representing the type III developmental pathway as identified by time-lapse tracking study in barley (de Maraschin et al. 2005; Planta 220:531). On the other hand, microspores with undifferentiated cytoplasm have been associated with the repression of the gametophytic pathway. Prior to induction of androgenesis, wheat and barley uninucleate microspores are characterized by the lack of specialized morphological structures in the cytoplasm and a thin intine layer. This suggests that, in wheat and barley, the microspores with undifferentiated cytoplasm and a thin intine layer after specific treatment, are associated with the repression of the gametophytic pathway (developmental types I and II). The maintenance of a thin intine layer after specific treatment seems to represent an early morphological marker for induced microspores in wheat. Tracking showed that the first developmental change associated with dividing microspores (developmental types I and II) was a star-like morphology, which was characterized as a transitory stage between vacuolated microspores after pretreatment and the initiation of cell division. Although the star-like morphology appears to be a morphological marker for the initiation of cell division in treated microspores, a star-like morphology per se does not assure that a microspore will ultimately commit to the embryogenic pathway. The occurrence of a star-like morphology is a dynamic process, in which the time of occurrence will depend on the type of treatment applied and the stage of microspore development. In wheat, microspores of developmental type I displayed the tendency to form a star-like morphology relatively later than type-II microspores.

The morphological markers identified from the time-lapse and ultrastructural studies have significantly improved the efficiency of getting viable calli from wheat microspore cultures, which in turn influence the number of putative transformants obtained per experiment. To obtain the optimal conditions for microspore-based transformations we transformed wheat microspores with constructs expressing three different marker genes [*green fluorescence protein* (*GFP*), *beta-glucuronidase (GUS*), and endochitinase] using different electroporation and culture conditions. A wide range of electroporation voltages ranging from 150–1,000 V were tested and a range of 250–500 V was found optimal with a peak ~375 V, at which maximum number of transformants were recovered. Similarly, pretreatment of immature spikes with $CuSO_4$ solution (500 mg/L) at 4°C for 10 days and incubation of microspores after transfection at 24°C resulted in recovery of large number green plants. Following these optimal conditions, we recently performed transformations of wheat microspores using *GUS*, *GFP*, and *endochitinase* expressing cassette and confirmed integration of these cassettes in the wheat genome by PCR followed by sequenceing of the PCR fragments.

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