lent in the eastern soft white winter wheat region, including leaf and stripe rusts, powdery mildew, Septoria tritici leaf blotch, Fusarium head blight, barley yellow dwarf virus, wheat soil-borne mosaic virus, and Hessian fly.

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The mission of the lab is two-fold: conduct milling, baking, and end-use quality evaluations on wheat breeding lines, and conduct research on wheat grain quality and utilization. Our web site: http://www.wsu.edu/~wwql/php/index.php provides great access to our research and methodology. Our research publications are available on our web site.

Morris and Engle lead the Pacific Northwest Wheat Quality Council, a consortium of collaborators who evaluate the quality of new cultivars and advanced breeding lines. We also conduct the U.S. Wheat Associates' Overseas Varietal Analysis Program for Soft White and Club Wheat. Our current activities and projects include grain hardness and puroindolines, waxy wheat, polyphenol oxidase (PPO), arabinoxylans, SDS sedimentation test, and soft durum wheat.

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Breeding celiac-safe wheat cultivars: a future market class of wheat.

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The gluten-induced disorders dubbed the 'gluten syndrome' collectively affect >7.5% of the U.S. population. So far, the only known therapy for gluten syndrome is a life-long adherence to an abstinence diet, which is difficult to follow, and shown to have an adverse influence on the diversity of gut microbiota in consumers making them vulnerable to a number of disorders including colon cancer. It has been demonstrated repeatedly that strict adherence to gluten-free diet result in an upward shift in the body mass index (BMI) of the consumers, predisposing them to many other disorders. Extensive efforts put into the analysis of a wide range of wheat genotypes, including diploid einkorn; tetraploid emmer; and hexaploid common wheat accessions, landraces, and old/new cultivars at best resulted in the identification of lines with reduced-toxicity. None of the wheats (including einkorn, Khorasan, or Kamut), barley, and rye genotypes are free of gliadins and, thus, cannot be considered celiac-safe for common use by all celiac patients. The present technology does not allow healthcare providers to make recommendations to the patients that they only have sensitivity for a specific group of gluten proteins and, thus, can consume a specific wheat variety/product lacking certain gluten protein(s)/epitope(s). Moreover, research on alternative sources of gluten-free grains, including cereals (such as rice, sorghum, oats, and maize) and pseudocereals (such as tef, quinoa, and buckwheat) revealed that certain varieties of maize, oats,

quinoa, and buckwheat induce immune reactions in susceptible individuals. On the other hand, with rice, there is a risk of increasing the glycemic index of the consumers and exposure to arsenic. In addition, cases of allergenicity to rice grain-storage proteins (other than prolamins and glutelins) were reported throughout the world, thus it is not an ideal choice for consumption by celiac patients. So far, sorghum and tef are the two noncontroversial choices, but sorghum is predominantly used as animal feed, thus more research is needed on its end-uses to introduce it in human diet, whereas tef, due to morphological similarities with wheat grains, is the fear of contamination. Furthermore, because wheat is a primary source of dietary fiber, protein, vitamins, antioxidative compounds, and mineral elements, the World Health Organization recommends eating bread several times a day, and a number of countries (depending upon the national food habits) recommend consuming 250–350 g of bread per day.

In summary, developing potential alternatives of a gluten-free diet by modifying the composition of wheat grain to make them less prone to inducing the most common of dietary disorders is imperative. Also evident from the above description is that no natural product makes a perfect dietary solution for celiac patients. Thus, this trait is an obvious candidate for genetic engineering.

Our research showed that it is possible to obtain wheat genotypes completely devoid of immunogenic prolamins while retaining their baking properties by silencing the wheat *DEMETER* (*DME*) homoeologues (Fig. 1). The DME genes are responsible for transcriptional activation of genes encoding gliadins and the low-molecular-weight glutenins (LMWgs) in the developing wheat endosperm. But, so far, after screening ~400 transformants expressing hairpin or artificial micro-RNAs targeting wheat *DME* homoeologues, one genotype showing 85.6% suppression in *DME* transcript abundance and up to 76.4% reduction in the amount of immunogenic prolamins could be identified. This low frequency of desired transformants can be attributed to the unpredictability of both the number and site of transgene integration(s) using the current transformation procedures, which has a consequential effect on the transcription level of the transgene.

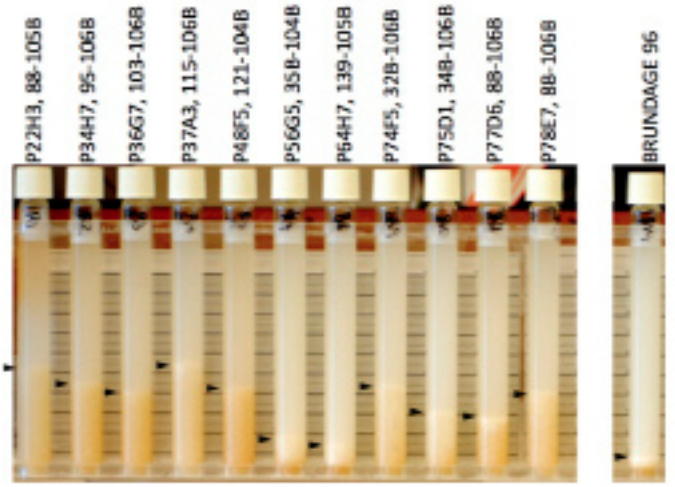

Fig. 1. Results of the SDS (sodium dodecyl sulfate) sedimentation test performed on a set of 11 T_2 transformants (whole-grain flour) and the wild-type control Brundage 96 as a preliminary test for grain quality. Because Brundage 96 is a soft white winter wheat, it showed a low sedimentation value as expected. Some of the transformants developed in Brundage 96 background showed high sedimentation values comparable to the hard wheat cultivars (generally used for bread making). Thus, suggesting that the changes in composition of grain-storage proteins resulted in a shift of market class from soft to hard in these transformants.

In view of the technical difficulties associated with current transformation procedures, we used a TILLING (Targeting Induced Local Lesions In Genomes) procedure to identify knockout or knockdown mutants in the wheat *DME* homoeologues. Screening of hexaploid wheat cultivar Express and tetraploid cultivar Kronos wheat TILLING populations for mutations in *DME* homoeologues resulted in identification of 191 mutants in the Express and 77 mutants in the Kronos background. These single mutants identified in DME homoeologues showed specific eliminations and/or reductions in the amount of prolamins. However, developing celiac-safe wheat genotypes requires pyramiding single mutations to obtain double mutations in the Kronos and triple mutations in the Express backgrounds. The nonsense mutations and splice-site variants in individual *DME* homoeologues with quantifiable effects on prolamin composition/content showed adverse effects on pollen viability and germination, imposing difficulties in pyramiding the effects of individual mutants in a single genotype. In view of these difficulties, an alternative approach employing a chimeric-hairpin construct capable of producing multiple interfering RNAs corresponding with the transcripts of the prolamin super-family genes was undertaken. This approach required aligning the sequences of the wheat gliadins (α/β-, γ- and ω-types) and LMWgs available in the public domain. The alignment results allowed identification of the five consensus sequences each representing a prolamin gene family, which were fused together to obtain the arms of the chimeric-hairpin. The chimeric-hairpin construct was used for wheat transformation of embryogenic-microspore and scutellar calli in the cultivars Louise, Hollis, WPB926, Farnum, Brundage 96, and Simon. The transformants showed sizable amount of reductions in the content of immunogenic

prolamins very similar to the transformants expressing *DME* hairpin or amiRNA constructs, which showed reductions and specific eliminations to variable degrees in the grain prolamin content. A compensatory increased in the amount of high-molecular-weight glutenins also was observed in some cases, which is a desirable characteristic for maintaining the end-use quality of the genotypes. Because none of the wheat transformants showed complete elimination of immunogenic prolamins, these genotypes with reduced gluten content are currently being crossed with an aim of pyramiding their effects on prolamin content or composition into a single wheat genotype.

We are currently using a site-directed procedure to achieve complete silencing of *DME* genes in the developing wheat endosperm to avoid the negative consequences of introducing point mutations in *DME* genes and obtain wheat genotypes showing complete elimination of immunogenic prolamins. To achieve this objective, we are attaching an engineered DNA binding domain of the *Xanthomons* transcription activator-like effector (TALE) to a universal *Arabidopsis* ethylene-responsive element binding factor-associated Amphiphilic Repression (EAR) domain that allows transcriptional suppression of the targeted gene(s). This DME-specific, TALE repressor will be introgressed in to the wheat genome via homologous recombination. In this procedure, the selected wheat transformants will be used as explant donors for retransformation with the expectation of a complete silencing of *DME* homoeologues. We are working in parallel on an alternative method of delivering recombinant TALEs in the plant cell using the type-III secretion system of *X. translucens*. This method does not require integration of foreign DNA in the plant genome to make desired genetic-modifications. Thus, this approach is expected to be the method of choice in future plant breeding efforts.

As a small number of celiac patients also were reported to show sensitivity to the HMW glutenins, we undertook another approach, which involves detoxification of gluten proteins by ectopic expression of gluten detoxifying enzymes (glutenases). For this purpose, a combination of a glutamine-specific, barley endoprotease B2 (EP-B2) and a post-proline cleaving prolyl endopeptidase from *Flavobacterium meningosepticum* (Fm-PEP) were selected and expressed in wheat endosperm. Wheat transformants showing sizable amount of reduction in Pro/Gln-rich peptides under simulated gastro-intestinal conditions were obtained. Because we intend to use wheat grains expressing glutenases as an ingredient of the daily bakery products, it is ideal to have thermostable enzymes that retain activity at and over 90°C. In order to achieve this objective, a site saturation mutagenesis approach was followed. The amino acid substitutions at residues 412, 413, 414, and 415 increased thermostability of Fm-PEP from 60°C to 90°C, while maintaining its catalytic properties under simulated gastro-intestinal conditions. Similarly, in view of increasing thermostability of EP-B2, a structure-based site directed mutagenesis approach was adapted and an increase in thermostability by 4°C was reported. The observed increase in the thermostability of EP-B2 is not substantial, but this pilot study demonstrated a possibility of obtaining thermostable variant of both Fm-PEP and EP-B2.

The solutions undertaken in the present research are based on developing wheat plants by genetic transformation. It is important to point out that most transgenic crops currently in use have been generated for the benefit of the producer, whereas crops that would lack immunogenic gluten proteins would benefit the consumer and the celiac populations in particular. They are thus acceptable to both regulatory authorities and the general public. Because these wheat transformants are developed in view of a medical purpose, they are exempt from labeling restriction in the State of Washington.

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