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WASHINGTON

WASHINGTON STATE UNIVERSITY
Department of Crop and Soil Sciences, & School of Molecular Bioscience, Pullman, WA 99164-6420, USA.

D. von Wettstein, S. Rustgi, C.G. Kannangara, N. Ankrah, S. Wen, R.A.T. Brew-Appiah, N. Wen, R. Gemini, R. Brueggeman, P. Reisenauer, and K.S. Gill, Department of Crop and Soil Sciences, Washington State University, Pullman, USA; B. Liu, J. Pang, and X. Wang, Institute of Cytology and Genetics Research, Northeast Normal University, Changchun, PR China; and M. Claar and G. Langen, K.H. Kogel, Research Centre for BioSystems, Land Use & Nutrition, Justus Liebig University of Giessen, Giessen, Germany.

A multipronged approach to develop nutritionally improved, celiac safe, wheat cultivars.

Wheat and its products are potential elicitors for two types of immune responses in human beings: the first being the immunoglobulin E (IgE)-mediated occupational responses (e.g., bakers' asthma) and the second being non-IgE-mediated responses due to ingestion of seed storage proteins of Triticeae (celiac disease). In general, wheat proteins also are poor in nutritional quality because of their imbalanced amino acid composition and deficiency of one of the essential amino acids, lysine. Among the known food allergy cases triggered by wheat and wheat products, most belong to celiac disease, constituting >24.4 million registered cases worldwide. The only effective therapy known to date is strict dietary adherence to a gluten-free diet, which often leads to nutritional deficiencies in celiac patients. In view of the above, we undertake a profound project with the ultimate objective of eliminating the prolamins from wheat grains that contain a majority of epitopes causing celiac disease. Eliminating these proteins also will address the issue of imbalance in the amino acid profile of wheat proteins.

Mapping and cloning of barley and wheat DEMETER homologues. DEMETER (DME) and its functional homologues ROS1, DML1, and DML2 were recently characterized from *Arabidopsis* and rice. DEMETER encodes a 5-methylcytosine DNA glycosylase that is involved in demethylation of genomic DNA in tissue and developmental specific manner as a short-patch, base excision repair pathway. Both the barley mutant Risø 1508 (lys3a) and *Arabidopsis* dme mutants prevent demethylation of gene promoters (von Wettstein 2009). We first identified a barley homologue (TA38047) of AtDME and designed primers to amplify it from barley genomic DNA and cDNA. The amplified product was used as probe to hybridize with the high-density filters of a barley BAC library. Gel-blot analyses allowed identification of a single BAC clone harboring the *HvDME* gene, which was then subcloned and sequenced to obtain full-length HvDME sequence and verified by cDNA sequencing. We used the barley DEMETER sequences (genomic DNA and cDNA) to identify wheat ESTs showing homology with the gene. The ESTs were assembled in contig. These ESTs were derived from ten different wheat cultivars, including 11 from Chinese Spring (CS), five from Recital, and four from Thatcher. The EST assembly was carefully examined for the presence of homoeologous sequence variants (HSVs) that allowed partitioning of the EST-contig into three sub-contigs. These sub-contigs virtually represent clusters of different homoeologous copies of the gene. We used these HSVs to tag our primers at their 3' ends, which allows us to amplify specific products from different subgenomes of bread wheat. We tested the primers on a complete set of nulli-tetrasomic lines, with CS as a control, to localize them to specific chromosomes and test their specificity. One of the primer pairs was assigned unambiguously to wheat chromosome 5B. We used the same set of primers on the genomic DNA of nulli-tetrasomic lines for group-5 chromosomes, deletion lines for long and short arms of chromosome 5B, and an interstitial deletion line *ph1b* to assign one of the DEMETER homoeologues (TaDME-B1) to a subchromosomal region. The analysis allowed localization of TaDME-B1 to the subcentromeric bin of 5BL, bracketed on either side by deletion break points of 5BL-12 (proximal) and 5BL-2 (distal). Two STS primers, derived from the RFLP probes co-localizing with the *lys3a* gene, also were localized to chromosome 5B using wheat aneuploid and deletion stocks. The subgenome specific primers developed as above were used to screen a CS genomic DNA library, leading to the identification of seven BAC clones that are currently being sequenced to get full-length gDNA sequences of wheat DEMETER homoeologues. HvDME genomic DNA and cDNA sequences also were blasted against CS genomic DNA sequences released recently in the public domain (http://www.cerealsdb.uk.net/search_reads.htm). More than 200 sequences showing similarity with HvDME were identified and are currently being utilized to assemble a contig spanning the whole gene sequence. The contig will be examined manually to identify subgenome-specific patterns and to develop specific primers for the D genome of bread wheat.

Establishment of a novel transformation procedure based on microspore culture and electroporation of binary Ti-vectors. We established a novel transformation procedure, where haploid microspores at uninucleate stage were selected, harvested, and purified by density-gradient centrifugation before transformation. The microspores were then transformed with binary Ti-vectors by electroporation using suitable transfection media followed by co-cultivation with ovaries on suitable culture media for induction of embryogenesis. The microspore-derived embryoids were then transferred to the selection media to weed out the nontransformants, and the survivors from there were selected using visible markers to eliminate false positives. Only the selected plantlets obtained from the true-transformants were then treated with colchicine to induced chromosome doubling leading to the production of doubled-haploid, homozygous transgenic lines. Three binary test plasmids were used to optimize the electroporation conditions with genes expressed and monitored in developing transformed embryoids, young seedlings, and maturing plants: (1) pJH271 and (2) pRBOV-hySFi-GFP expressing the green fluorescing protein GFP with the CaMV 35S promoter and (3) pYW300 expressing the *Trichoderma harzianum* endochitinase that can be monitored by UV-induced fluorescence upon cleavage of 4-methylumbelliferyl-β-D-N,N',N''-triacetylchitotrioside substrate. The transformants obtained using each of above three binary vectors were tested for their respective visual phenotypes and with gene specific primers for the integration of respective transgenes in their nuclear genomes. Both of the above genotypic and phenotypic screens confirmed the integration of transgenes in the nuclear genome of the transformants.

Silencing wheat DEMETER genes using artificial microRNAs (amiRNAs) and hairpin constructs. We have amplified a 981-bp fragment of bread wheat covering the active site of DEMETER and a 300-bp fragment from the N-terminal first exon (covering the bipartite nuclear localization signal). These fragments were analyzed by the Web MicroRNA Designer (WMD: http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) for the most suitable sequences for amiRNAs. For the fragment covering the active site region, eight sequences were suggested suitable, and for the fragment spanning the N-terminal domain, only one sequence was suggested suitable by the software. From the suggested sequences, we selected three sequences, two from the active site region (DME1 and DME2) and one from the N-terminal domain (TADMEStart) for constructing the first amiRNAs. The artificial miRNA-containing precursors of the DME1, DME2, and TADMEStart have been generated on the pNW55-OsaMIR528 of *Oryza sativa* following fusion PCR reactions. These amiRNAs will

be expressed under the control of the D-Hordein (D-Hor) promoter of barley and/or HMW-glutenin (HMWg) promoter of wheat and will be cloned in pGreen binary vector. Similarly, hairpin constructs were designed from the above two DEMETER fragments and will be incorporated in pHELLSGATE vector using homologous recombination. The hairpin constructs will be expressed under the control of D-Hor and/or HMWg promoters.

Cloning and expression of prolyl endopeptidase. Prolyl endopeptidase (PREP) or prolyl oligopeptidase is a cytosolic enzyme that belongs to a distinct class of serine peptidases. The enzyme cleaves peptide bonds at the C-terminal side of proline residues. Its activity is confined to action on oligopeptides of less than 10 kDa. The PREP enzyme has been shown to decrease the propensity of gluten-containing wheat products by detoxifying the peptides causing celiac disease. In view of the above, we used the PREP sequence of *Flavobacterium meningosepticum*, optimized its codon composition, had it synthesized by GenScript Inc., U.S., and cloned it in pUC57 using the *Eco*RV restriction site. The insert cloned in pUC57 was flanked by the restriction sites of *Eco*RI and *Apa*I, these restriction sites were specifically selected to digest the plasmid and to take out the insert, which will then be cloned in the Pichia expression vectors using the same restriction sites (pPICZ A, Invitrogen Inc., U.S.). The above experiment will allow us to test the PREP functionality and activity in the eukaryotic system (yeast). Once the codon optimized PREP sequence is tested for its functionality and activity in the yeast, it will be introduced in wheat under the control of HMWg promoter through our microspore transformation technique. The transforments thus obtained will then be examined for PREP activity and gluten content.

***In vitro* examination of DEMETER activity.** We were able to obtain full-length sequences of DEMETER from barley mutant Risø 1508 (lys3a) and its parent variety Bomi. Wild type and mutant DEMETER cDNA clones were expressed in *E. coli* with a his-tag. The resultant proteins will be purified on a Ni2+-NTA column, and their activity tested with methylcytosine containing double-stranded oligonucleotides. The recombinant protein expressed in *E. coli* is used to raise antibodies against the DEMETER protein, which is used in quantification of DEMETER protein in TILLING mutants.

Acknowledgements. Financial support by NIH grants 1R01 GM080749-01A2 and a Mercator Professorship from the German Research Foundation is gratefully acknowledged.

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USDA–ARS, WESTERN WHEAT QUALITY LABORATORY
E-202 Food Science & Human Nutrition Facility East, P.O. Box 646394, Washington State University, Pullman, WA 99164-6394, USA.
http://www.wsu.edu/~wwql/php/index.php

Craig F. Morris, B. Beecher, A.D. Bettge, D.A. Engle, G.E. King, M. Baldridge, P.K. Boyer, E.P. Fuerst, B. Paszczynska, G.L. Jacobson, W.J. Kelley, M.J. Lenssen, J. Luna, E. Wegner, S. Vogl, S. Sykes, D. Ramseyer, H. Ramseyer, N. von Sauer, E. Coburn, F. Burgos, and A. Hansen.

The mission of the Western Wheat Quality Laboratory 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, including a database of wheat cultivars relating kernel hardness and puroindoline alleles. Our research publications are available on our web site.

We are serving as curator of the grain hardness, puroindoline, and *GSP-1* gene sections of the Catalogue of Gene Symbols in Wheat. Several new alleles have been documented in *Ae. tauschii*, synthetic hexaploids from CIMMYT, and other diploid taxa. 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. Our current activities and projects include grain hardness and puroindolines, waxy wheat, polyphenol oxidase (PPO), arabinoxylans, SDS sedimentation test, and soft durums. Beecher and Luna currently are researching the genetic basis for noodle dough color stability. Bettge currently is researching the influence of oxidative gelation on flour end-use functionality. As such, he is developing a laboratory-scale method for pancake-making in order to provide an end-use test for flour functionality in batter systems.

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