SCIENTIA DANICA · SERIES B · BIOLOGICA · VOL. 3

# Natural dietary therapies for the 'gluten syndrome'

*By Sachin Rustgi, Nuan Wen, Claudia Osorio, Rhoda A.T. Brew-Appiah, Shanshan Wen, Richa Gemini, Jaime H. Mejias, Nii Ankrah, Charles P. Moehs & Diter von Wettstein*

Det Kongelige Danske Videnskabernes Selskab
The Royal Danish Academy of Sciences and Letters

DET KONGELIGE DANSKE VIDENSKABERNES SELSKAB
udgiver følgende publikationsrækker:
THE ROYAL DANISH ACADEMY OF SCIENCES AND LETTERS
issues the following series of publications:

| | AUTHORIZED ABBREVIATIONS |
|---|---|
| Scientia Danica. Series B, Biologica<br>*Formerly: Biologiske Skrifter*, 4°<br>(Botany, Zoology, Palaeontology, general Biology) | Sci.Dan.B |
| Scientia Danica. Series H, Humanistica, 4<br>*Formerly: Historisk-filosofiske Skrifter*, 4°<br>(History, Philosophy, Philology, Archaeology, Art History) | Sci.Dan.H.4 |
| Scientia Danica. Series H, Humanistica, 8<br>*Formerly: Historisk-filosofiske Meddelelser*, 8°<br>(History, Philosophy, Philology, Archaeology, Art History) | Sci.Dan.H.8 |
| Scientia Danica. Series M, Mathematica et physica<br>*Formerly: Matematisk-fysiske Meddelelser*, 8°<br>(Mathematics, Physics, Chemistry, Astronomy, Geology) | Sci.Dan.M |
| *Oversigt, Annual Report*, 8° | Overs.Dan.Vid.Selsk. |

**Correspondence**
Manuscripts are to be sent to

The Editor
Det Kongelige Danske Videnskabernes Selskab
H. C. Andersens Boulevard 35
DK-1553 Copenhagen V, Denmark.
Tel: +45 33 43 53 00
E-mail: kdvs@royalacademy.dk.
www.royalacademy.dk

Questions concerning subscription to the series should be directed to the Academy

**Editor** Marita Akhøj Nielsen

Natural dietary therapies for the 'gluten syndrome'

## Abstract

Wheat supplies about 20% of the total food calories consumed worldwide and feeds approximately half of the global demand for dietary proteins. Besides being a major source of energy and nutrition it is also a major cause of frequent diet-induced health issues especially celiac disease, gluten sensitivity, and food allergies, collectively known as the 'gluten syndrome'. Gluten-intake in the sensitive individuals can elicit various reactions, which in combination with their respective genetic constitutions lead to diverse symptoms from gastrointestinal or neurological to fatal. Among these disorders celiac disease is one of the most devastating and affects ~1% of the global population. Despite of its prevalence in all tested populations the only effective therapy so far is strict dietary abstinence from glutenous grains. We therefore have undertaken three approaches to develop natural dietary therapies for these diet-induced disorders: i) epigenetic elimination of the immunogenic prolamins using transgenic and non-transgenic procedures, ii) post transcriptional silencing of immunogenic prolamins via RNA interference and iii) post-translational detoxification of prolamins by ectopic expression of 'glutenases' in wheat grains for degradation of prolamins in the human gut after consumption. The present communication describes the progress made in these directions.



Sachin Rustgi[1], Nuan Wen[1], Claudia Osorio[1], Rhoda A.T. Brew-Appiah[1], Shanshan Wen[1], Richa Gemini[1], Jaime H. Mejias[2], Nii Ankrah[1], Charles P. Moehs[3] & Diter Von Wettstein[1,3,4,5]

[1]Department of Crop & Soil Sciences, Washington State University, Pullman, WA 99164, USA, [2] InserInstituto de Investigaciones Agropecuarias INIA, Camino Cajón-Vileún km 10, Vilcún, Chile. [3]Arcadia Biosciences, Seattle, WA 98104, USA, [4]School of Molecular Biosciences & [5]Center for Reproductive Biology, Washington State University, Pullman, WA 99164, USA

Email: rustgi@wsu.edu (SR) & diter@wsu.edu (DvW)

# Natural dietary therapies for the 'gluten syndrome'

*By Sachin Rustgi, Nuan Wen, Claudia Osorio, Rhoda A.T. Brew-Appiah, Shanshan Wen, Richa Gemini, Jaime H. Mejias, Nii Ankrah, Charles P. Moehs & Diter von Wettstein*


Scientia Danica. Series B, Biologica · vol. 3

DET KONGELIGE DANSKE VIDENSKABERNES SELSKAB

Natural dietary therapies for the 'gluten syndrome'


Printed in Denmark by Specialtrykkeriet Viborg a-s
ISSN 1904-5484 · ISBN 978-87-7304-376-9


Submitted to the Academy August 2013
Published March 2014

# Table of Contents

# Introduction

Celiac disease is one of the most common food-sensitive enteropathological conditions in humans and it is caused by an autoimmune reaction against certain wheat, barley and rye grain storage proteins. In human leukocyte antigen (HLA) DQ2- or DQ8-positive individuals exposure to these "gluten" proteins can lead to a painful chronic erasure of the microvilli of the epithelium in the intestine and to a permanent intolerance of dietary prolamins. The autoimmune response results from the resistance to digestion of certain proline/glutamine rich peptides (epitopes) in the prolamins by gastric, pancreatic and brushborder membrane proteases. Peptides like PFPQPQLPY are taken up through the intestinal mucosa into the lamina propria and initiate the autoimmune response. Celiac disease is commonly detected in congenital cases with severe symptoms in early childhood. In many and increasing numbers of patients, symptoms arise only later in life as a result of bread and pasta consumption. If untreated celiac disease may cause increased morbidity and mortality. Despite its prevalence in most populations comprising 24.4 million registered celiac individuals worldwide, the only effective therapy is strict dietary abstinence from these food grains.

However, because of the multiple presentations of the disease, many sufferers of this disease have not been formally diagnosed with it and estimates suggest that for every registered celiac there are 50 unrecognized individuals.

It is the purpose of this project to eliminate from wheat grains the prolamins containing majority of epitopes causing celiac disease. Eliminating these proteins will address also the other major quality problem for the consumer of wheat products: the imbalance in the amino acid profile of wheat proteins. Wheat grain is especially low in lysine, which is the first limiting amino acid in cereal proteins for humans and monogastric animals. Because the prolamin protein families that we are targeting are very lysine poor, their elimination will lead to a considerable increase in grain lysine content with concomitant improvement in the nutritional quality, which will be beneficial for all consumers of wheat products. The highly homologous storage proteins of wheat, barley and rye called prolamins fall into two groups: one group represented by the lysine poor gliadins and low molecular weight (LMW) glutenins of wheat that contain the overwhelming majority of the protein domains (epitopes) causing the celiac response are dispensable for baking and therefore need to be removed. The other group, represented by the wheat high molecular weight (HMW) glutenins, are alone required for dough formation and baking and therefore have to be retained. The molecular structure of these two types of prolamins is very different and their genes are turned on and off by two fundamentally different mechanisms, which provides the bases for elimination of the gliadins and LMW glutenins but preservation of the HMW glutenins. The genes for gliadins and LMW glutenins are silenced by DNA methylation of their promoters in vegetative tissues. The promoters have to be de-methylated at the beginning of endosperm development to permit their transcription and the synthesis of the encoded prolamins. The aim is to silence these genes permanently in the endosperm by mutations or using RNA interference. The six wheat genes encoding the elastic fibrils of the HMW glutenins that form dityrosine cross-links during dough formation and baking and pasta production are protected against DNA methylation in all tissues by a CpG island in their promoter. They are turned on alone by synthesis of transcription factors or removal of repressor proteins at the beginning of grain filling. Thus two categories of promoters for endosperm specific gene expression can be distinguished; one, that is silenced by methylation in vegetative tissues and has to be demethylated before activation of transcription can take place; the other is solely dependent on removal of repressors or induction of transcription fac-

tors specific for the endosperm (cf. Wen et al., 2012). Hexaploid wheat has in each genome on the long arm of chromosome 1 two linked genes encoding HMW glutenins. They are unmethylated in the developing endosperm and the other organs of the wheat plant. This provides the rationale for investigating, if in wheat the gliadins and LMW glutenins can be eliminated by transcriptional silencing of the 5-methyl cytosine DNA glycosylase (i.e. *DEMETER*) genes without affecting the synthesis of the HMW glutenins.

In addition successful implementation of RNA interference (RNAi) for silencing of α/β-, γ- and ω-gliadins in wheat (Gil-Humanes et al., 2010) formed the basis of another approach for silencing of genes encoding immunogenic gliadins and LMW glutenins. In order to synchronously silence a large number of prolamin genes expressed in the wheat endosperm we decided to test the possibility of developing a chimeric hairpin construct derived from short conserved stretches of DNA sequences identified from the sequence alignment of individual prolamin families. Such a hairpin is expected to provide simultaneous suppression of different prolamins. This approach has similar advantages as described for the previous strategy.

Furthermore in order to have a therapy for the patients sensitive to the HMW glutenin derived epitopes yet another but well-documented post-translational prolamin detoxification approach (cf. Bethune & Khosla, 2012) is adapted. This approach takes advantage of the biochemical properties of endo-proteases and endo-peptidases that preferentially cleave at proline and glutamine residues, which make them capable of degrading prolamins to non-immunogenic peptides. This approach has an added advantage for the healthy individuals as it expedites and improves the digestion of the gluten proteins, thus increase bioavailability of nutrients to the consumer and improve their general health.

# Statement of Progress

## 1. Objective 1

The 'high lysine' viable mutation *lys3a* inhibits the transcription of the barley hordein C, B, and γ prolamin genes by hypermethylation of the promoter and 4 CpGs from the adjacent coding region. The hypermethylation is characteristic for these prolamin genes in leaves but for their transcription during endosperm development they have to be demethylated. The structural gene for high molecular weight hordein D on the other hand is hypomethylated in all tissues due to the presence of a CpG island and therefore is expressed normally in the *lys3a* mutant. Hordein D is homologous to the high molecular weight (HMW)

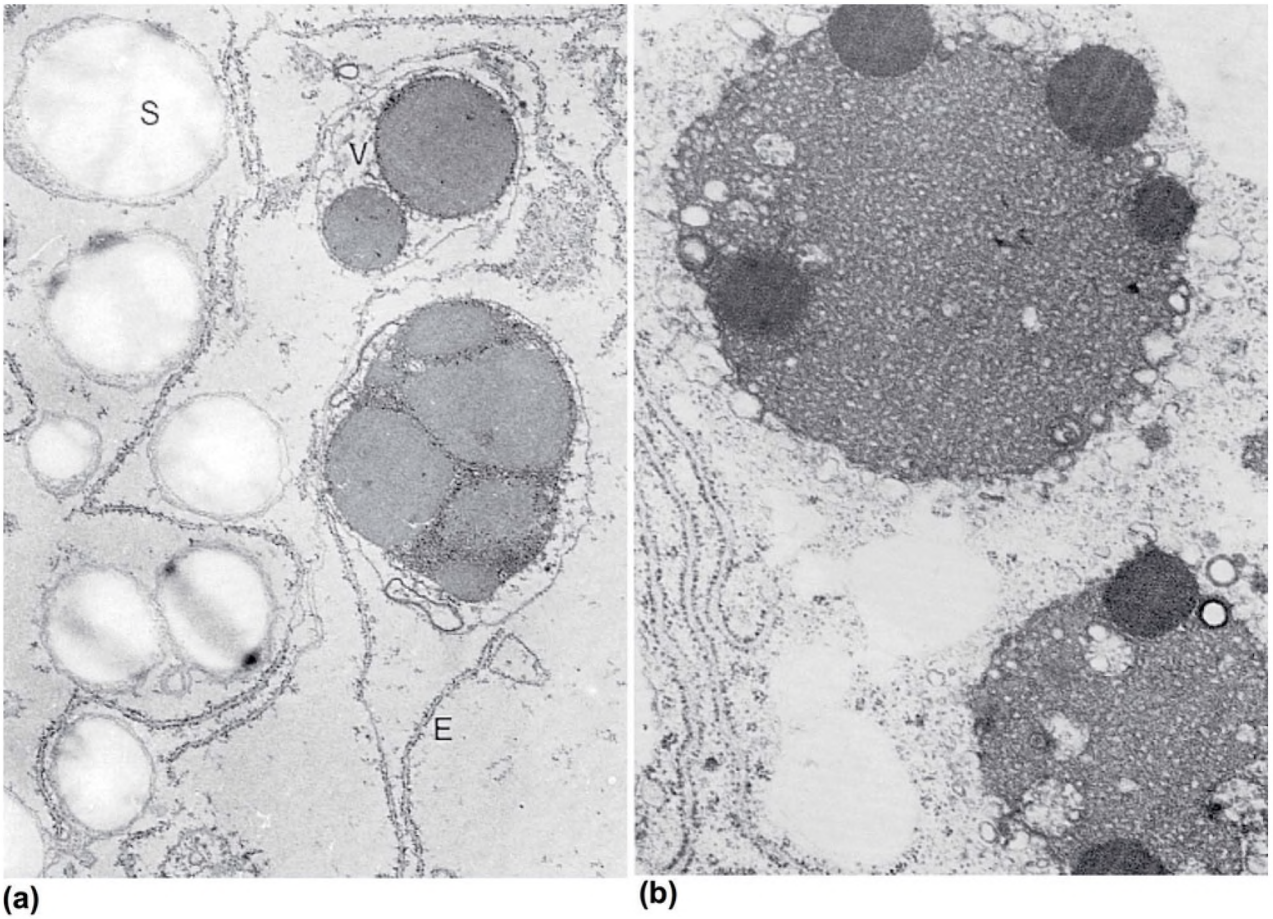



Figure 1.
(a) Transmission electron micrograph of thin section through wild type developing endosperm of barley, showing developing starch grains in the amyloplasts - S, extensive endoplasmic reticulum - E and depositions of prolamins in the storage vacuoles - V. These are of two types: The compact component contains the B, C and γ hordeins, which are homologues of the gliadins. The interspersed elastic spiral fibrils are the D-hordeins, the high molecular weight glutenins of barley (x 6000). (b) Comparable micrograph of the developing endosperm of the high lysine mutant *lys3a*. It contains only the elastic spiral fibrils of the D-hordein in the storage vacuoles. The globules are phytate, which are also present in the wild type but not contained in the micrograph of A (x 15000).

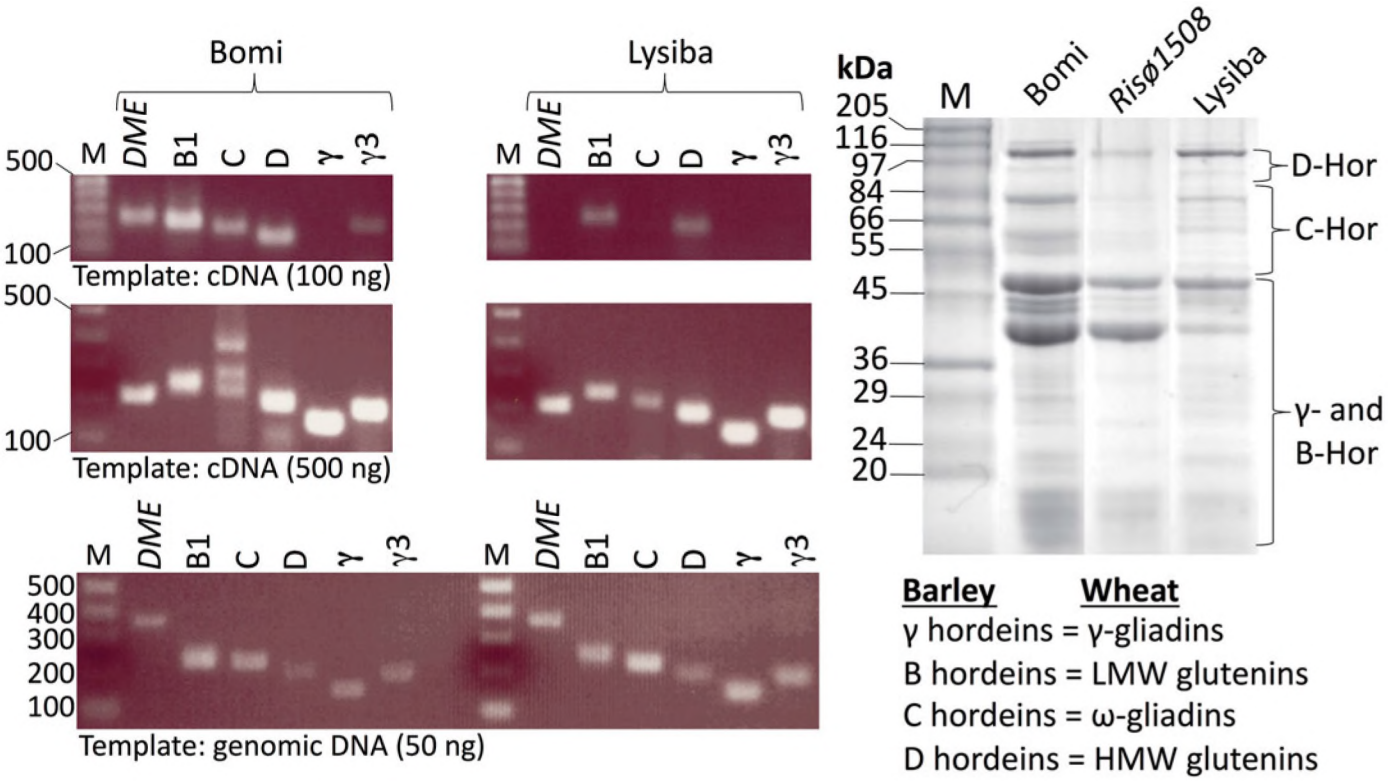



Figure 2.
Genomic DNA, transcript and protein profiling of wild type Bomi and high lysine barley cv. Lysiba (a derivative of Risø 1508). Analysis of genomic DNA with gene-specific primers for B1-, C-, D-, γ- and γ3-hordeins showed intact copies of these genes both in Bomi and Lysiba (lower left). Transcript profiling with the gene-specific primers using cDNAs derived from the developing grains of Bomi and Lysiba: i) At the lower initial template concentration showed expression of all hordeins in case of Bomi and only B1- and D-hordeins in case of Lysiba, and ii) at the higher initial template concentration showed minor differences between the expression profiles of Bomi and Lysiba (top left). Profiling of prolamins with SDS-PAGE revealed complete elimination of C-hordeins and reduced accumulation of B- and γ-hordeins in Risø 1508, whereas reintroduced of some of these prolamins was observed in Lysiba (right), which might be a consequence of breeding for increased grain yield.

glutenin of wheat. We want to test the hypothesis that the transcriptional inhibition is complete enough to eliminate all known celiac T-cell stimulating epitopes from the prolamins in the grain, with the exception of those in the HMW glutenins.

## 2. Results

Ultrastructural information provided by electron microscopy on the development of protein bodies in the barley endosperm of wild type, 'Bomi' and the high lysine mutant Risø 1508 suggested that the mutant produces practically no B- and C-hordeins (Fig. 1). However, profiling of prolamins (known as hordeins in barley) from 'Bomi', Risø 1508 and a derivative cultivar Lysiba on SDS-PAGE elucidated that Risø 1508 having *lys3a* allele is a mutant with near complete elimination of C-hordeins and revealed reduced accumulation of B and γ-hordeins (Fig. 2). Furthermore amplification of wild type Bomi and Lysiba genomic DNAs with the primers designed from genes encoding B1-, C-, D-, γ- and γ3-hordeins amplified product of expected size for each one of them confirming no insertion/deletion in the structural copies of these

genes. In addition sequence analysis of the PCR products revealed no variation in the sequence of any analyzed prolamin gene.

Contrarily the transcript profiling of Bomi vs. Lysiba using 100ng of the template cDNA in RT-PCR confirmed transcription of all genes in Bomi (except for γ3-hordein) and only B1 and D-hordeins in Lysiba. Albeit increasing the initial template concentration to 500ng showed amplification of all prolamin genes from Lysiba, whereas transcript abundance of C-hordeins is relatively low (Fig. 2). To characterize the most prevalent proteins retained in Risø 1508, gel slices with these proteins were excised and analyzed by Dr. Peter Roepstorff at the University of Southern Denmark, Odense. Proteins isolated from the gel slices were digested with trypsin, and the obtained peptides were sequenced via MALDI-TOF/TOF. The analysis of peptides revealed that the band #1 represents B3-hordein (gi|123459) and band #2 represents γ3-hordein (gi|288709). As B- and C-hordeins respectively constitute 70% and 24% of the total hordeins accumulated in barley endosperm it is worthwhile to test Risø 1508 mutant showing complete elimination of C-hordeins and reduced accumulation of B-hordeins for its immunotoxicity. In view of the above Dr. L.M. Sollid at University of Oslo, Norway performed the T-cell activity assays by isolating and purifying hordeins from 10 g samples of whole meal flour from field grown seeds of Risø 1508 and wild type Bomi. To test the immunogenic potential purified hordeins derived from Risø 1508 and Bomi were added individually to the T-cells isolated from blood samples of volunteers with celiac disease 6-days after commencing oral barley challenge. The test involved measuring the number of T-cells which produced gamma-interferon following overnight incubation with the purified protein, using an antibody assay for the level of gamma-interferon. The level of gamma-interferon was an indication of the extent of toxicity of the proteins in the grain. This measure of the celiac toxicity of the flour was then plotted as a function of the fresh weight of flour obtained from the grain. The analysis revealed 18-fold reduced immunotoxicity in Risø 1508 in comparison with the wild type Bomi.

To identify the gene underlying the *Lys3* locus and to elucidate its molecular function the following two approaches were undertaken: (i) genomics-guided functional cloning and (ii) candidate gene-based cloning.

### *2.1. Genomics-guided functional cloning of* Lys3 *locus*

Using translocation lines the *Lys3* locus was initially assigned to barley chromosome 7(5H) by Jensen in 1979. This genetic map with limited number of morphological markers was aligned with available 'gene maps' of chromosome 5H in GrainGenes (http://wheat.pw.usda.gov/cmap/) and also with the high density DNA marker based Bin map developed by Kleinhofs and coworkers (http://barleygenomics.wsu.edu/all-chr.pdf). The comparative mapping allowed assignment of *Lys3* locus to the Bin 3 on the genetic linkage map of barley chromosome 7 (5H) (Fig. 3a). Based on its assignment to the Bin 3 we used 6 DNA markers (*Abg705*, *Cdo669b*, *Abg064*, *Abg708*, *Abg497* and *Psr326*) mapping to the same recombination bin to track location of *Lys3* on corresponding wheat group 5 chromosomes using a unique set of wheat lines carrying characterized terminal chromosomal deficiencies. The cytogenetic mapping assigned two of the 'associated' markers, *Abg705* and *Psr326* to the sub-telomeric bin flanked by the deletion breakpoints of 5BS-5 on the proximal end and 5BS-6 on the distal end of chromosome 5BS (Fig. 3b).

Using wheat ESTs mapping to this deletion bin as queries and rice genomic DNA sequences as surrogate the corresponding region was determined on barley chromosome 5H. In view of narrowing down the region of interest a genomic-guided microarray based approach was followed. To identify differentially expressing genes between Risø 1508 (*lys3a*) and its parent variety Bomi, RNA was extracted from immature grains (17 days post-anthesis), converted to cDNA and used for hybridization on 44k barley Agilent chip. The experiment has resulted in identification of about 2,872 genes exhibiting more than two fold (p-value 0.05) expression level differences between Risø 1508 and Bomi. In comparison with Bomi 1,428 genes were

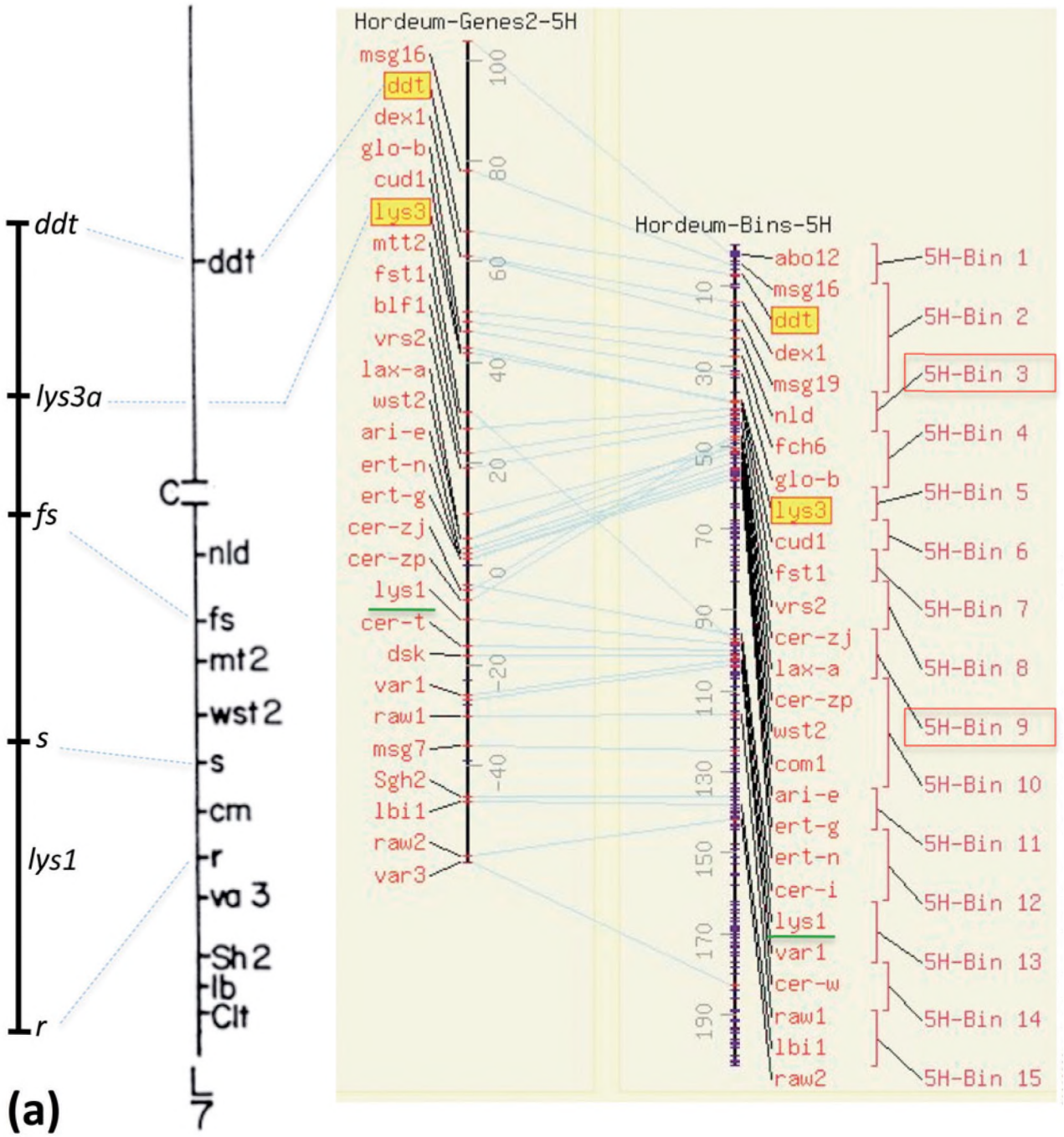
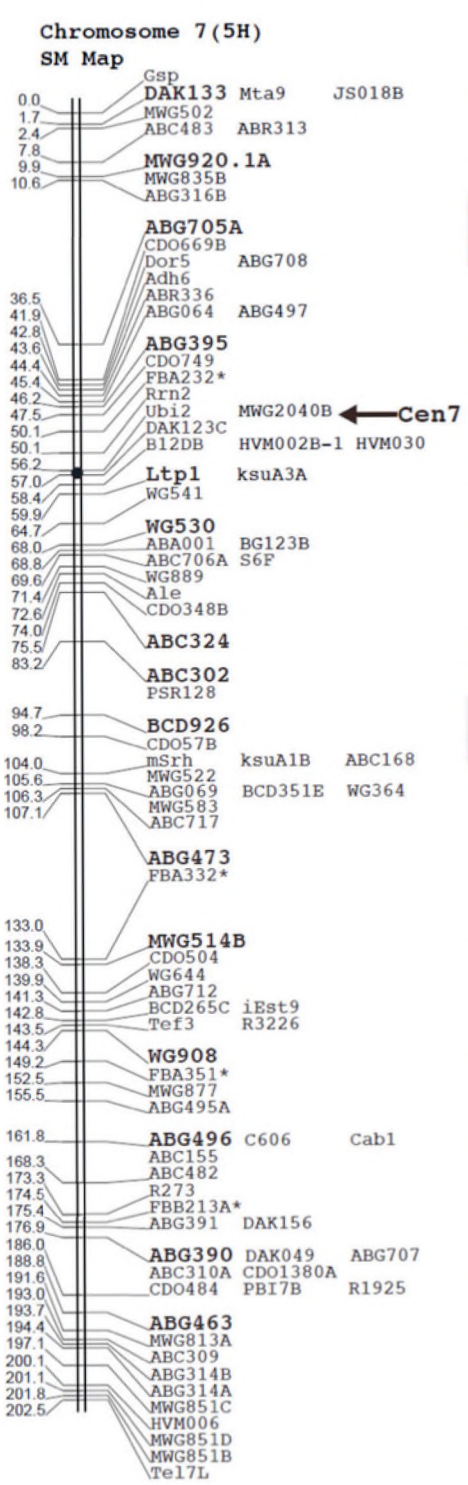



Figure 3.
(a) Comparative mapping of *Lys3* locus by aligning maps prepared using morphological and DNA based markers. Analysis allowed assignment of *Lys3* locus to barley chromosome 5H genetic Bin 3. (b) Assignment of *Lys3* locus to wheat chromosome 5B using barley chromosome 5H bin3 specific markers.




| | Markers mapped in other crosses and located to the Bin | | | |
|---|---|---|---|---|
| Bin1 | PSR1204 | ksuD4 | Tel7S | MWG618 |
| | Act8B | Lox1A | | |
| Bin2 | ABG610 | AWBMA12 | CDO1335A | CDO665C |
| | ABG615B | | | |
| Bin3 | cud1 | PSR326 | RphTR | BG140C |
| Bin4 | lax-a | AWBMA1 | AWBMA33 | AWBMA32 |
| | Acl1 | OPR3 | BCD410B | CDO460 |
| | Ym3 | | | |
| Bin5 | CDO460 | | | |
| Bin6 | nar2 | nar5 | cMWG770 | cMWG717 |
| | MWG596 | MWG526 | MWG561B | |
| Bin7 | ABG461D | Mlj | WG564 | RSB001A |
| Bin8 | | | | |
| Bin9 | var1 | lj | MWG923 | MWG956 |
| | MWG2121 | MWG624 | MWG604 | PBI39 |
| | MWG2237 | MWG2191 | CDO675B | CDO771A |
| | ksuE2A | WG364 | WG1026 | |
| Bin10 | MWG894 | RZ404 | Ugp3 | Xa21like |
| | HVDHN009 | MWG914 | MWG549B | MWG553A |
| | MWG550 | WG583 | WG1026 | MWG2230 |
| | mRaw1 | | | |
| Bin11 | cMWG781 | R3226 | ABC159B | ABG003B |
| | ABG702B | MWG533 | cMWG646B | cMWG716A |
| | MWG2092 | MWG2227B | MWG2224 | MWG862 |
| | MWG933 | MWG900 | Sgh2 | Dhn2A |
| Bin12 | DAK111 | Xyl | TubA3 | MWG922 |
| | CDO583 | | | |
| Bin13 | AMP744C | ARD065 | Aga5 | ARD578.3 |
| | ARD188.3 | CDO419C | PSR370 | ABC170C |
| | DAK200A | cMWG740 | ABC622 | cMWG654 |
| | C1257 | cMWG701A | MWG2020 | MWG2088 |
| | MWG827 | MWG850 | MWG2037A | CDO113A |
| | CDO213 | BCD298A | Ch1 | ARD032 |
| | E10757A | rpg4 | JS005C | |
| Bin14 | cMWG650 | C9A | MWG2193 | CDO484 |
| Bin15 | MWG632C | ABC718 | ABG057B | MWG891 |
| | MWG2249 | CDO506 | | |

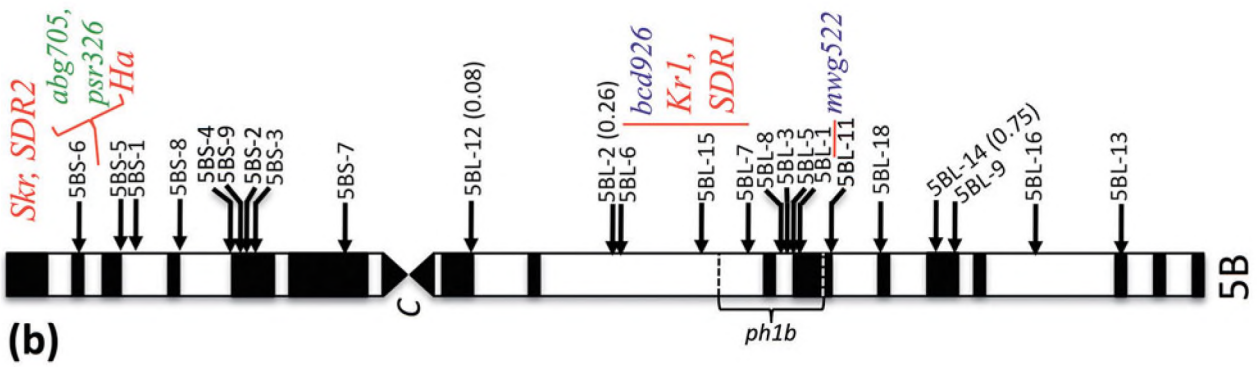



Figure 3 b

down regulated and 1,444 genes up regulated in Risø 1508. Sequences for these differentially expressed genes were retrieved from the HarvEST database (http://www.harvest-web.org/hweb/bin/srchEST.wc?Wsize =1255x572), and used to blast against rice genomic DNA sequences (http://gramene.org/Multi/blastview) and mapped wheat ESTs (http://wheat.pw.usda.gov/GG2/blast.shtml). Out of 2,872 sequences used for analysis against rice pseudo-chromosomes, 1,707 (59.44%) sequences detected corresponding loci on 12 rice chromosomes. Out of the above 1,707 sequences 424 (24.85%) sequences detected hits on rice chromosomes 3, 9 and 12 that correspond to barley chromosome 5H. And 89 of 424 differentially expressed genes specifically map to rice chromosome 12 that corresponds to barley chromosome arm 5HS carrying the gene of interest. Later based on the mapping effort of Druka and coworkers near isogenic lines (NILs) developed for a number of morphological mutants including one for *lys3a* gene (BW496) showed polymorphism exclusively on chromosome 1HS (rice chromosome 5) (Druka et al., 2011).

This analysis for the first time showed the possibility that the gene responsible for the high lysine content in Risø 1508 exists on chromosome 1HS instead of 5HS as was initially thought based on the previous mapping results (Jensen, 1979). Our analysis of differentially expressing genes between Risø 1508 and Bomi showed that 146 differentially expressed genes map to the corresponding rice chromosome 5 and out of these 146 genes 14 genes belonging to 5 rice BAC clones fall within the region flanked by the single nucleotide polymorphism (SNP) markers bracketing the polymorphic regions in barley NIL. Thus we identified a small region of barley chromosome 1H potentially harboring *lys3a*. Scientists at IPK, Gatersleben provided information of barley BAC clones mapping in this region. This BAC scaffold in barley is represented by 21 BAC contigs and spans 10.21 Mb of sequence which is 13.36 times larger than that of rice BAC scaffold (763.841 kb) lining the region of interest (Fig. 4). The genes are currently being predicted and annotated in this sequence. The predicted gene will be blasted against genes showing differential expression between Risø 1508 and Bomi in the microarray experiment. All genes mapping to this region (including the once showing differential expression) will be classified on the basis of their function, and the likely candidates will be functionally characterized using RNA interference and/or virus induced gene silencing, which will ultimately allow identification of the underlying gene.

### 2.2. *Candidate gene-based cloning of* Lys3 *locus*

In parallel to the former approach a candidate gene based approach was followed to clone *Lys3* gene. Based on the functional similarity between the barley *Lys3* and *Arabidopsis DEMETER* (*DME*) genes, we undertook cloning of barley and wheat *DME* genes.

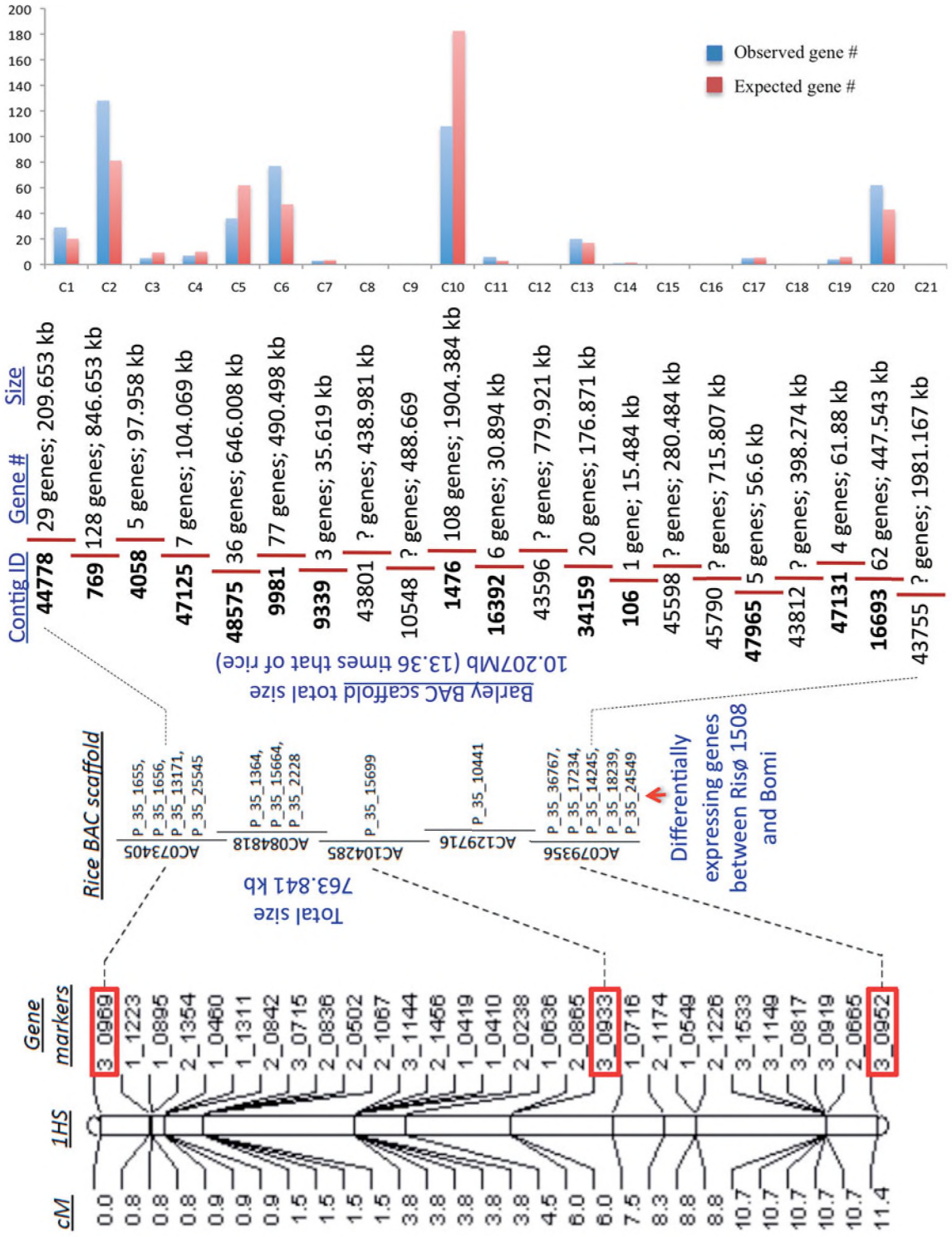



Figure 4.
Single nucleotide polymorphism (SNP)-based map of a specific region on chromosome 1H showing polymorphism between Bowman and a NIL ($BC_5$), BW496 for *lys4* locus in Bowman background (left). BAC scaffold of the corresponding region on rice chromosome 5 showing location of 14 differentially expressing genes between Risø 1508 and Bomi. Twenty-one BAC contigs represent the corresponding region in barley. The number of putative genes identified in these BAC clones is shown on right. The observed and expected number of genes identified in each BAC contig is shown on extreme right.

| Accession | Genotype | HSVs 5537bp | 5652bp | 5821bp | 5850bp | 6019bp | 6048bp | 6070bp | |
|---|---|---|---|---|---|---|---|---|---|
| >TA52016_4565 | | C | C | C | G | C | A | C | |
| >CJ666546 | CS | C | C | C | G | C | A | C | |
| >TC304399 | | C | C | C | G | C | A | C | |
| >CD888432 | recital(reversed) | C | C | C | G | C | A | C | |
| >BE446194 | CS | C | C | C | G | C | A | C | |
| >EB514264 | CS | | | C | G | C | A | C | **Haplotype 1** |
| >CJ540593 | kitaKEI1354(reversed) | | | | G | C | A | N | |
| >BE515735 | Brevor | C | C | C | G | | | | |
| >WHE0603_A07_B13ZA | Brevor | C | C | C | G | | | | |
| >BE443993 | CS | | C | C | G | | | | |
| >WHE1123_H05_P09ZS | CS | | C | C | G | | | | |
| >GH728100 | recital | T | T | T | T | T | A | C | |
| >TA51414_4565 | | T | T | T | T | T | A | C | |
| >CJ727182 | Valuevskaya | T | T | T | T | T | A | C | |
| >TC284057 | | T | T | T | T | T | A | C | |
| >CJ896441 | Thatcher(reversed) | | T | T | T | T | A | C | **Haplotype 2** |
| >CJ623341 | Valuevskaya(reversed) | | T | T | T | T | A | C | |
| >CK208658 | Norstar | | | T | T | T | A | C | |
| >CJ857516 | Scout66 | | | T | T | T | A | C | |
| >CJ870300 | Scout66(reversed) | | | T | T | T | A | C | |
| >BJ314693 | CS | C | C | C | T | C | | | |
| >CA643900 | | | | C | T | C | G | T | |
| >CD886960 | recital | | | C | T | C | G | T | |
| >CJ684257 | CS | | | C | T | C | G | T | |
| >BJ320180 | CS(reversed) | | | C | T | C | G | T | **Haplotype 3** |
| >CJ894494 | Thatcher(reversed) | | | | T | C | G | T | |
| >CJ577643 | CS(reversed) | | | | T | C | G | T | |
| >CJ882700 | Thatcher | | | | T | C | G | T | |

Figure 5.
List of expressed sequence tags (ESTs) showing three haplotypes corresponding with the three homoeologous copies of *DEMETER* represented by the respective BAC clones. HSVs = homoeologous sequence variants; CS = Chinese Spring. [Source: Wen et al., 2012 Proc. Natl. Acad. Sci. U.S.A., Copyright (2012)]

### 2.2.1. Cloning of the barley DEMETER gene

A barley transcript assembly TA38047_4513 (TIGR database) of 2312 bp showing 77.47% nucleotide identity and 56.52% coverage of *Arabidopsis DME* gene was used to design DEMETER-specific primers. The primers TA38047_F: 5'-TGTGCGTCTTTT-GACACTCC-3'and TA38047_R: 5'-GCTCGTA-CAATGTCCGTTGA-3' were used to amplify the desired fragments from the barley genomic DNA and a cDNA library prepared from the developing grains. PCR products were visualized by gel electrophoresis. The PCR reactions using the above primers yielded 342 and 187 nucleotide fragments respectively from barley genomic DNA and cDNA. The sequence of the genomic fragment covered introns no. 6 and 7 and adjacent domains with a nucleotide identity of 91.6%

TABLE 1. List of primers used for chromosomal localization of *DEMETER* homoeologues.

| Primer designation | Primer sequence (5'-3') | Product size (bp) | Primer allocation |
|---|---|---|---|
| 5A_2159_1:F | CACAATTAGTTGAGACGGGAAT | 200 | Intron13 |
| 5A_2159_1:R | CCTTCAAGCCTGATTGATGC | | |
| 5A_2159_2:F | ACCTGAAGTTCCTGCTGACA | 303 | Exon3-Intron3 |
| 5A_2159_2:R | TGCCCACATGTCCATATGACTA | | |
| 5B_1946_1:F | TCAAAAAGCAAATTCTGAACTCC | 370 | Exon3-Intron3 |
| 5B_1946_1:R | TTGCCCACATGTCTAAATACAGAAC | | |
| 5B_1946_2:F | ACAGAAATACCTCTGGCCTCGATTATGC | 546 | Exon7-Intron9 |
| 5B_1946_2:R | AAATATCATCAGTCGCTGCCGTCAAG | | |
| 5D_2106_1:F | ACAAAACTAGTGGGAACAGCAG | 528 | Introns13-15 |
| 5D_2106_1:R | CGTGAATTAATCCATGGAGTAGAT | | |
| 5D_2106_2:F | TCAAGACCCTATGAGTCCATAAC | 370 | Intron4 |
| 5D_2106_2:R | AGACCTATCACAAGAAAACTAATGG | | |

[Source: Wen et al., 2012 Proc. Natl. Acad. Sci. U.S.A., Copyright (2012)]

between rice and barley. The fragment was radioactively labelled and hybridized to the filters of the barley BAC library of cv. Morex. The probe hybridized to a single BAC clone 27314i4, which has been subcloned and sequenced. For sequencing the transposon EZ-Tn5™<KAN-2>Insertion Kit (Epicentre® Biotechnologies, Madison WI) was used. The BAC clone 27314i4 was subcloned with *Eco*RI and *Hin*dIII into pBluescript and fingerprinted. The selected clones with the fragments were incubated for 2 h with EZ-Tn5<KAN-2>transposon and EZ-Tn5 transposase and the reaction mixture transformed into competent *recA E. coli* cells. Kanamycin resistant colonies were isolated, the clones sequenced and the contigues assembled (Genbank accession no. FM164415.1). A full-length cDNA clone of barley *DME* gene was also obtained by reverse transcriptase polymerase chain reaction (RT-PCR) followed by restriction digestion and ligation of 2 fragments obtained by RT-PCR. The resultant fragment (>5.9 kb) was then cloned in pNEB206A (New England BioLabs, Inc. Cat. # N5502L).

### 2.2.2. Cloning of the three wheat DEMETER genes

A pair of *DME*-specific primers (DME-F1: 5'-TGTGCGTCTTTTGACACTCC-3' and DME-R1: 5'-GCTCGTACAATGTCCGTTGA-3') potentially amplifying a 356 bp fragment from the homoeologous copies of the *DME* gene (nucleotide pos. 13263-13618 on FM164415.1) was designed, and used for amplification of wheat genomic DNA. The fragments thus obtained were used as probe to screen the Chinese Spring BAC library (consisting of 1.3 million clones, representing 7x coverage of the genome) through macroarray hybridizations (mean insert size of clones is 130 kb), which lead to the

TABLE 2. List of structural changes observed between *DEMETER* homoeologues.

| | Length in bp | | | Average | HSVs | |
|---|---|---|---|---|---|---|
| | 1946 | 2159 | 2106 | (bp) | Observed* | Expected |
| Exon1 | 2836 | 2815 | 2833 | 2828 | 78 (43) | 65.21 |
| Exon2 | 91 | 91 | 91 | 91 | 2 | 2.10 |
| Exon3 | 1543 | 1543 | 1543 | 1543 | 34 (16) | 35.58 |
| Exon4 | 80 | 80 | 80 | 80 | 0 | 1.84 |
| Exon5 | 85 | 85 | 85 | 85 | 2 | 1.96 |
| Exon6 | 92 | 92 | 92 | 92 | 3 | 2.12 |
| Exon7 | 69 | 69 | 69 | 69 | 1 | 1.59 |
| Exon8 | 37 | 37 | 37 | 37 | 0 | 0.85 |
| Exon9 | 83 | 83 | 83 | 83 | 2 | 1.91 |
| Exon10 | 444 | 444 | 444 | 444 | 4 | 10.24 |
| Exon11 | 37 | 37 | 37 | 37 | 1 | 0.85 |
| Exon12 | 58 | 58 | 58 | 58 | 1 | 1.34 |
| Exon13 | 140 | 140 | 140 | 140 | 1 | 3.23 |
| Exon14 | 66 | 66 | 66 | 66 | 0 | 1.52 |
| Exon15 | 115 | 115 | 115 | 115 | 2 | 2.65 |
| Exon16 | 39 | 39 | 39 | 39 | 0 | 0.90 |
| Exon17 | 134 | 134 | 134 | 134 | 4 (1) | 3.09 |
| Total | 5949 | 5928 | 5946 | 5941 | 135 | |

*Number of HSVs causing amino acid substitutions is given in parenthesis.
[Source: Wen et al., 2012 Proc. Natl. Acad. Sci. U.S.A., Copyright (2012)]

identification of 7 BAC clones that were partially sequenced using *DME*-specific primers, allowed identification of three unique BAC clones. The three BAC clones (1946D08, 2106P11 and 2159B03) identified were sequenced at >60-fold coverage by 454 sequencing method at the DNA Sequencing Core, Washington State University, Pullman, WA. A total of 38.9 Mb of high quality sequence was obtained for three BAC clones namely 1946D08, 2106P11 and 2159B03. Ninety-four percent of 13.4 Mb sequence obtained for 1946D08 was assembled into 10 large contigs covering 147 kb of the BAC insert. Similarly, 88% of 14.5 Mb of the sequence obtained for 2106P11 and 87% of 11 Mb of the sequence obtained for 2159B03 were assembled into 5 and 6 large contigs respectively covering 157 kb and 102 kb of the BAC inserts. Analysis of sequences obtained from the above three BAC clones revealed that each of them harbors a full-length *DME* sequence.

Comparison of the full-length wheat *DME* sequences with all available wheat ESTs in the public domain revealed that the three homoeologues are transcriptionally active (Fig. 5). The ESTs showing homology with *DME* homoeologues were assembled in a contig. These ESTs were derived from 10 different wheat cultivars [including 11 ESTs from Chinese Spring (CS), 5 ESTs from Recital, 4 ESTs each from Thatcher, etc.]. The ESTs assembled in a contig were carefully examined for the presence of homoeologous sequence variants (HSVs) allowing partitioning of the EST-contig into three sub-contigs. These sub-contigs virtually represent transcripts derived from three

| | Length in bp | | | Average | HSVs | |
|---|---|---|---|---|---|---|
| | 1946 | 2159 | 2106 | (bp) | Observed | Expected |
| Intron1 | 227 | 226 | 227 | 226.67 | 11 | 20.36 |
| Intron2 | 97 | 100 | 100 | 99.00 | 10 | 8.89 |
| Intron3 | 135 | 147 | 139 | 140.33 | 21 | 12.60 |
| Intron4 | 1709 | 1791 | 1725 | 1741.67 | 92 | 156.43 |
| Intron5 | 78 | 79 | 79 | 78.67 | 3 | 7.07 |
| Intron6 | 78 | 77 | 77 | 77.33 | 3 | 6.95 |
| Intron7 | 83 | 83 | 83 | 83.00 | 4 | 7.45 |
| Intron8 | 80 | 80 | 81 | 80.33 | 5 | 7.22 |
| Intron9 | 431 | 432 | 428 | 430.33 | 27 | 38.65 |
| Intron10 | 146 | 155 | 154 | 151.67 | 19 | 13.62 |
| Intron11 | 86 | 85 | 86 | 85.67 | 16 | 7.69 |
| Intron12 | 89 | 88 | 88 | 88.33 | 6 | 7.93 |
| Intron13 | 1335 | 1334 | 1330 | 1333.00 | 101 | 119.73 |
| Intron14 | 109 | 109 | 109 | 109.00 | 3 | 9.79 |
| Intron15 | 1430 | 1141 | 1059 | 1210.00 | 232 | 108.68 |
| Intron16 | 568 | 568 | 565 | 567.00 | 31 | 50.93 |
| Total | 6681 | 6495 | 6330 | 6502 | 584 | |

*DME* homoeologues (Fig. 5). The virtual transcript profiling of wheat *DME* homoeologues suggested that the three homoeologues contribute almost equally to the transcript abundance, and are expressed in various different tissues including grains, anthers, developing and mature spikes and roots.

In addition, comparison of wheat *DME* homoeologues with mapped wheat ESTs showed high homology (e-value = 0.0 and Score = 731) with BE471039, and allowed their assignment to the long arm of wheat group *5* chromosomes (5AL, 5BL and 5DL; Fig. 6). Assignment of *DME* homoeologues to the specific sub-genomes of bread wheat was performed using homoeologue-specific primers, designed by tagging their 3'-ends at homoeologue sequence variants (HSVs) (Table 1). Each of the homoeologue-specific primer pairs was used on the genomic DNA of diploid wheat progenitors [*Triticum urartu* (AA), *Aegilops speltoides* (BB), and *Ae. tauschii* (DD)] with Chinese Spring [*T. aestivum* (AABBDD)]. These primers allowed unambiguous assignment of 2159B03 to A sub-genome, 1946D08 to B sub-genome and 2106P11 to D sub-genome of common wheat (Figs. 6 & 7). Additionally, the chromosomal and sub-genome assignment of *DME* homoeologues was further validated by the use of wheat group *5* nulli-tetrasomic lines (Fig. 6). Whereas, sub-chromosomal location of 5B *DME* homoeologue to the subcentromeric-bin bracketed by the deletion breakpoints of 5BL-12 [fraction length (FL)-0.08] and 5BL-2 (FL-0.26), encompassing 98.78 Mb of genomic DNA on 5BL, was determined using 5B specific terminal and interstitial deletion lines (Fig. 6).

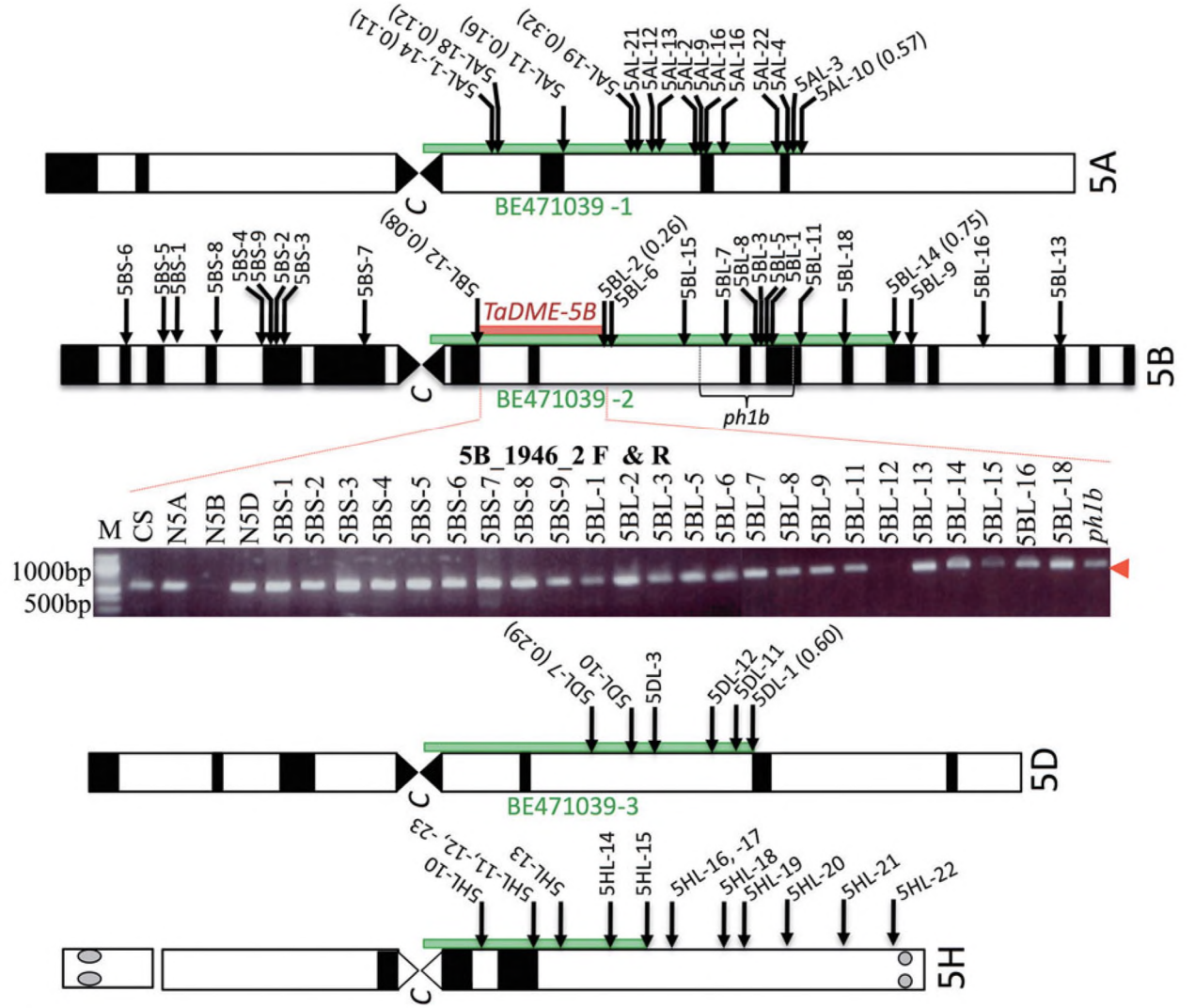



Figure 6.
Cytogenetic deletion map of wheat and barley *DME* homologues on wheat group 5 (5A, 5B and 5D) and barley 5H chromosomes. The green bars show gross chromosomal locations (based on homology with mapped wheat ESTs and/or synteny and colinearity in case of barley) and the red bar shows precise location (based on wheat nulli-tetrasomic and deletion lines). Sub-chromosomal localization of wheat *DME* homoeologue on 5B was determined using sub-genome specific primers 5B_1946_2 F and R (Table 1) on the genomic DNA of wheat cultivar Chinese Spring (control), nulli-tetrasomic lines for group *5* chromosomes, deletion lines for long and short arms of chromosome 5B, and an interstitial deletion line *ph1b*. *TaDME-5B* localizes to the sub-centromeric bin of the long arm of chromosome 5B (5BL). Specific product indicated by arrow head; M = 100bp ladder. [Source: Wen et al., 2012 Proc. Natl. Acad. Sci. U.S.A., Copyright (2012)].

Genomic location of the barley *DME* homologue (*HvDME*) was determined using rice as a proxy, where wheat ESTs mapped to specific chromosomal bins were used to identify syntenous region on rice chromosome 9, and rice BAC/PAC sequences were searched against mapped barley ESTs. The analysis allowed localization of *HvDME* to chromosome 5H between centromere (*C*) and 5HL-15 (Fig. 6).

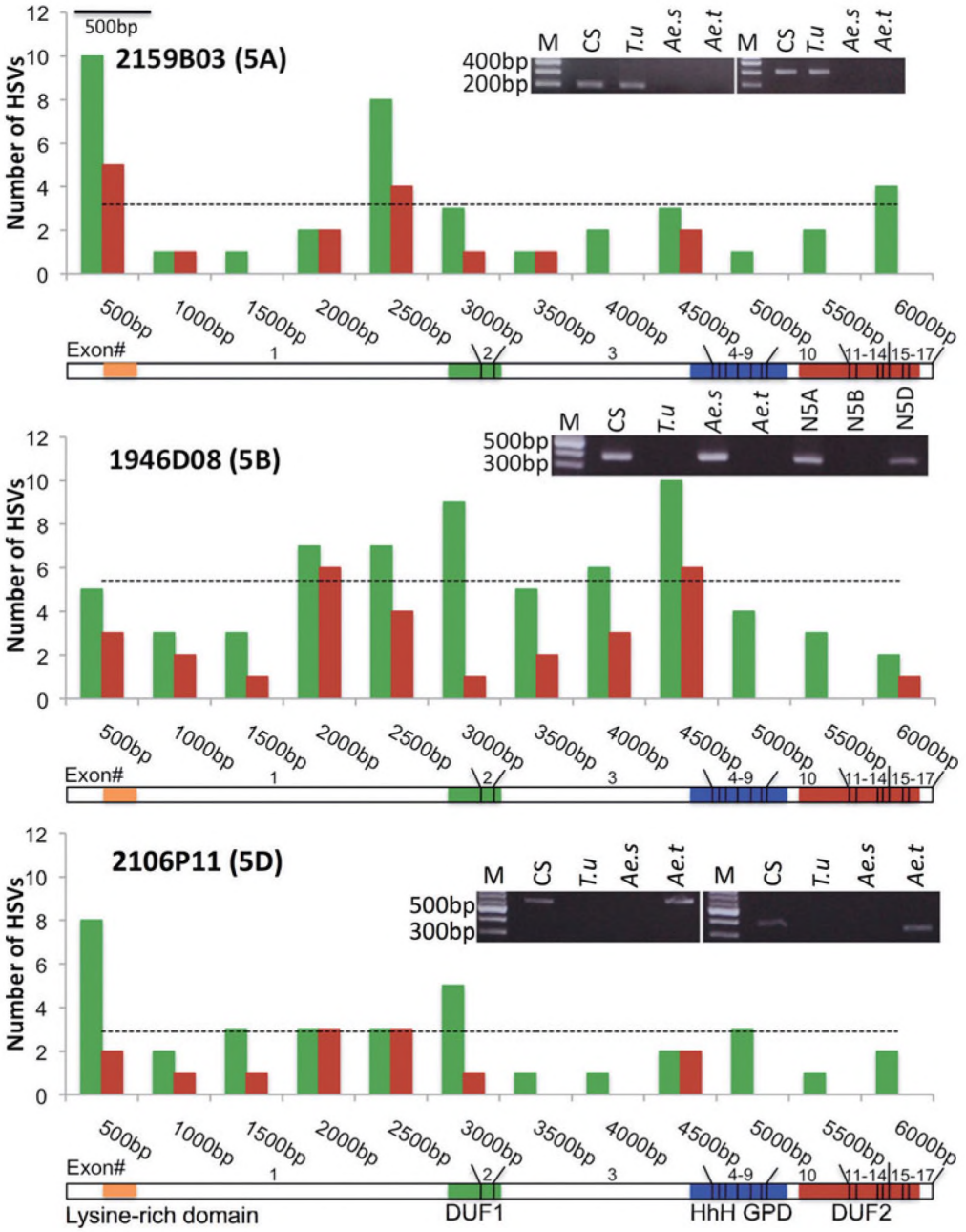



Figure 7.
Bar diagrams showing observed and expected (dotted line) frequencies of homoeologous sequence variants (synonymous changes in green and non-synonymous changes in red) across the length of wheat *DME* homoeologues. Location of different exons and four functional domains were shown below each bar diagram. PCR amplification profiles obtained using sub-genome specific primers on the genomic DNAs extracted from diploid wheat progenitors (*T.u* = *T. urartu* 'A' sub-genome donor, *Ae.s* = *Ae. speltoides* most likely 'B' sub-genome donor, and *Ae.t* = *Ae. tauschii* 'D' sub-genome donor) and 'Chinese Spring' (AABBDD) was shown at the top of each bar diagram. To confirm assignment to wheat *DME* homoeologues two primer pairs each from BAC clones 2159B03, 1946D08 and 2160P11 were designed and tested (Table 1). The primers for 1946D08 were additionally validated on wheat nulli-tetrasomic lines for group 5 chromosomes. [Source: Wen et al., 2012 Proc. Natl. Acad. Sci. U.S.A., Copyright (2012)]

TABLE 3. List of structural differences observed between DEMETER homologues.

| Species | Gene | Accession # | Lysine-rich domain | Linker region 1 | DUF1 | Linker region 2 | HhH-GPD | Linker region 3 | DUF2 | Genomic location |
|---|---|---|---|---|---|---|---|---|---|---|
| *Triticum aestivum* (5A) | *TaDME-5A* | JF683316 | 72aa | 714aa | 108aa | 447aa | 214aa | 41aa | 274aa | Chromosome 5A |
| *Triticum aestivum* (5B) | *TaDME-5B* | JF683317 | 72aa | 721aa | 108aa | 447aa | 214aa | 41aa | 274aa | Chromosome 5B |
| *Triticum aestivum* (5D) | *TaDMA-5D* | JF683318 | 72aa | 720aa | 108aa | 447aa | 214aa | 41aa | 274aa | Chromosome 5D |
| *Hordeum vulgare* | *HvDME* | CAQ58412.1 | 72aa | 720aa | 108aa | 447aa | 214aa | 44aa | 271aa | Chromosome 5H |
| *Oryza sativa* | *OsDME* | BAF04322.1 | 75aa | 694aa | 111aa | 435aa | 214aa | 45aa | 245aa | Chromosome 1R |
| *Sorghum bicolor* | *SbDME* | JF683319 | 93aa | 708aa | 110aa | 410aa | 191aa | 45aa | 271aa | Chromosome 4 |
| *Arabidopsis thaliana* | *AtDME* | NP_001078527.1 | 93aa | 564aa | 108aa | 394aa | 214aa | 43aa | 272aa | Chromosome 5 |

[Source: Wen et al., 2012 Proc. Natl. Acad. Sci. U.S.A., Copyright (2012)]

### 2.2.3. Nucleotide diversity among wheat DEMETER homoeologues

The three *DME* sequences differ in length from each other ranging from 12.27kb for 2106P11 to 12.63kb 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 (collectively referred as HSVs) between *DME* homoeologous also exist in exons, and contribute to the observed diversity in the protein sequences. A total of 135 HSVs giving a frequency of 22.7 HSVs/kb in exons and 584 HSVs giving a frequency of 90 HSVs/kb in introns was observed. Out of 135 HSVs 60 (44.44%) contribute to amino acid substitutions in at least one of the three *DME* homoeologues (Table 2). When expected and observed HSV frequencies were plotted against the distance in nucleotides for three *DME* homoeologues, interestingly, highest level of nucleotide diversity was observed in *TaDME-5B* followed by *TaDME-5A* and *TaDME-5D* (Fig. 7).

For *TaDME-5A*, total number of HSVs peaked in the beginning and end of exon 1 and was kept lower than the expected number of HSVs until exon 15. The HSVs causing amino acid changes follow the same trend keeping the nucleotide diversity low in vicinity of 4 functional domains (including lysine rich domain, DUF1, HhH-GPD + FES, and DUF2). For *TaDME-5B* the frequency of HSVs gradually increased from the middle of exon 1 persisted high till exon 3 and then gradually declined thereafter. However, frequency of HSVs causing functional changes followed the same trend as observed for *TaDME-5A*, keeping nucleotide diversity low in vicinity of all functional domains. Similarly, *TaDME-5D* also followed the same trend of nucleotide diversity as observed for *TaDME-5A* (Fig. 7). These observations indicated a natural selection against non-synonymous point mutations (selective sweep) in these domains, and also reflected towards their functional importance.

### 2.2.4. Phylogenetic analysis of DEMETER

Comparison of amino acid sequences of DME revealed high levels of similarity within the grass lineage as well as with *Arabidopsis* (Fig. 8a). The sizes of five functional domains and the linker regions connecting various domains were kept highly conserved between plants belonging to very distant taxonomic groups. Specifically, sizes of domain of unknown function 1 (DUF1) ranged from 108-111 amino acids

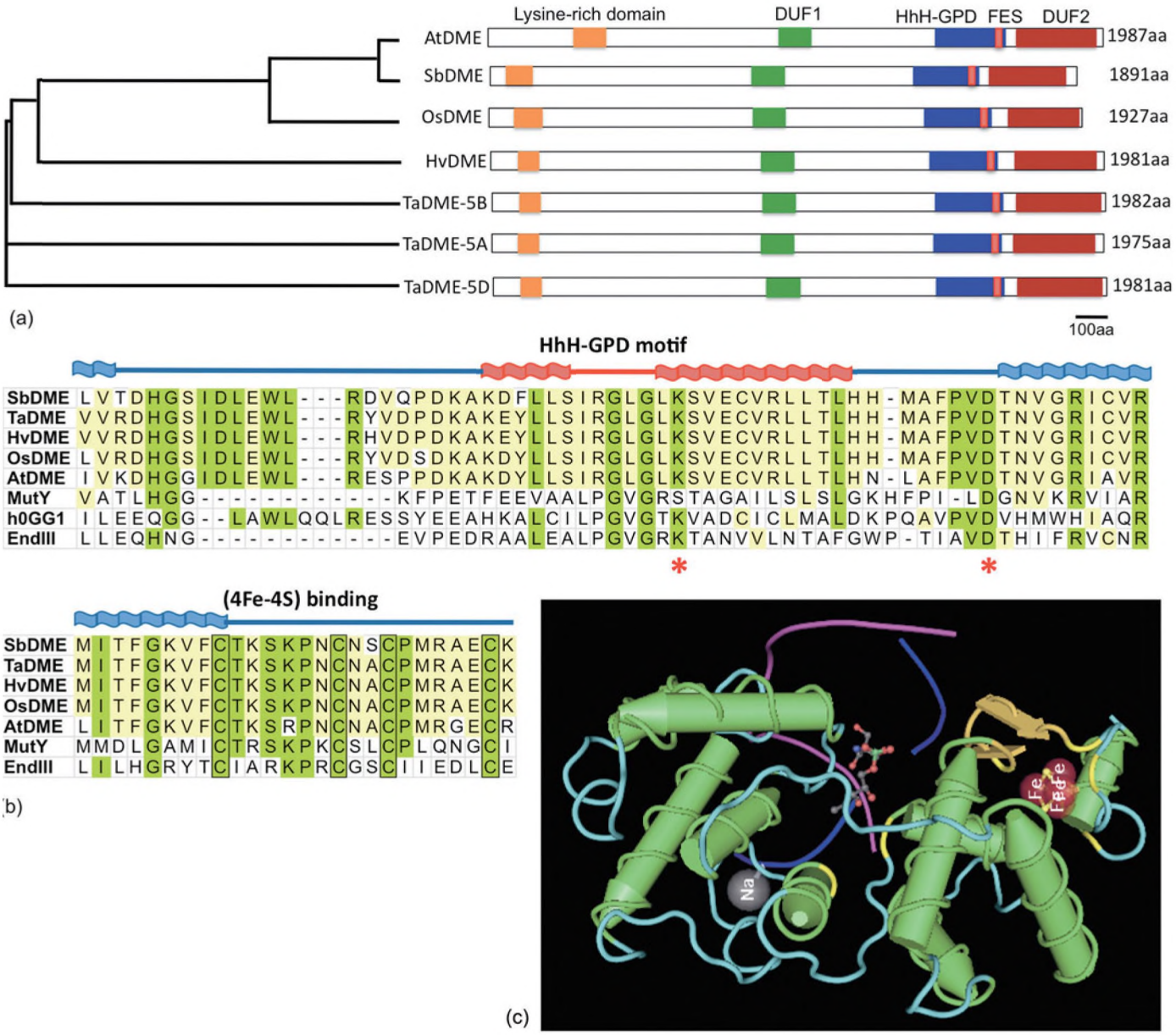



Figure 8.
(a) Phylogenetic analysis of DME homologues from *Arabidopsis* (NP_001078527.1), rice (BAF04322.1), sorghum (JF683319), barley (CAQ58412.1) and wheat (JF683316-JF683318) showing high level of conservation at sequence as well as structural levels. (b) Diagrammatic representation of DME protein showing four conserved domains with a magnified view of helix-hairpin-helix domain and iron sulfur cluster underlying the active site of the enzyme showing homology among 5-methylcytosine DNA glycosylases obtained from different organisms. (c) 3D structural model of the glycosylase domain of EndoIII in complex with DNA to show close functional conservation among different glycosylases. [Source: Wen et al., 2012 Proc. Natl. Acad. Sci. U.S.A., Copyright (2012)]

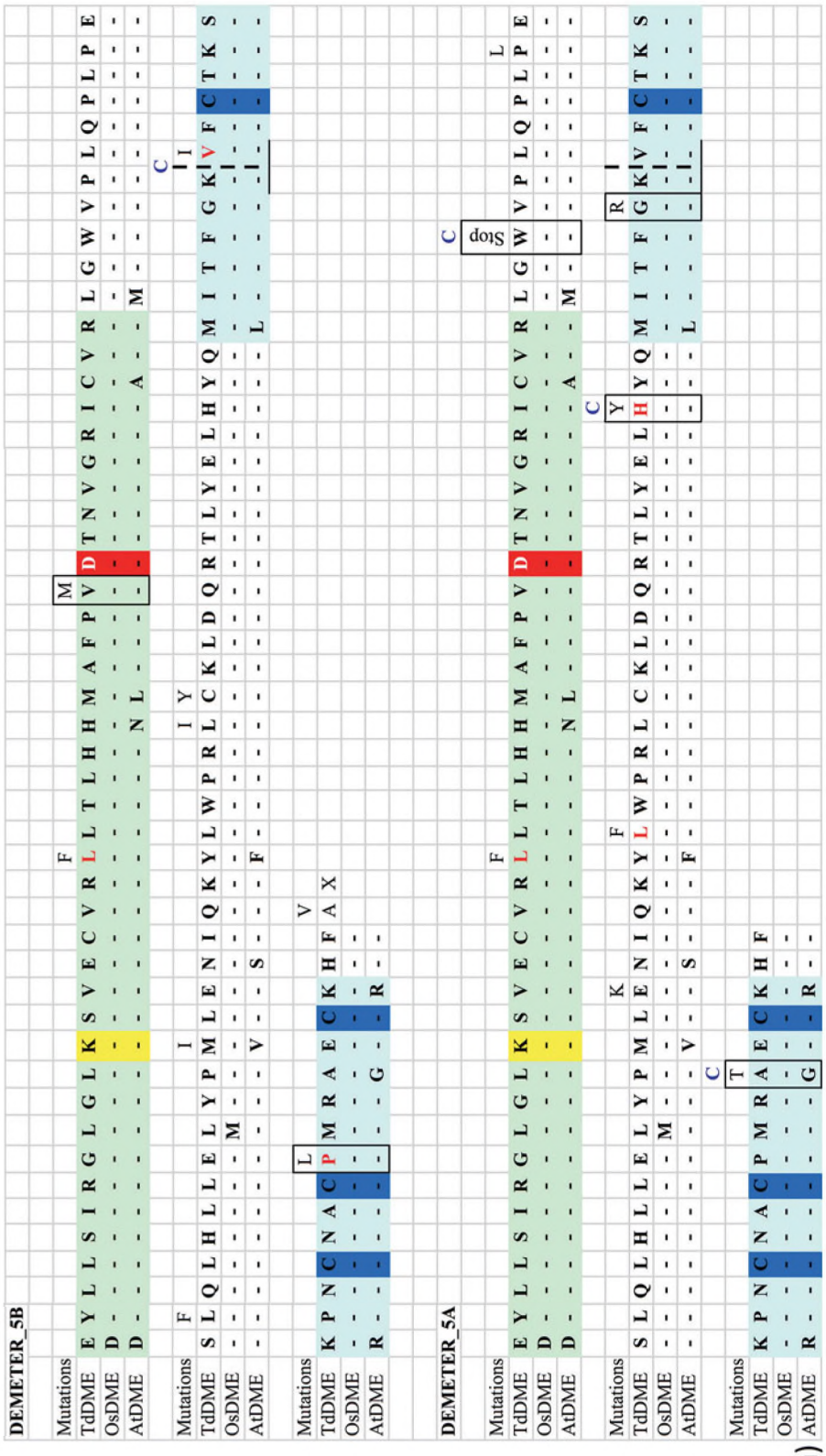



Figure 9.
Diagrammatic representation of the mutations identified in the active site of wheat *DEMETER* homoeologues in (a) 'Express' and (b) 'Kronos' backgrounds. The glycosylase domain including helix-hairpin-helix-Gly/Pro rich loop followed by a conserved aspartate and iron-sulfur cluster were respectively highlighted by light green and light blue colors. Conserved lysine 'K', aspartic acid 'D' and cysteine 'C' residues are respectively highlighted by yellow, red and blue colors. Splice site variants are indicated by dashed line. Mutations analyzed further for validation of mutant genotype and zygosity are boxed or underlined in case of splice site variants. Bold red font = mutations previously reported in Arabidopsis and shown to disrupt enzyme activity (Mok et al. 2010); C = mutants used for crossing to obtain *DEMETER* double mutations.

DEMETER_5D
DEMETER_5B
DEMETER_5A
Mutations
TaDME
OsDME
AtDME
Stop

(b)

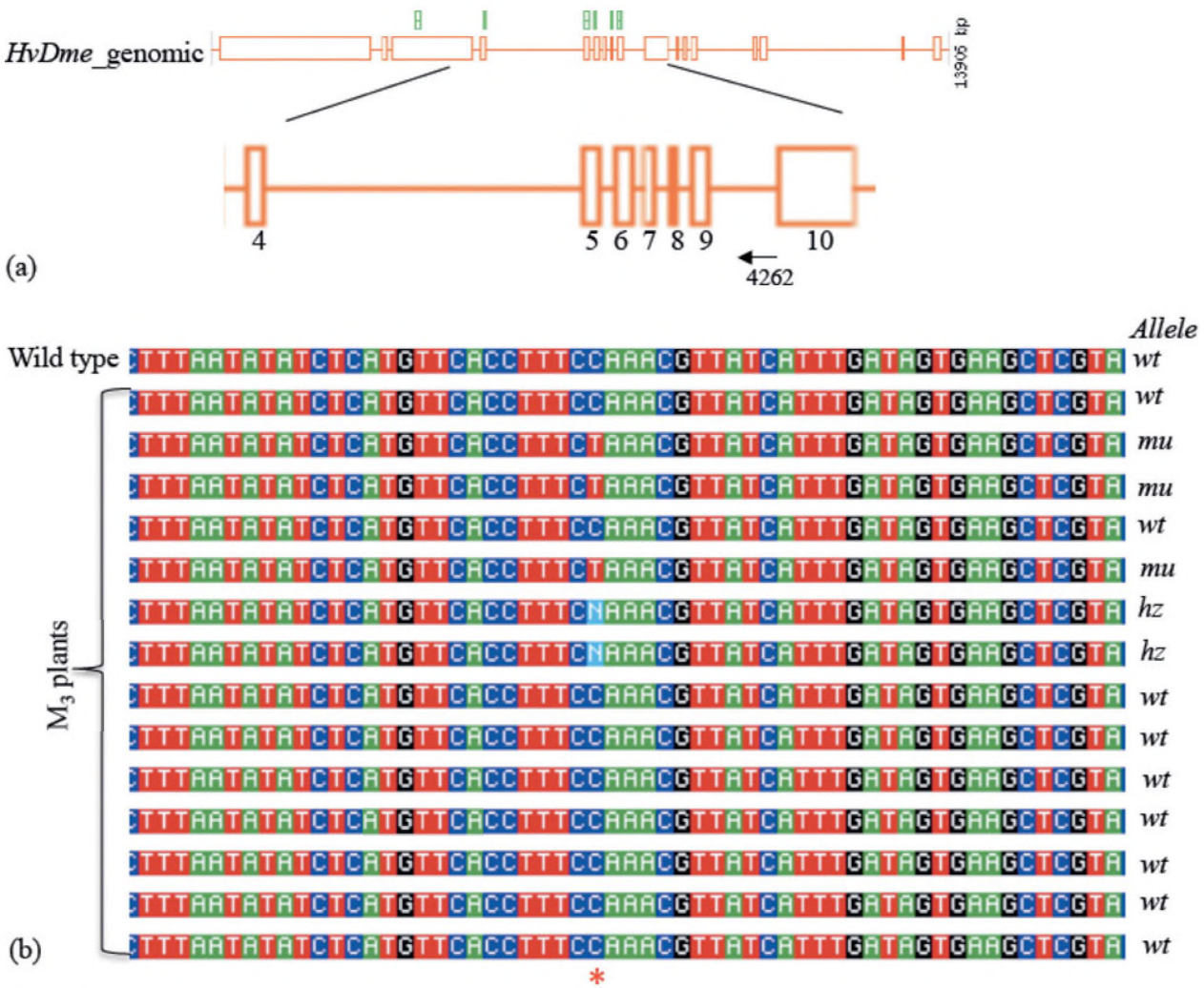
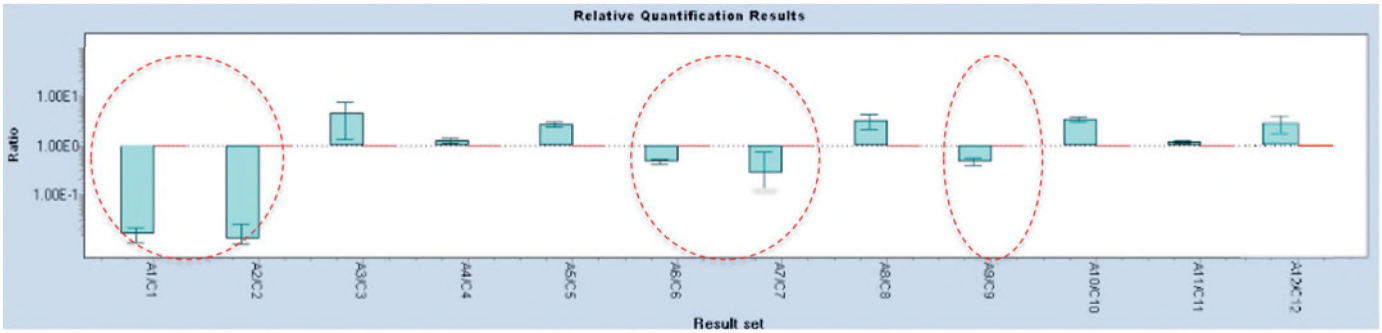



Figure 10.
Diagrammatic representation of *DEMETER* gene sequence **(a)** highlighting exons 4 to 10 (rectangular boxes) showing position of *DME* 'B' homoeologue specific primer (4262) used for sequencing of PCR products obtained from $M_3$ plants in 'Express' background. **(b)** Results of sequencing confirmed the zygosity of $M_3$ plants obtained from heterozygous $M_3$ seeds for the G/C to A/T mutation changing Gly to Arg in the enzyme sequence. Wt = wild type, Mu = mutant and Hz = heterozygote.

(AAs), glycosylase domain [including HhH-GPD and iron-sulfur cluster (FeS) motifs] ranged from 191-214 AAs, linker region 3 and DUF2 ranged from 41-45 AAs and 245-274 AAs, respectively among different plant species (Fig. 8a; Table 3). The HhH-GPD domain showed conservation across kingdoms, indicating towards the functional conservation of a short-patch DNA base excision repair (BER) pathway for these proteins (Fig. 8b,c).

## 3. Conclusion

Profiling of prolamins from Risø 1508 (*lys3a*) and a derivative cultivar Lysiba revealed that Risø 1508 is a mutant with near complete elimination of C-hordeins and partial elimination of B- and γ-hordeins. T-cell based assay was performed to determine level of prolamin immunotoxicity in this mutant. In comparison with the wild type Bomi the mutant revealed 18-fold




| Sample Name | Targets | References | Mean Cp | Mean Cp |
|---|---|---|---|---|
| 1 D8K-42773 | DME | Actin | 30.38 | 24.38 |
| 6 D8K-33412* | DME | Actin | 29.84 | 23.56 |
| 66 D8E-20396 | DME | Actin | 23.56 | 25.68 |
| 80 D8E-50322 | DME | Actin | 24.72 | 25.04 |
| 81 D8E-50325 | DME | Actin | 24.23 | 25.65 |
| 88 D8E-50324 | DME | Actin | 25.89 | 24.80 |
| 110 D8E-21599 | DME | Actin | 27.34 | 25.49 |
| 130 D7K-36505 | DME | Actin | 23.86 | 25.52 |
| 132 D7K-36505 | DME | Actin | 24.75 | 23.64 |
| 138 D7K-32166 | DME | Actin | 24.04 | 25.76 |
| K Kronos | DME | Actin | 24.79 | 25.03 |
| E Express | DME | Actin | 24.04 | 25.76 |

Figure 11.
Expression profiling of *DEMETER* using RNA extracted from the immature $M_4$ grains of homozygous mutant plants using Roche Light Cycler 480. *DEMETER* mRNA level was normalized to *Actin*. Dotted circles indicate $M_4$ plants with suppression of *DEMETER* transcripts.

reduction in immunotoxicity. The level of suppression is quite encouraging instigating us for molecular characterization of this mutant using two different approaches: i) A genomics-guided functional cloning approach that allowed us to track the location of *Lys3* gene to the short arm of barley chromosome 1H, and ii) a candidate gene based approach that allowed us to clone and map wheat and barley homologues of *Arabidopsis DEMETER* (a 5-methylcytosine DNA glycosylases/lyase) gene. Cytogenetic mapping of wheat and barley *DME* genes allowed their assignment to the long arms of wheat group 5 chromosomes and barley chromosome arm 5HL, respectively. The results indicated that albeit *Lys3* and *DEMETER* control expression of immunogenic prolamins (low molecular weight glutenins and gliadins) by active demethylation of their promoters in the developing endosperm however, they are different genes. This is evident from the localization of *Lys3* gene on chromosome arm 1HS and of *DME* gene on chromosome arm 5HLhowever, detailed molecular characterization of the *Lys3* gene requires further investigation.

## 4. Objective 2

We want to test the hypothesis that transcription of wheat gliadin genes are regulated by the same mechanism. Since the *lys3a* gene is not cloned, we wish to select "high lysine" *lys3a* ortholog mutants in wheat $M_2$ generations of mutagenized seed and analyze their promoter methylation state during endosperm development. Both approaches have the potential to obtain barley and wheat lines that lack the majority of the known and potential celiac T-cell stimulating epitopes. The resulting plants will limit the requirement of elimination of celiac causing peptides to the single HMW glutenin in barley and to the six wheat HMW glutenins that are encoded by two adjacent

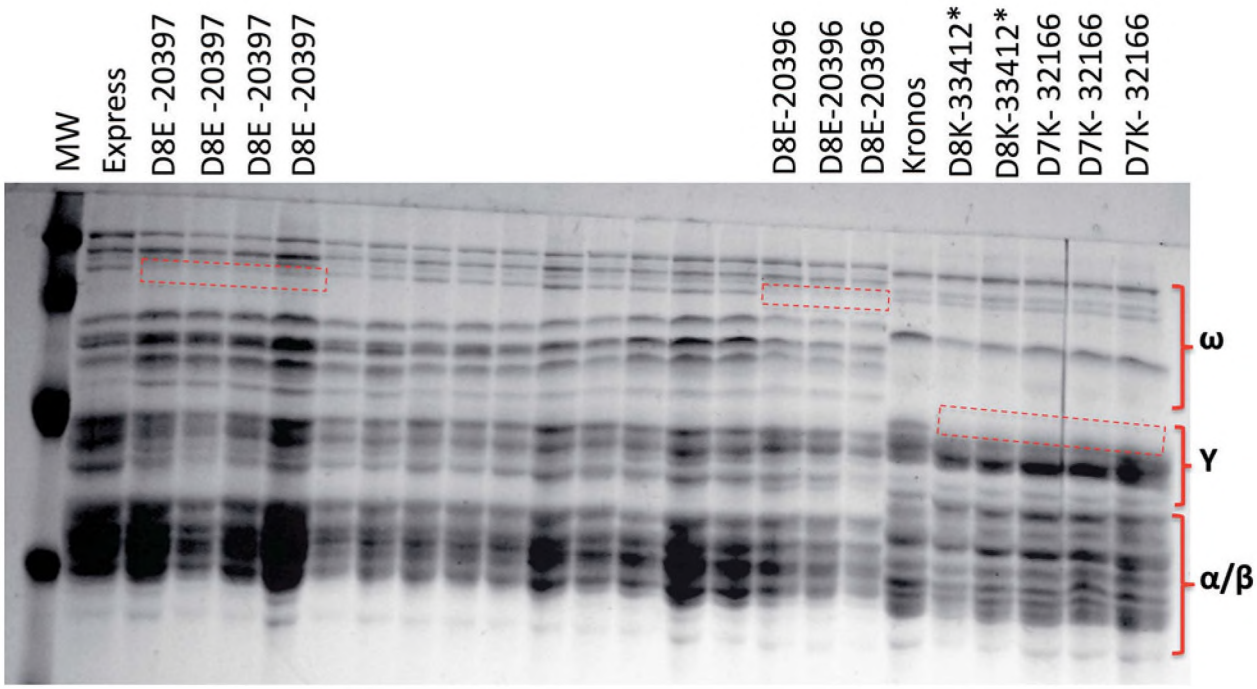



Figure 12.
Profiling of gliadin-fraction extracted from the mature grains of homozygous $M_3$ plants on SDS-PAGE. Dotty rectangular boxes mark the position of missing bands.

genes in each of the three wheat genomes. These proteins are to the best of our knowledge alone decisive for baking quality.

## 5. Results

The following experiments were undertaken to assure conservation of mechanism for transcriptional regulation of prolamin genes of wheat and barley, and to ascertain if *DEMETER* regulates accumulation of immunogenic prolamins in the developing endosperm of wheat grains similar to high-lysine barley mutant Risø 1508 (*lys3a*).

### *5.1. CpG-islands in the wheat prolamin genes*

High homology between the promoters (85-89%) and transcribed (71-79%) regions of wheat high molecular weight glutenin (HMWg) and barley D-hordein genes, and the presence of CpG-islands (CpGis) bracketing the transcription start site (TSS) of the D-hordein genes preventing their methylation and subsequent silencing, instigated us to examine wheat and barley prolamin sequences for the presence of CpGis. Out of 47 HMWg sequences derived from different wheat cultivars and representing various HMWg alleles, CpGis could be predicted in 38 cases leaving 9 cases where no island could be detected (Appendix 1). All HMWg sequences that escaped prediction of a CpGi belong to the *Glu-D1* locus except for the one case where information is not available. In contrast a CpGi was always predicted in the sequences belonging to *Glu-A1* and *Glu-B1*, but in both cases at two different locations i) bracketing the TSS and ii) after the TSS (within 120 bp). The prediction of CpGi after TSS could be argued due to the lack of sequence information and not because of their sheer locations. But the validity of two kinds of predictions can be advocated by the cases where enough sequence information is available (e.g., EU137874). The distribution of CpGi is even more diverse for sequences belonging to the *Glu-D1* locus, where CpGis were predicted at the

following 3 locations or not at all i) in 5'-untranslated region (UTR), ii) after TSS, and iii) at the most common location, i.e., bracketing the TSS. The diverse locations of CpGi in the D sub-genome copies of HMWgs indicate an intricate pattern of their transcriptional regulation that might have evolved after the second round of polyploidization in common wheat and/or as a consequence of breeding. The HMWg sequences lacking CpGi when compared with the sequences with presence of CpGi showed significant homology with each other except for the regions where CpGis are predicted. Close examination of the sequences showed presence of multiple C to T substitutions and InDels in the CpGis predicted regions. This leads to the overall reduction in the CG content of these regions causing them to escape CpGi prediction. When sequences were retrieved for LMWgs and gliadins no CpGis could be detected for any of the 31 LMWgs and 30 gliadins (Appendix 1) except for two cases (both belonging to α-gliadins). Interestingly when 5¢-end of some of the HMWg gene sequences were examined carefully they showed high homology with α-gliadin sequences. This throws light on the evolution of α-gliadin genes with CpGi involving illegitimate recombination followed by selection.

### *5.2. TILLING of DEMETER homoeologues in tetraploid and hexaploid wheat*

We screened tetraploid 'Kronos' and hexaploid 'Express' TILLING populations for *DEMETER* knockout/-down-expressed mutations. Average mutation density in an ethyl methanesulfonate (EMS) mutagenized $M_2$ population of Kronos was 1 mutation every 40kb DNA, and of Express was 1 mutation every 24kb DNA. The mutations are mostly single nucleotide polymorphisms or small deletions. Two runs, one each with a set of sub-genome specific primers were executed on Kronos and Express $M_2$ DNA-bulks. The sub-genome specific *DEMETER* primers amplified 1050 bp from 'A' sub-genome and 1044 or 855 bp respectively from the 'B' sub-genome of Kronos and Express (Table 4). The amplified region represents exon 5 to exon 9 of the *DME* gene, which includes the active site of the enzyme. In total 42 mutations in the 'A' sub-genome and 35 mutations in the 'B' sub-genome of the *T. durum DEMETER* homoeologues (*Td-DEM*) were detected. Out of 42 mutations detected for the 'A' homoeologue and 35 mutations detected for 'B' homoeologue, 4 and 5 mutations respectively coincided with the conserved motifs jointly forming

TABLE 4. List of homoeologues-specific *DEMETER* primers for screening of Kronos and Express TILLING libraries.

| Primer designation | Primer sequence (5'-3') | Product size (bp) | Primer allocation |
|---|---|---|---|
| DME_A_4259F | GGGAAGTTTGCATGGTTGACTGAAATAA | 1050 (K; E) | Intron 4-9 |
| DME_A_4261R | GGGAAATATTATCAGTCGATGCCATCAAA | | |
| DME_B_4260F | TAAAAGGGTTATTCTAAAAGTTATATTATGCG | 1045 (K) | Intron 4-9 |
| DME_B_3910F | TGTGTGCGTCTTTTGACACTCCAT | 855 (E) | Exon 5-Intron 9 |
| DME_B_4262R | AAATATCATCAGTCGCTGCCGTCAAG | | |
| DME_D_1555F | CCTTAGATGAATGTTTTTGGCGAAC | 1008 | Intron 4-9 |
| DME_D_2514R | ATACACAGTTCCACAGGAAACTCGA | | |

TABLE 5. Summary of mutations detected in tetraploid and heaxploid wheats via TILLING.

| Cultivar | Sub-genome | Indivs. screened | Total (unique); frequency | Coding (unique); frequency | Nonsynonymous/ nonsense (unique) | Synonymous (unique) | Noncoding (unique); frequency | Active site | Between domains |
|---|---|---|---|---|---|---|---|---|---|
| Kronos | A | 3456 | 42 (36); 1/86kb | 17 (15); 1/213kb | 9 (8) | 8 (7) | 25 (21); 1/145kb | 3 (S) | 5 |
| | B | 3840 | 35 (26); 1/115kb | 17 (16); 1/236kb | 10 (9) | 7 (7) | 18 (10); 1/223kb | 4 (S) | 5 |
| Express | A | 3456 | 42 (37); 1/86kb | 18 (16); 1/202kb | 9 (9) | 9 (7) | 24 (21); 1/151kb | 5 (S) | 4 (S) |
| | B | 3456 | 56 (44); 1/53bp | 35 (26); 1/84kb | 25 (20) | 10 (6) | 21 (18); 1/141kb | 4 (S) | 16 |
| | D | 13824 | 93 (63); 1/150kb | 56 (34); 1/249kb | 28 (19) | 28 (15) | 37 (29); 1/377kb | 11 (S) | 8 |

S = splice site variant

the glycosylase domain [helix-hairpin-helix (HhH) motif and a glycine/proline-rich loop with a conserved aspartic acid (GPD)] of the DEMETER enzyme. A mutation resulting in a premature stop codon, falling between the two conserved motifs was also detected in 'A' homoeologue of *TdDEM*. Similarly, 42 mutations in 'A' sub-genome and 56 mutations in 'B' sub-genome of the *T. aestivum DEMETER* homoeologues (*TaDEM*) were detected (Fig. 9; Table 5). Out of 42 mutations detected for the 'A' homoeologue and 56 mutations detected for the 'B' homoeologue, 6 and 5 mutations respectively coincided with the conserved motifs, whereas 4 mutations two each detected in 'A' and 'B' homoeologues resulted in either a premature stop codon or splice-site variants, fall between the two conserved motifs. Similarly, a 'D' sub-genome specific primer pair amplifying a 1008bp fragment from the wheat genome was used to screen Express $M_2$ DNA-bulks. Three rounds of screening allowed identification of 93 mutations in 'D' homoeologue of *TaDEM*. Out of these 93 mutations 11 coincided with the glycosylase domain (Fig. 9; Table 5). One mutation resulted in a premature stop codon, fell between the two conserved motifs forming the active glycosylase domain. Interestingly a point mutation at the conserved aspartic acid residue, which repeatedly had been shown to disrupt enzyme activity in *Arabidopsis*, was also recovered.

A total of 344 $M_3$ plants including 130 plants respectively representing 3 and 7 (either in homozygous or heterozygous state) mutations in 'Kronos' and 'Express' backgrounds, underlying the conserved domains of B subgenome *DEMETER* homoeologue and 214 plants representing 5 and 9 mutants respectively in 'Kronos' and 'Express' backgrounds underlying the conserved domains of A subgenome *DEMETER* homoeologue were planted in glasshouse. Similarly, a total of 44 $M_3$ plants representing 9 (either in homozygous or heterozygous state) mutations in 'Express' background, underlying the conserved domains of D subgenome *DEMETER* homoeologue were propagated in glasshouse. DNA was extracted from the leaves of 2 weeks old plants, used for amplification of subgenome-specific *DEMETER* products, and to determine zygosity of plants by sequencing of the PCR products (Fig. 10a, b). The analysis allowed identification of at least one homozygous $M_3$ plant for each heterozygous $M_2$ stock, except for the four cases

**Express**

| A♂ \ B♀ | 66 | 76/78 |
|---|---|---|
| 45 | 22 | 7 |
| 67 | 36 | 49 |

| A♀ \ B♂ | 66 | 76/78/80 |
|---|---|---|
| 45 | 13 | 7 |
| 67 | 44 | 31 |

45: D7E20548, V29M, A sub-genome; 67: D7E12680, stop codon, A sub-genome;
66: D8E20396, stop codon, B sub-genome; 76: D8E50322, M89I, B sub-genome;
78: D8E50322, M89I, B sub-genome; 80: D8E50322, M89I, B sub-genome

Note: Plants involving 78:D8E50322 in crosses are also segregating for a background striata mutant (green and yellowish-white striped leaves).

**Kronos**

| A♂ \ B♀ | 20 |
|---|---|
| 26 | 1 |
| 138 | 21 |

| A♀ \ B♂ | 20 |
|---|---|
| 26 | 58 |
| 129 | 11 |
| 138 | 81 |

20: D8K33412, splice site, B sub-genome; 26: D7K29375, A111T, A sub-genome;
129: D7K36505, stop codon, A sub-genome; 138: D7K32166, H86Y, A sub-genome

Figure 13.
Diagrammatic representation of crosses made between the TILLING mutations identified in the 'A' and 'B' sub-genome *DEMETER* homoeologues. Numbers in the grids represent total number of seeds obtained per cross.

where no homo/heterozygous mutant plant could be recovered (Table 6). Immature seeds from the spikes of the homozygous $M_3$ plants were collected and used for RNA extraction to study suppression of *DEMETER* transcripts by real-time quantitative PCR (qRT-PCR). Out of 19 homozygous $M_3$ plants 10 showed suppression at transcript level (Fig. 11). Ten of the 19 homozygous mutants were also analyzed for their efficiency to prevent synthesis of gliadin and low molecular weight glutenins by protein profiling of mature grains using SDS-PAGE. Interestingly, 4 of the 10 analyzed mutants showed elimination of specific bands either from ω or γ-gliadin fractions (Fig. 12). Although we do not expect elimination of bands in these mutant lines as they only contain mutations in one of either two or three *DEMETER* homoeologues in tetraploid or hexaploid wheat, respectively.

Single mutations in 'A' and 'B' sub-genome *DEMETER* homoelogues of bread and durum wheat identified as above were crossed in combinations to obtain *DEMETER* double mutants. All crosses were made reciprocally and in duplicates. The $F_1$ and later on $F_2$ seeds were obtained for 3 and 8 different mutant combinations respectively in Kronos and Express backgrounds (Fig. 13). The mutations for crossing were selected on the basis of type (substitution, splice site variation or premature stop codon), respective location in the *DEMETER* active site, and level of suppression at transcriptional and translational levels. The $F_2$ grains obtained from the aforementioned crosses were propagated in 48 well flats and are currently being checked for homo-/heterozygous double mutations by PCR followed by sequencing (Fig. 14). The preliminary analysis allowed identification of 20 double mu-

TABLE 6. List of selected mutants checked for their genotypes and zygosities at $M_3$ stage. Immature and mature grains were harvested from the homozygous mutants to be checked for their transcriptional and protein profiles.

| ID | Mutation | Effect | Zygosity determined at $M_2$ | # of plants checked | Zygosity determined at $M_3$ | Protein | qRT-PCR |
|---|---|---|---|---|---|---|---|
| **DME_5A_Kro_36505**[b] | G354A | W42* | Het | 10 | Mutant, wildtype and heterozygote | | supression |
| **DME_5A_Kro_32166** | C642T | H86Y | Het | 20 | Mutant and wildtype | gamma gli | |
| DME_5A_Kro_17186 | G663A | G93R | Het | 20 | Mutant, wildtype and heterozygote | | |
| DME_5A_Kro_33322 | G669A | splice junction | Het | 10 | Wildtype and heterozygote | | |
| **DME_5A_Kro_29375** | G797A | A111T | ? | 20 | Mutant, wildtype and heterozygote | | |
| DME_5B_Kro_42773[d] | G308A | V29M | Hom | 10 | Mutant | | supression |
| **DME_5B_Kro_33412** | G667A | splice junction | Het | 20 | Mutant, wildtype and heterozygote | gamma & alpha gli | supression |
| DME_5B_Kro_36182 | C787T | P108L | Hom | 5 | Wildtype | | |
| **DME_5A_Exp_20548** | G313A | V29M | Hom | 5 | Mutant | | |
| **DME_5A_Exp_12680** | G354A | W42* | Het | 20 | Mutant, wildtype and heterozygote | | |
| DME_5A_Exp_15945 | G480A | splice junction | Het | 20 | Mutant, wildtype and heterozygote | | |
| DME_5A_Exp_15246 | G653A | M89I | Het | 20 | Mutant, wildtype and heterozygote | | |
| DME_5A_Exp_22471 | C658T | T91M | Het | 20 | Mutant and wildtype | | |
| DME_5A_Exp_14773 | G664A | G93E | Het | 20 | Mutant and wildtype | | |
| DME_5A_Exp_46066 | G669A | splice junction | Hom | 5 | Wildtype | | |
| DME_5A_Exp_46332 | G795A | R110K | Het | 20 | Wildtype | | |

| ID | Mutation | Effect | Zygosity determined at $M_2$ | # of plants checked | Zygosity determined at $M_3$ | Protein | qRT-PCR |
|---|---|---|---|---|---|---|---|
| DME_5A_Exp_46354 | G669A | splice junction | Hom | 4 | Mutant | | |
| DME_5B_Exp_22914 | G307A | splice junction | Het | 20 | Mutant, wildtype and heterozygote | | |
| DME_5B_Exp_20397 | C500T | Q67* | Hom | 5 | Mutant | omega gli | |
| **DME_5B_Exp_20396**[b] | C500T | Q67* | Het | 20 | Mutant, wildtype and heterozygote | omega gli | |
| **DME_5B_Exp_50322**[a] | G651A | M89I | Hom | 5 | Mutant | | |
| DME_5B_Exp_50325 | G651A | M89I | Hom | 5 | Mutant | | |
| DME_5B_Exp_50324[c] | G651A | M89I | Het | 20 | Mutant, wildtype and heterozygote | | supression |
| DME_5B_Exp_21599 | G661A | G93R | Het | 20 | Mutant, wildtype and heterozygote | | supression |
| DME_5D_Exp_13105 | C221T | L9F | Hom | 2 | Mutant | | |
| DME_5D_Exp_49004 | C278T | P28S | Hom | 2 | Mutant | | |
| DME_5D_EXP_19793 | C273T | A26V | Hom | 2 | Mutant | | |
| DME_5D_Exp_22244 | G363A | D30N | Het | 8 | Wildtype | | |
| DME_5D_Exp_50449 | G363A | D30N | Het | 6 | Mutant, wildtype and heterozygote | | |
| DME_5D_Exp_50503 | G197A | E1K | Het | 6 | Mutant, wildtype and heterozygote | | |
| DME_5D_Exp_51464 | C367T | T31I | Het | 6 | Mutant, wildtype and heterozygote | | |
| DME_5D_Exp_49066 | C367T | T31I | Het | 6 | Mutant, wildtype and heterozygote | | |
| DME_5D_Exp_15603 | C203T | L3F | Het | 6 | Mutant, wildtype and heterozygote | | |

Red font = stocks showing incorrect genotypes in $M_3$ generation (not corresponding with the genotypes determined in $M_2$)
Blue font = stocks selected for crossing
a = striata mutation in background
b = necrotic mutation in background
c = mutation causing supernumerary spikelets in background
d = mutation causing male sterility in background

TABLE 7. List of $F_2$ plants selected for propagation in glasshouse till maturity for further analysis.

| Express Plant # | DME_5B D8E50322, M89I, G651A [D3] | DME_5A D7E20548, V29M, G313A [D2] | Class |
|---|---|---|---|
| 72 | MM | MW | aaBb |
| 73 | MM | MM | aabb |
| 80 | MM | MM | aabb |
| 85 | MM | MW | aaBb |
| 86 | MM | MW | aaBb |
| 96 | MM | MM | aabb |
| 75 | MM | MW | aaBb |
| 76 | MW | MW | AaBb |
| 83 | MW | MM | Aabb |
| 87 | MW | MW | AaBb |
| 88 | MW | MM | Aabb |
| 90 | MW | MW | AaBb |
| 91 | MW | MW | AaBb |
| 94 | MW | MW | AaBb |
| **Kronos Plant #** | **DME_5B D8K33412, splice site, G667A [D3]** | **DME_5A D7K29375, A111T, G797A [D3]** | **Class** |
| 145 | MM | MM | aabb |
| 148 | MM | MM | aabb |
| 175 | MW | MM | Aabb |
| 176 | MW | MM | Aabb |
| 196 | MW | MM | Aabb |
| 204 | MW | MM | Aabb |
| 205 | MW | MM | Aabb |
| 209* | MW | MM | Aabb |
| 210 | MW | MM | Aabb |
| 213 | MM | MM | aabb |
| 218 | MW | MM | Aabb |
| 227 | MM | MW | aaBb |
| 228 | MM | MM | aabb |
| 238 | MM | MM | aabb |
| 241 | MW | MM | Aabb |
| 244 | MM | MW | aaBb |
| 251 | MW | MW | AaBb |
| 253 | MM | MM | aabb |
| 254 | MW | MW | AaBb |
| 256 | MM | MM | aabb |

*Plant died at seedling stage

D2 = HhH-GPD domain

D3 = 4Fe-4S binding domain

M = mutant allele; W = wild type allele

tations in Kronos background and 14 double mutants in Express background (Table 7). These selected double mutants were transferred to larger pots and raised till maturity to obtain $F_3$ grains. Spikes of 19 *DEMETER* double mutants identified in Kronos background were collected to extract RNA from immature grains followed by qRT-PCR analysis. Results of qRT-PCR showed reduction in *DEMETER* transcript abundance in relation to *Actin* and *GAPDH* in 8 cases. However, we do not expect transcriptional suppression in the double mutants, as single mutants used to obtain these double mutants are either amino acid substitutions or splice site variants, which in principle should not lead to transcriptional suppression. Albeit we expected reduction in amount of immunogenic prolamins accumulated in the endosperm. A very little reduction in amount of immunogenic prolamins was observed with the double mutants, which in all confirmed cases showed pyramiding of amino acid substitutions.

Interestingly, in contrast to the barley high-lysine mutants *DME* mutants carrying premature stop codons or splice site variants especially in tetraploid wheat background showed reduction in anther size, locule size, number of fertile pollens, and pollen germination rate (Fig. 15). Reduced level of pollen germination adversely affected competitiveness of the mutant pollen relative to the wild type pollen causing severe segregation distortion. In fact analysis of >2000 $F_2$ plants from crosses between A and B sub-genome *DME* mutants with premature stop codons have so far yielded either single mutants or wild type alleles at both homoeologues. A reduction in seedling vigour of the *DME* mutants with stop codons was also observed. Both of these observations indicated the vital role of *DME* during microspore/pollen development and seed germination. This suggested an RNAi based approach, expressing *DEMETER* silencing microRNAs in a spatio-temporal fashion as an alternative.

### *5.3. Transcriptional suppression of DEMETER homoeologues and its influence on prolamin accumulation*

A RNA interference based approach was followed to develop DEMETER deficient wheat lines with no in-

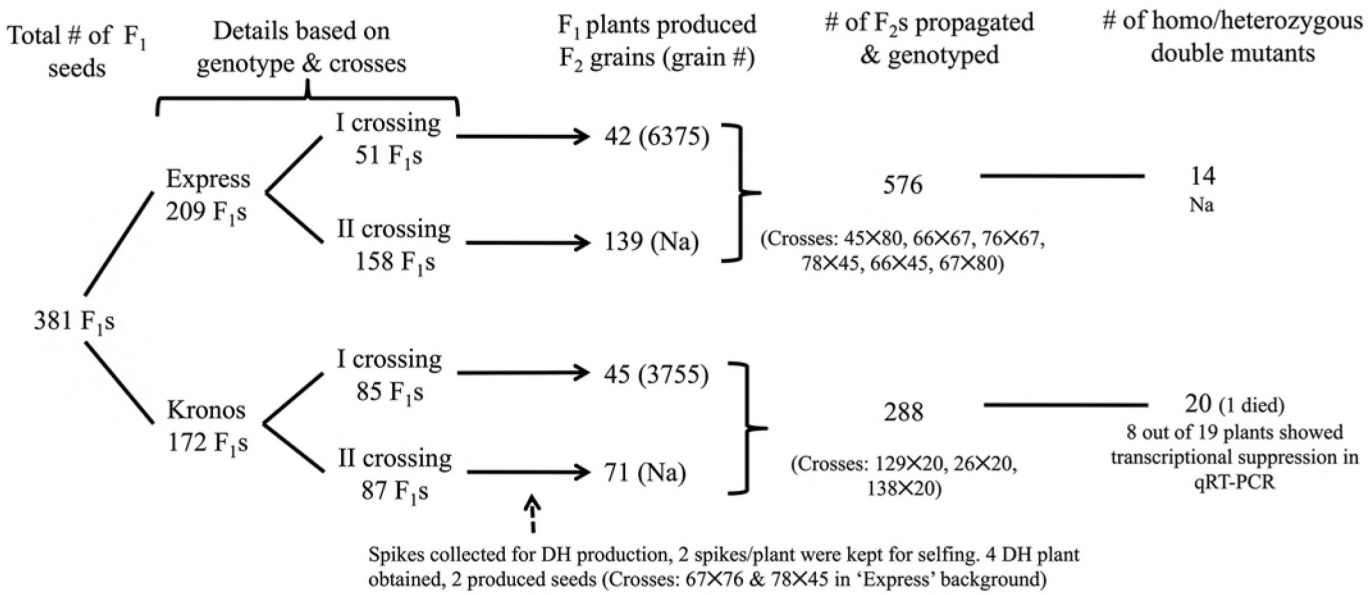



Figure 14.
The sequence of events after crossing of *DEMETER* single mutants obtained in Express and Kronos backgrounds for 'A' and 'B' sub-genome homoeologues. Na = data not available at the time of reporting. I crossing = first crossing event and II crossing = second crossing event (duplication of the first event).

fluence on other vital plant processes. In order to achieve this objective transformants exclusively expressing *DEMETER* targeting micro/small interfering RNAs in wheat endosperm where developed. Both conventional biolistic and a novel microspore electroporation based approaches were used for this purpose.

### 5.3.1. Transformants obtained using biolistic approach

A total of 333 candidate transformants were obtained by four rounds of biolistic transformations using three constructs expressing artificial microRNAs (pRB104, pRB105 and pRB106) and two constructs expressing hairpin RNA (p728 and pDRB6). All of these single cassette vectors were co-transformed in 2:1 proportion with another single cassette vector (pDPG165) expressing the *Bar* gene under the control of a *35S* promoter and a *Nos* terminator, using biolistic transformation of scutellar calli derived from two soft white winter wheat cultivars Brundage 96 and Simon. The co-transformation provides the opportunity to eliminate false positives by regeneration of calli on selective media with increasing quantities of herbicide (bialaphos; up to 5 µg/ml). In principle, independent integration of two constructs in the wheat genome provides greater chances of permitting removal of the undesired marker gene by segregation in subsequent generations. The plants recovered from the tissue culture were transferred to soil and vernalized for 8-12 weeks. After vernalization the plants were transplanted to 6-inch pots, and their leaves painted with 2% Ignite solution containing the active ingredient bialaphos. Data were recorded for injury on a 0-5 scale, where 0 represents no injury and 5 dead tissue. Two weeks after transplantation, leaf tissue was collected from candidate transformants ($T_0$), and DNA was extracted to study integration of ami/hpRNA expressing the cassettes in the wheat genome. RNA was also extracted from developing $T_1$ seeds [harvested at 17 ±3 days post anthesis (DPA)] of the putative transformants. The results of qRT-PCR showed suppression of *DME* transcript with an abundance ranging from 3.0% to 85.2% in different transformants.

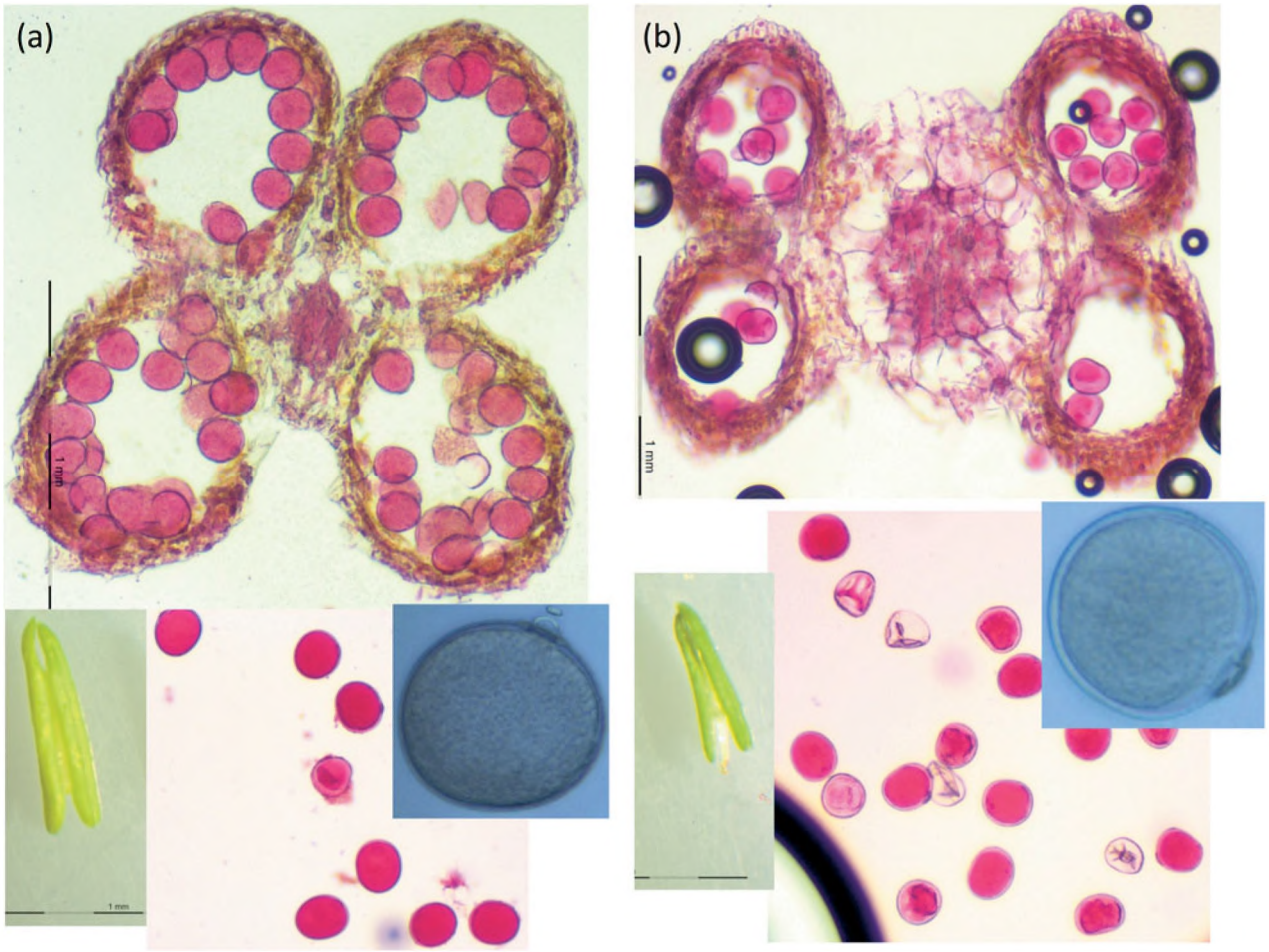



Figure 15.
Differences observed between the anthers and pollen grains of a splice site variant (a), and a mutant with premature stop codon (b). Size of anthers was reduced to almost half in mutant, 'D7K36505' carrying a stop codon in A sub-genome *DEMETER* homoeologue in Kronos background. To determine pollen viability transversely sectioned anthers and isolated pollen grains were stained with a solution containing 10 mL of 95% alcohol, 1 mL of malachite green, 25 mL of glycerol, 5 mL of acid fuchsin, 0.5 mL of Orange G, and 4 mL of glacial acetic acid in 54.5 mL of distilled water. Pollens showing deep magenta colour represent viable pollens. The isolated pollens were also cultured on liquid pollen growth medium (Boavida and McCormick 2007) containing azadecalin-1 (a pollentube growth promoting substance). Pollen germination was recorded 3 h after depositing pollen on the growth medium. The pollen grains from splice-site variant 'D8K33412' (mutation in B sub-genome *T. durum DME* homoeologue) showed removal of operculum and extrusion of pollen tube after 3 h incubation, but no signs of germination was observed in D7K36505 pollen grains.

On the basis of the results of PCR and qRT-PCR analysis a set of 52 transformants ($T_0$) was selected for further analysis (Figs. 16 to 18). Out of the above 52 plants 50 gave seeds ($T_1$) which 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 soluble fraction) and glutenins (soluble in aqueous alcohol with reducing agents) were extracted from the $T_1$ grains of each transformant, and analyzed using denaturing SDS-PAGE.

The extracted proteins were first quantified using

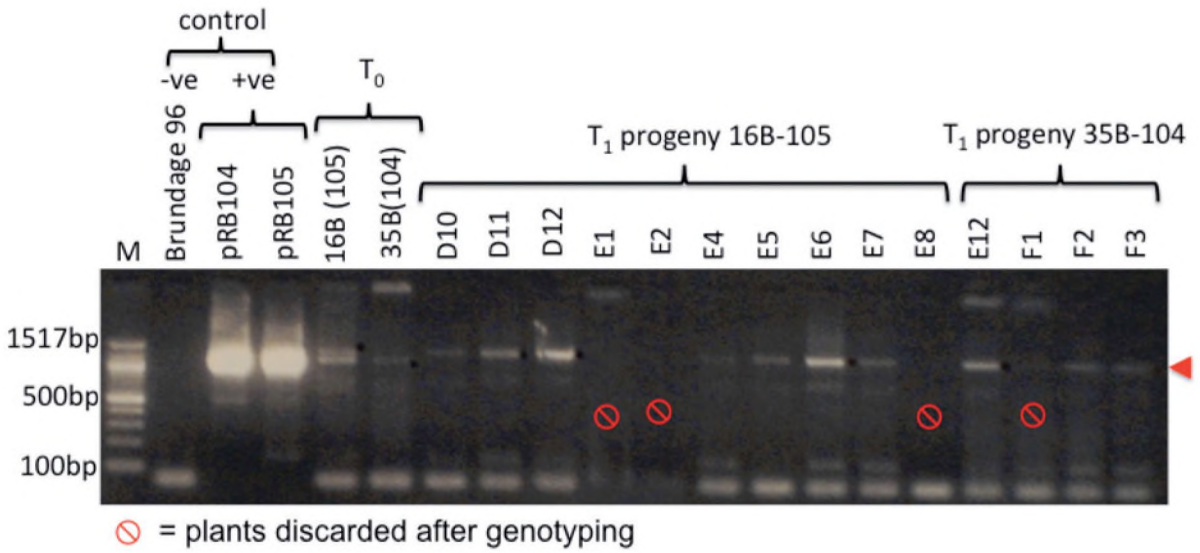



Figure 16.
PCR analysis of $T_0$ transformants using pRB104 and pRB105 expressing artificial microRNAs designed from the active site of *DEMETER* with specific primers. M = 100 bp ladder (New England BioLabs Inc. Cat # N3231S).

Bradford colorimetric assay followed by quantitative analysis (i.e., 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 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 (Fig. 19).

To improve resolution of the analysis 3 HPLC traces (based on retention times) were collected from gliadin fraction and 5 HPLC traces were collected from glutenin fraction, and used for detailed mass spectrometric analysis on MALDI-TOF-MS at the Proteomics Core Facility, WSU, Pullman (Figs. 20 & 21).

Elimination of specific prolamins instead of gross eliminations was attributed to the bulk harvest of all $T_1$ grains from individual $T_0$ plants, which were most likely chimeric in nature, and have resulted in dilution effect in the protein gels and RP-HPLC profiles. In order to deal with the problem of chimerism we propagated 2,620 $T_1$ progeny plants in glasshouse and more than three times the number of plants at the Cook Agronomy Farm, WSU, Pullman (Fig. 22).
The $T_1$ plants were sown in glasshouse in 7 batches of eight 48 well flats each, planting was staggered over a period of 7 weeks (from Jul. 14-Aug. 18, 2011). The staggering was done in view of providing enough time to collect leave samples and extract DNA from the previous batch. Each batch included eight 48 well flats corresponding with four 96 well plates. DNA was extracted using Biosprint Plant DNA extraction kit (Qiagen, Valencia, CA) following manufacturer's instructions. Once the leave samples were collected for DNA extraction the flats were transferred to the vernalization chamber (from Aug. 2-Sep. 1, 2011) maintained at 4°C for twelve weeks (from Nov. 1-Dec. 15, 2011). The period during vernalization was used for DNA quantification and genotyping, using construct-specific primer to confirm inheritance of transgene integrations (expressing *DEMETER* silencing hp/amiRNAs). Analysis using construct specific primers: M13 5'-TCACACAGGAAACAGCTATGAC-3' and 3'Phor-Gus-NosR.2 5'-GATCTAGTAACATAGATGACACCGC-3' (first 8 cycles at 62°C followed

TABLE 8. List of transformants showing faithful inheritance of transgene, results of qRT-PCR on T1/T2 grains sion on accumulation of gliadins and total amount of immunogenic prolamins (LMWgs and gliadins).

| Genotype | GN | TKW | $T_1$ % suppression | $T_1$ grains propagated |
|---|---|---|---|---|
| 10_728 | 1000 | 20.0 | 36.6 | 105 |
| 18_728* | 657 | 22.8 | 65.8 | 66 |
| 17_728* | 1000 | 18.0 | 78.1 | 109 |
| 25_728* | 558 | 16.1 | 51.7 | 56 |
| 36b_728* | 710 | 18.3 | - | 71 |
| 20_728 | 175 | 22.9 | - | 18 |
| 26_728 | 143 | 21.0 | 77.1 | 14 |
| 29_728 | 662 | 27.2 | - | 66 |
| 30_728 | 0 | - | - | - |
| 46_728* | 352 | 14.2 | 85.2 | 35 |
| 28_728* | 166 | 12.0 | 54.9 | 16 |
| 17b_728* | 106 | 9.4 | 79.9 | 10 |
| 19_728* | 784 | 19.1 | 78.4 | 79 |
| 22_728* | 594 | 18.5 | - | 60 |
| 38_728* | 622 | 27.3 | 55.5 | 62 |
| 16_728 | 666 | 19.5 | 4.6 | 66 |
| 30b_728 | 126 | 7.9 | 16.4 | 13 |
| 40_728 | 561 | 28.5 | 3.0 | 56 |
| 140_728 | 891 | 23.6 | 10.6 | 89 |
| 34_728 | 142 | 14.1 | 0.0 | 14 |
| 29b_728 | 494 | 16.2 | 62.6 | 50 |

*HPLC analysis was also conducted on the gliadin and glutenin fractions derived from $T_1$ grains.
[Source: Wen et al., 2012 Proc. Natl. Acad. Sci. U.S.A., Copyright (2012)]

by 38 cycles at 56°C) in case of pRB104/105/106 transformed plants, and Pri_15 5'-TGTTCTTTCCTGCGTTATGCCC-3' and Pri_16 5'-TGTCACCTAAATAGC TTGGCGAAT-3' (annealing temperature = 58°C) in case of p728 transformed plants, showed faithful inheritance of transgene in a total of 522 cases (Figs. 16 & 17). Out of 522 cases 242 represent $T_1$s expressing one of the three amiRNAs (namely pRB104, pRB105 and pRB106), and 280 represent $T_1$s expressing the hpRNA (p728). The $T_1$ plants showing inheritance of transgene integration(s) in hemi-/homozygous state were transferred to individual pots and the spikes carrying immature $T_2$ grains (collected after 17±3 days post anthesis) were collected from glasshouse to analyze for transcriptional suppression of *DEMETER* homoeologues. And the mature $T_2$ grains were collected from these transformants to study effect of *DEMETER* suppression/silencing on prolamin accumulation.

DNA analysis of the $T_1$ plants showed faithful inheritance of the transgene in 2.8 to 71.7% of progeny plants belonging to different transformants (Figs. 16 & 17). A wide range of plants showing transgene inheritance in the $T_1$ progeny confirmed chimeric origin of these plants. Similarly the results of qRT-PCR analysis performed on the cDNAs prepared from the im-

and data collected on grain number (GN) and thousand kernel weight (TKW). Effect of *DEMETER* suppres-

| PCR +ve | $T_2$s studied | $T_2$ % suppression | % reduction gliadins | % reduction LMW+gliadin |
|---|---|---|---|---|
| 54 (51.4%) | 52 | 57.2-85.6 | 56.3-74.8 | 60.4-68.0 |
| 13 (19.7%) | 13 | 71.9 | 61.5 | 63.1 |
| 10 (9.2%) | 10 | 78.4 | - | - |
| 6 (10.7%) | 6 | 73.5 | 59.9 | 65.6 |
| - | - | - | - | - |
| 9 (50%) | - | - | - | - |
| 2 (14.3%) | 1 | - | - | - |
| 3 (4.5%) | 3 | 71.3 | 67.6 | 64.1 |
| - | - | - | - | - |
| 10 (28.6%) | 10 | 81.7 | 76.0 | - |
| 10 (62.5%) | 6 | 78.2 | 68.8 | 68.0 |
| 7 (70%) | 6 | 68.0 | 79.4 | 70.6 |
| 50 (63.3%) | 50 | 78.6-83.9 | 43.1-76.4 | 56.2-76.4 |
| 43 (71.7%) | 36 | 75.0-76.7 | 66.1-77.5 | 63.1-69.3 |
| 19 (30.6%) | 13 | - | - | - |
| 5 (7.6%) | - | - | - | - |
| 7 (53.8%) | 4 | - | - | - |
| 3 (5.4%) | 3 | - | - | - |
| 17 (19.1%) | 17 | 59.9-82.6 | 25.3-71.5 | 45.2-74.1 |
| 6 (42.9%) | 6 | 75.1 | 67.5 | 64.9 |
| 11 (22%) | 10 | 74.5-74.7 | 67.2-71.4 | - |

mature $T_2$ grains belonging to different transformant families showed a wide suppression range from 3.5% to 85.6% (Table 8; Appendix 2).

The selected transformants showing suppression in *DME* transcript abundance close to or more than 50% were examined for the accumulation of immunogenic prolamins in their endosperm by sequential extraction of seed storage proteins followed by SDS-PAGE and RP-HPLC. The PAGE and HPLC results revealed that different transformants show elimination of different prolamin groups (Figs. 23-26), for instance P32F2 and P31D12, progeny of $T_0$ transformant 10-728 showed reduced accumulation of α-gliadins respectively by 56.2 and 37.6% and γ-gliadins by 21.8% and 20.7%. These lines also show reduced accumulation for LMWgs, which together with gliadins account for ~67% reduction in the amount of total immunogenic prolamins (Appendix 2). However a slight increase in the amount of ω-gliadins and a significant increase in the amount of HMWgs was also observed in these lines, which will be beneficial in maintaining the rheological properties of these lines (Fig. 23). Not only reduction in amount of immunogenic prolamins but also total elimination of specific gliadins and/or LMWgs was also observed in different transformants (Figs. 24-26).

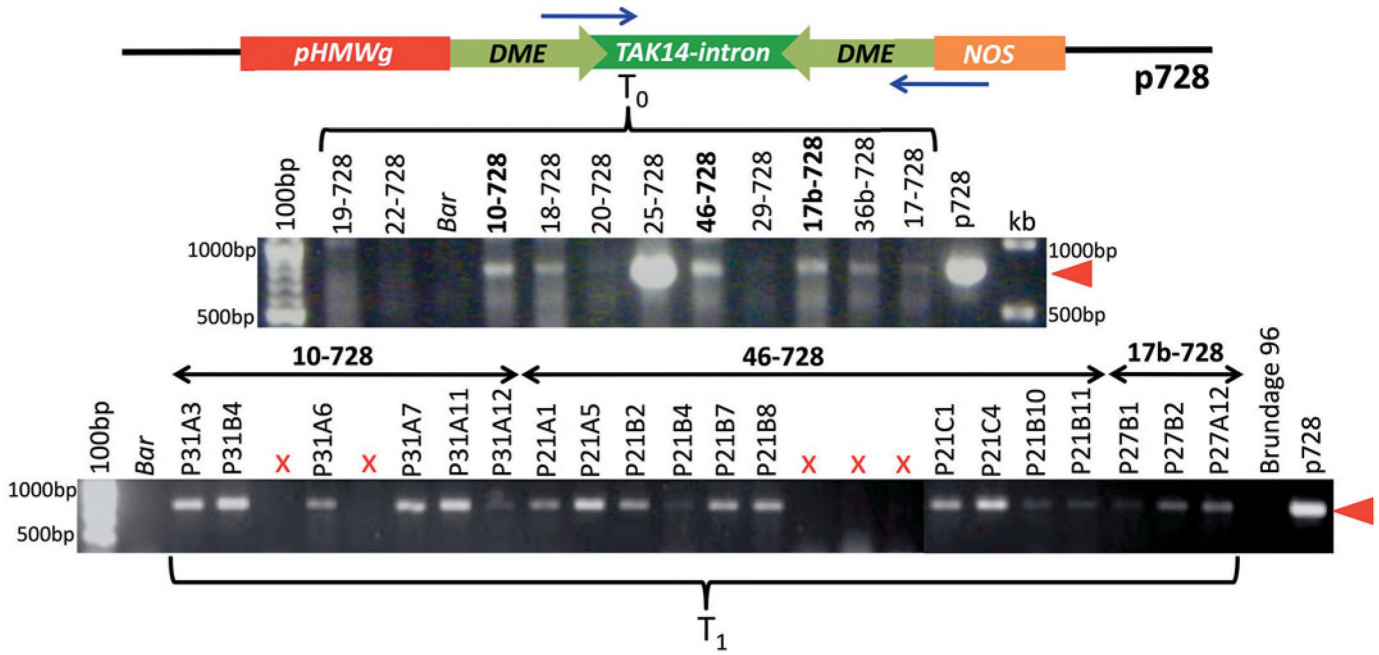



Figure 17.
PCR-based confirmation of transgene integration in putative transformants obtained by biolistic transformation of *scutellar calli* with *DEMETER* silencing hairpin construct (p728). Primers used for analysis are Pri-15 and -16 (see below). M = 100 bp ladder (NEB Cat # N3231S). [Source: Wen et al., 2012 Proc. Natl. Acad. Sci. U.S.A., Copyright (2012)]

The level of reduction for immunogenic prolamins (LMWgs and gliadins) calculated using the area covered by specific prolamin groups on the HPLC chromatograms ranged from 45.2-76.4%, and used as independent variable to regress on the values obtained for *DME* transcript level expressed as percent suppression (Table 8; Appendix 2). The two variables regressed very well on each other with a $r^2$ value of 0.877 suggesting 87.7% correspondence among the two variables indicating that level of *DME* transcript determines the amount and type of immunogenic prolamins accumulated in the wheat grain (Fig. 27).

In summary, the qRT-PCR analysis with immature $T_2$ grains followed by protein profiling with 3 individual mature grains per transformant showed reduced accumulation of immunogenic prolamins in 48 transformants expressing amiRNAs (representing 22 transformation events) and 50 transformants expressing hpRNA (representing 13 transformation events). Out of these 98 transformants 19 $T_2$s (12 expressing amiRNA and 7 expressing hpRNA) representing a total of 15 transformation events (10 with amiRNA and 5 with hpRNA), showed highest level of reduction in the amount of immunogenic prolamins, which were selected for propagation in glasshouse (Fig. 28a, b). Whereas a larger set of 92 $T_2$s representing 30 transformation events are currently under propagation at the Cook Agronomy Farm WSU, Pullman. In view of multiple integrations and zygosity of transgene at each integration site ten rows with 10 grains per $T_2$ was propagated in field and 10 grains per $T_2$ were propagated in glasshouse. The plants propagated in glasshouse will be converted to doubled haploids (DHs) using the microspore culture method standardized in our laboratory. The plants are expected to attain the desired developmental stage (i.e., Feeke's stage 10-10.1) for microspore collection by early May 2013. Once obtained each DH line will be evaluated for its prolamin content us-

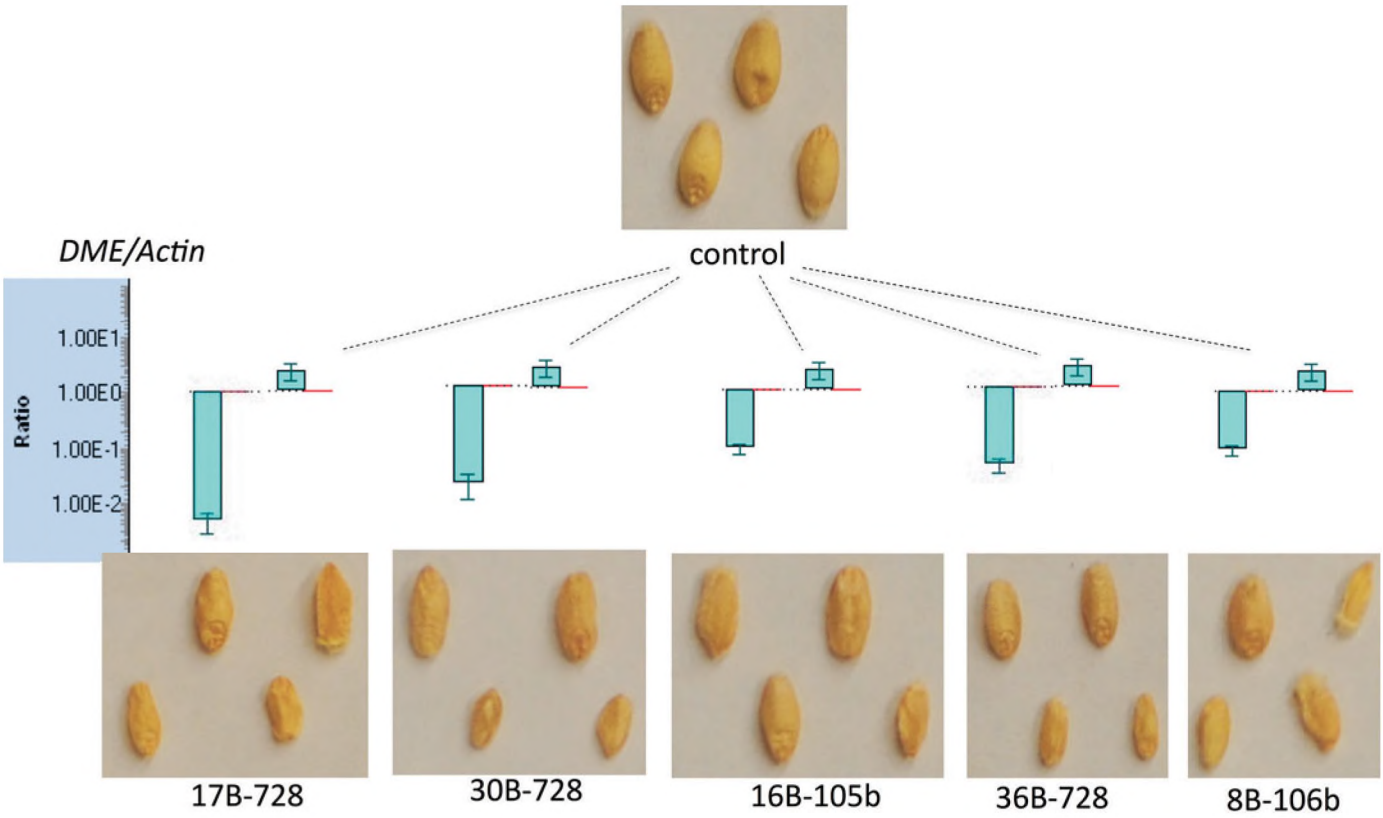



Figure 18.
Phenotypic (seed morphology) and expression level differences (by qRT-PCR; normalized to *Actin*) observed in different transformants in comparison with what the control plants (Brundage 96) have shown.

ing SDS-/A-PAGE followed by RP-HPLC. The DH lines showing elimination of different prolamin families will be crossed to pyramid their effect in a single genotype.

With the current transformation procedures there is no control on the number and site of transgene integrations, which determine the level of transgene expression, and in this particular case, expression of *DEMETER* silencing hpRNA/amiRNAs and its concomitant influence on the accumulation of prolamins. In addition from our previous experience working with wheat and barley transformations we observed that the desired transformation events usually occur in a proportion of 1 in 100. Thus, it is necessary to screen more transformants in order to identify desired transformation event(s) with highly reduced prolamin (≤90%) levels. It has been demonstrated in several previous studies that in-general celiac patients can tolerate 20 to 100 ppm (mg/kg) gluten in their diets, thus according to the latest recommendations of the Codex Alimentarius Commission any food item containing 20 ppm gluten can be treated as 'zero gluten' or 'gluten free'.

In view of the above we screened 175 additional transformants including 62 and 113 $T_0$s respectively expressing hpRNA and amiRNAs targeting wheat *DEMETER* homoeologues. RNA was extracted from the immature $T_1$ grains and *DEMETER* transcript suppression was observed in 74 cases including 45 transformants expressing hpRNA and 29 expressing amiRNAs (Fig. 22). As we are aware that the plants obtained using the biolistic approach suffers from chimerism (i.e., the whole plant is not evenly transformed) individual spikes from each plant were collected separate-

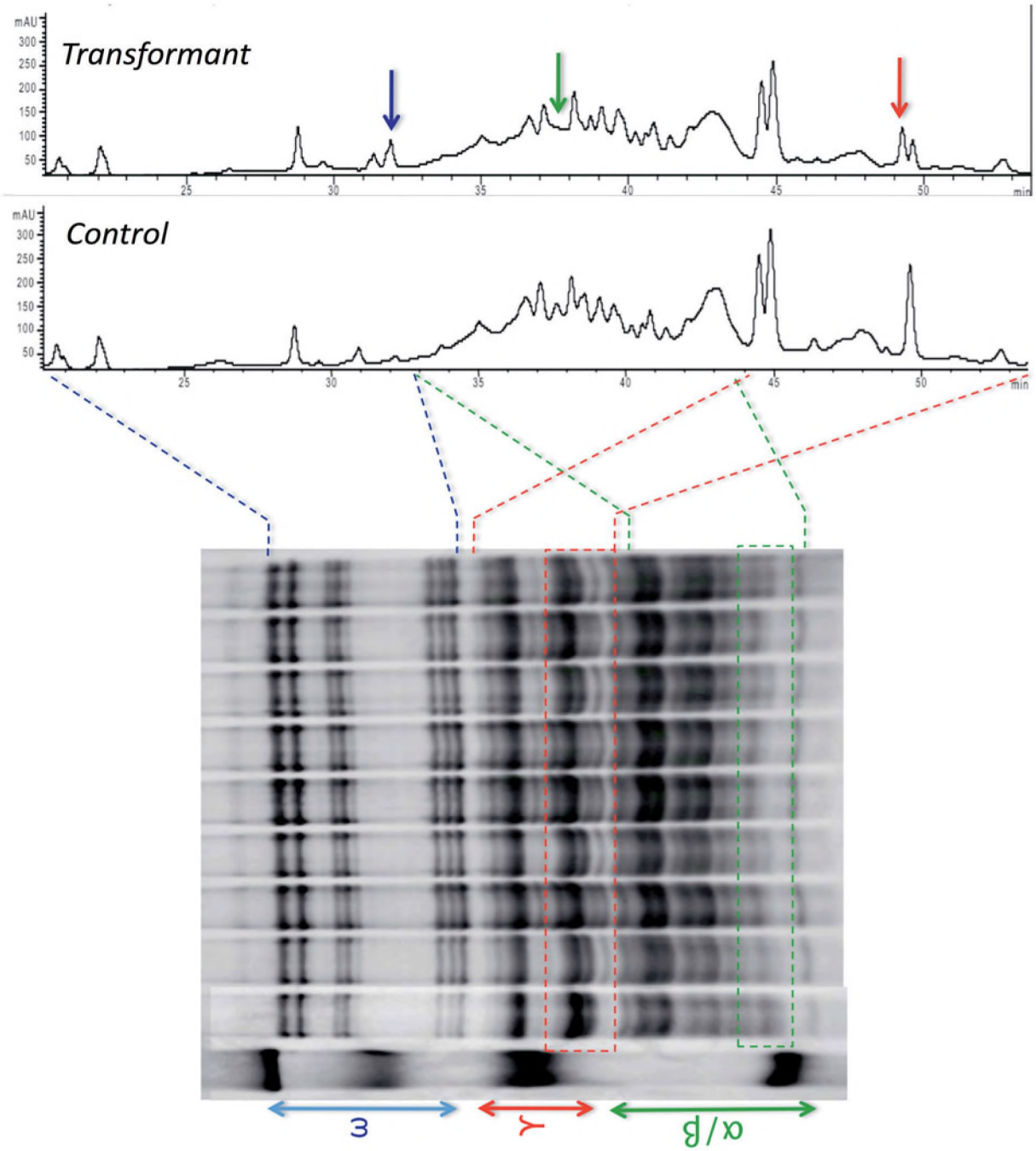



Figure 19.
SDS-PAGE gel and RP-HPLC analysis of gliadin fraction extracted from the mature grains of various transformants and Brundage 96 (first lane in gel). Specific proteins eliminated in transformants were boxed on the PAGE gel and were marked with arrowhead on the HPLC profile.

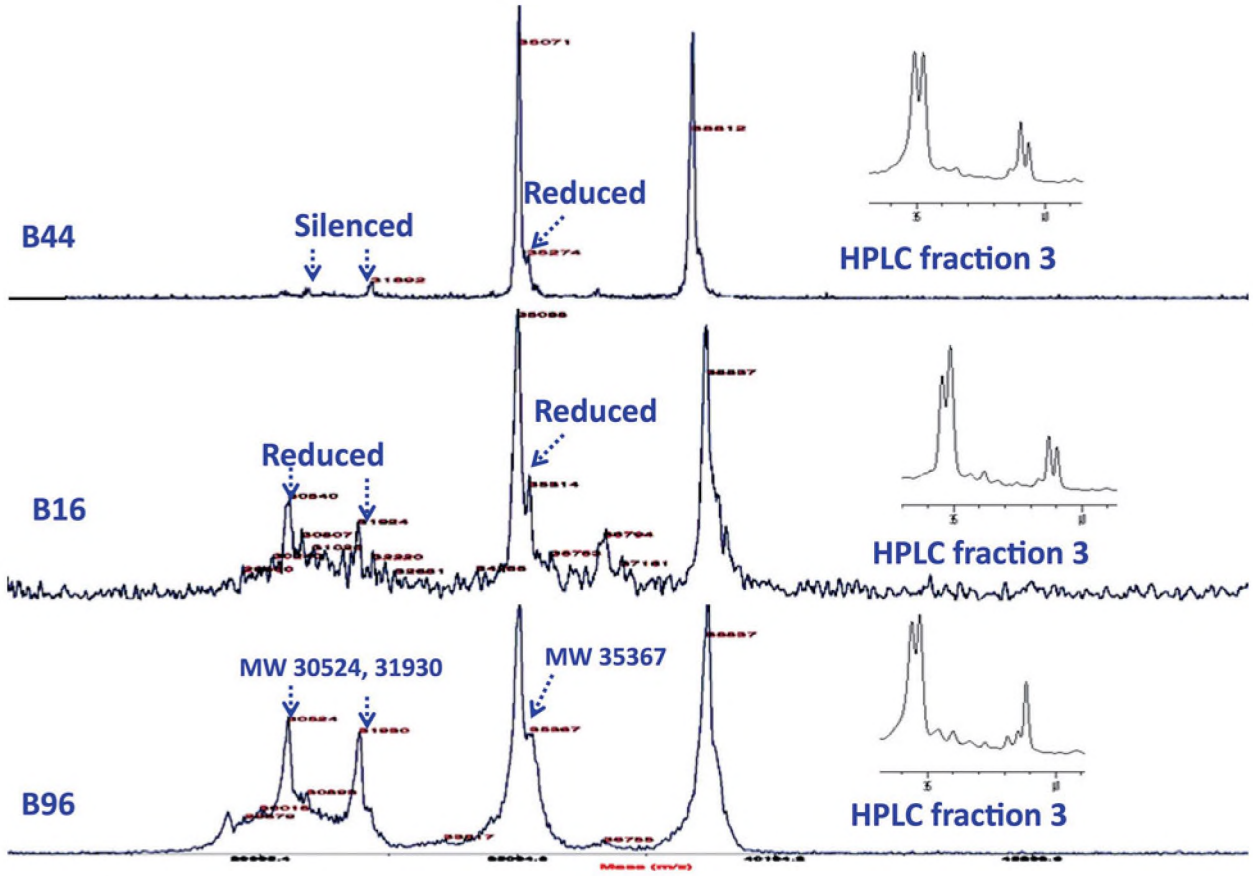



Figure 20.
Mass spectrum of HPLC fraction 3 of gliadins (γ-gliadins) extracted from Brundage96, 16B (pRB105) and 44B (pRB105).

ly. And seeds from 2 spikes per transformant were initially screened for their prolamin profiles (Fig. 29a-h). The initial screen showed reduction in amount of prolamins in 15 transformants expressing hpRNA and 9 transformants expressing amiRNAs. Seeds from the remaining spikes of these selected transformants are currently being analyzed for their prolamin profiles. And seeds from the selected spikes will be propagated in glasshouse to obtain $T_2$ grains.

### 5.3.2. Transformants obtained using microspore-electroporation based approach

The $T_1$ grains of the transformants produced using microspore-electroparation approach were screened for the accumulation of immunogenic gliadins using A-PAGE. In the previous experiments conducted using the constructs expressing different marker genes encoding endochitinase, β-glucuronidase (GUS) and green fluorescence protein (GFP) we observed that the doubled haploid transformants also suffer from the problem of chimerism. This is unexpected because the calli regenerating into a plant are expected to derive from a single cell (microspore). However, in wheat the harvested embryogenic microspores turned out to be a population of uni/bi-nucleate microspores with generative and vegetative nuclei that could lead to chimerism. Since we have not applied any antibiotic selection during propagation of transfected microspores, it is possible that nascent embryonic cells formed after a few divisions, in the absence of selection pressure, produced cells lacking the foreign DNA fragment (carrying gene encoding for *DEMETER* si-

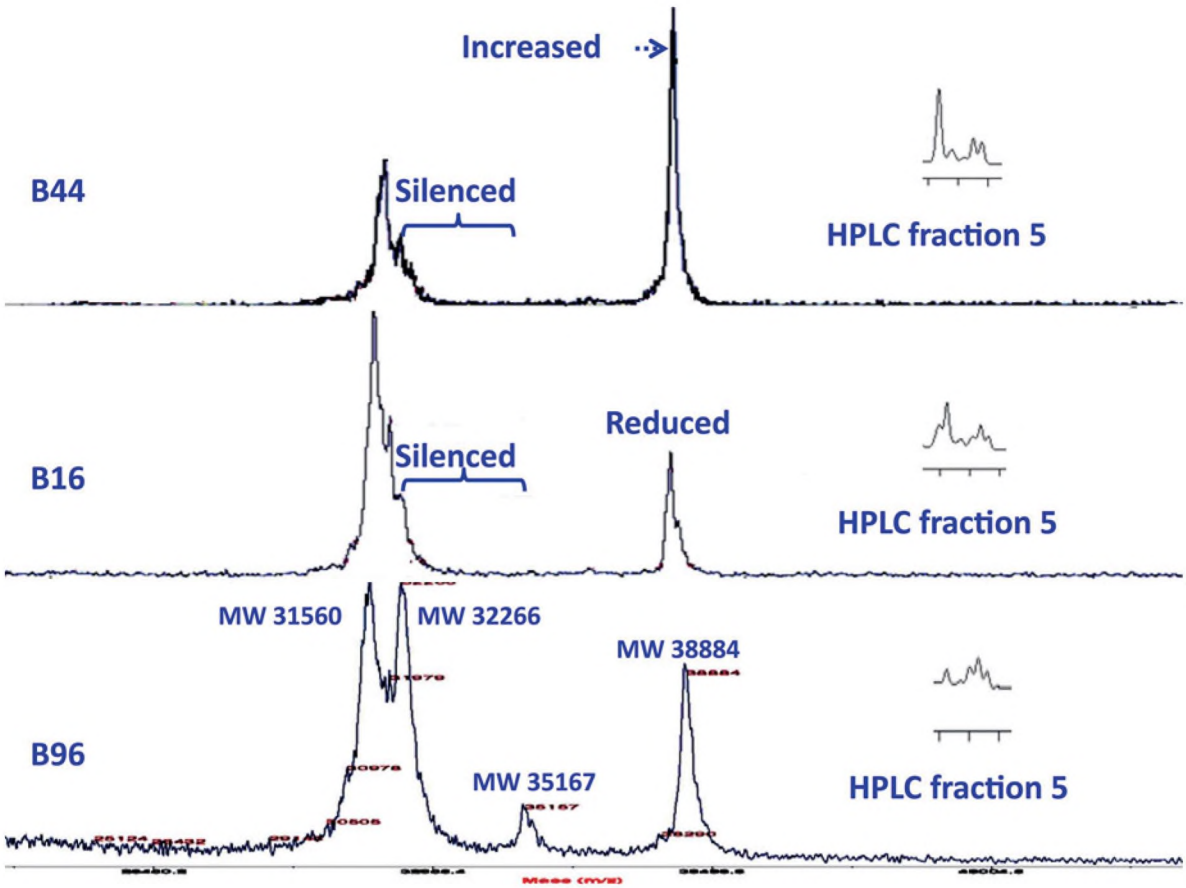



Figure 21.
Mass spectrum of HPLC fraction 5 of glutenins (including low molecular weight glutenins) extracted from Brundage96, 16B (pRB105) and B44 (pRB105).

lencing amiRNA) and led to the formation of chimeric calli, but the exact reason behind chimerism is unknown and requires more research.

In view of the above we harvested 1-27 spike separately from each of the 18 $T_0$ transformants, and analyzed them individually for transgene integration and prolamin content. These transformants are in three different genetic backgrounds, two hard red spring wheat cultivars Hollis and WB926 and one hard red winter wheat cultivar Farnum. Microspores derived from these varieties were induced and electroporated with pRB105 construct encoding *DEMETER* silencing amiRNA. Out of the 18 $T_0$s reduced accumulation of gliadins was observed in 1-7 spikes collected from 11 $T_0$s (Fig. 30).

## 6. Conclusion

Analysis of prolamin gene promoters from wheat revealed similar pattern of differential regulation in LMW glutenins and gliadins by active demethylation in the developing endosperm and of HMW glutenins via expression of specific transcription factors. This similarity in transcriptional regulation of the prolamin genes prompted us to characterize mutations in the *Lys3* candidate gene *DEMETER*. Efforts to pyramid *DME* mutants to obtain double and triple mutants respectively in tetraploid and hexaploid wheats revealed that *DME* serves a vital role in pollen development and seed germination. In view of that an RNAi based approach was adapted to silence

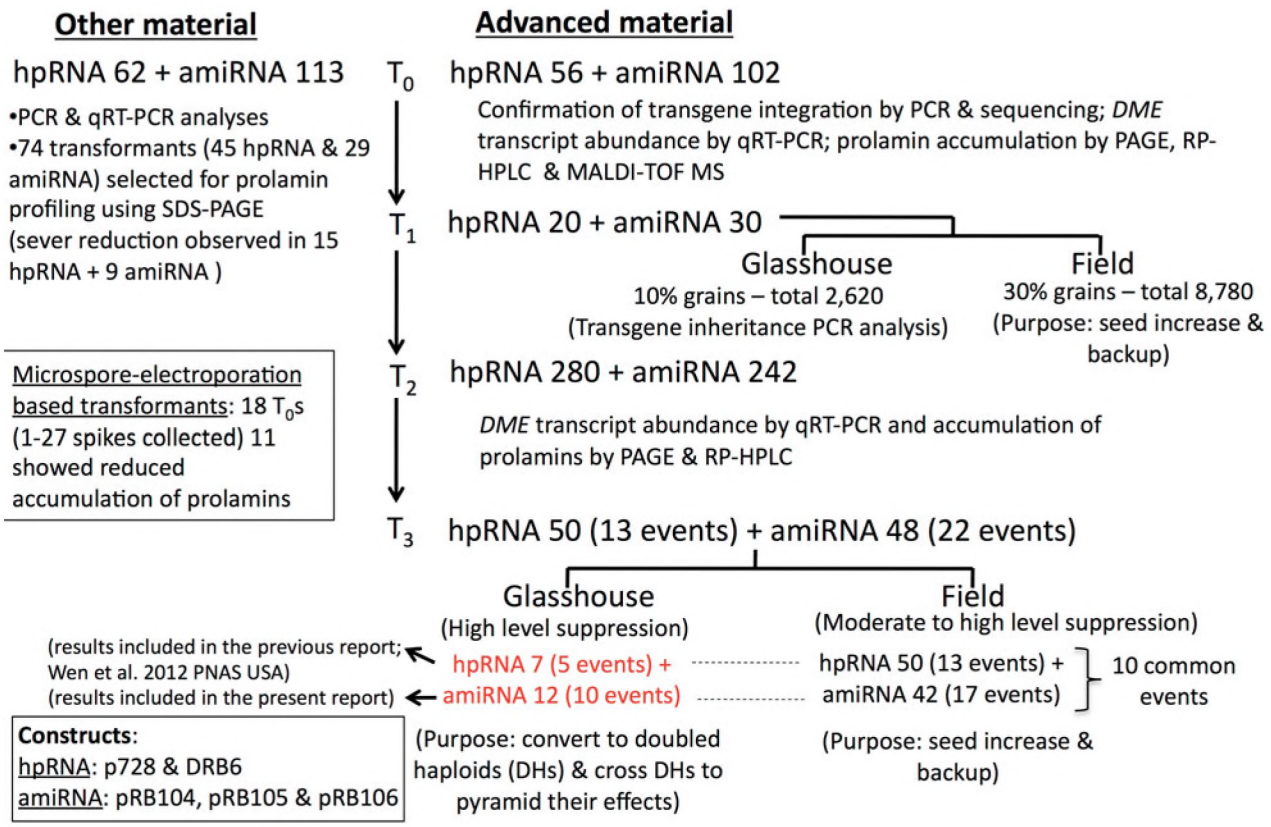



Figure 22.
Current status of research on wheat transformants expressing *DEMETER* silencing hpRNA or amiRNAs in the developing endosperm.

*DME* homoeologues exclusively during endosperm development. The reduction of the immunogenic gluten proteins has reached in some transformants 76.4% indicating that a complete hereditary elimination is achievable. When wheat plants with complete elimination of the immunogenic storage proteins has been achieved analysis of the gliadin-specific T-cells will be carried out as previously agreed with Dr. L.M. Sollid.

[Note: Dr. Sollid's laboratory is one of the few who maintains the T-cell cultures. He does however not like to carry out analysis before at least 90% elimination of the immunogenic proteins has approached.]

## 7. Objective 3

Post-transcriptional silencing of the members of four prolamin families, simultaneously, using RNA interference (RNAi).

## 8. Results

For the post-transcriptional silencing of 71-79 immunogenic prolamins a novel hairpin comprised of a chimeric stem derived from small interfering RNAs each selected from the sequence alignment of the members of the individual prolamin families, and a 100 bp loop derived from the second intron of wheat *TAK14* gene was synthesized (Fig. 31). Later the efficiency of this chimeric hairpin was tested by cloning it in the γ sub-genome of the barley streak mosaic vi-

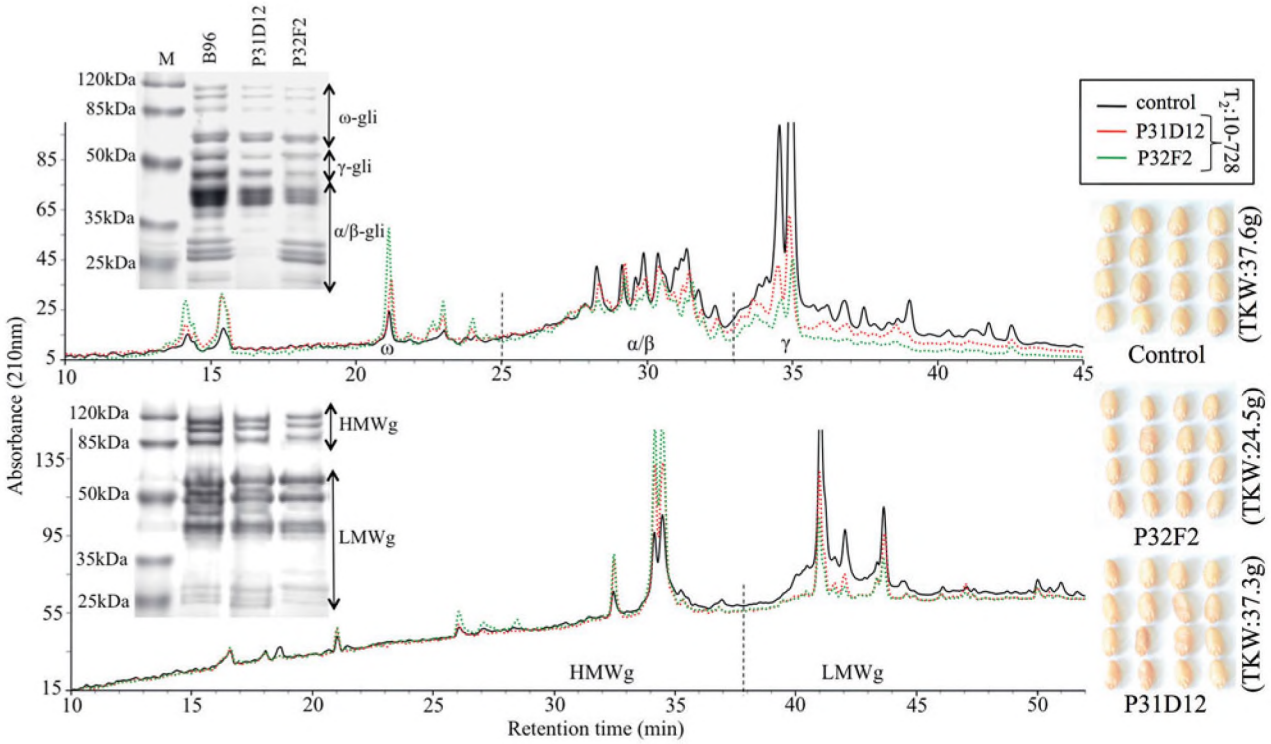



Figure 23.
Reverse phase high performance chromatography (RP-HPLC) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of gliadins and glutenin fractions extracted from the $T_2$ grains of the two progeny plants (P31D12 and P32F2) of transformant 10-728 (Table 8) expressing *DME* silencing hairpin RNA and the wild-type 'Brundage 96' (B96). A random sample of $T_2$ grains with their respective thousand kernel weights (TKW) was shown at right hand side. [Source: Wen et al., 2012 Proc. Natl. Acad. Sci. U.S.A., Copyright (2012)]

rus (BSMV), and by infiltrating the recombinant disarmed BSMV into the flag leaves of the wheat cultivar Chinese Spring. The results of this transient transformation also known as virus induced gene silencing (VIGS) showed reduced accumulation of gliadins and LMW glutenins in the BSMV infested plants (Fig. 31). The hairpin was also provided with the endosperm specific HMW glutenin *1Dy* promoter and *nos* terminator, and introduced into the wheat genome by biolistic approach or via microspore electroporation based transformation method. The $T_1$ grains of the selected transformants are currently being examined for their prolamin content. However, the preliminary analyses of the $T_1$ grains for the prolamin content showed very encouraging results (Fig. 31).

## 9. Conclusion

The results of transient and stable transformation analyses suggested that chimeric hairpin constructs can be successfully used to silence gene families, and adequate screening of putative transformants will allow identification of transformants showing complete/near-complete elimination of immunogenic prolamins.

## 10. Objective 4

Proline/glutamine rich peptides containing T-cell stimulating epitopes of wheat can be detoxified by prolyl endopeptidases and the barley cysteine endoprotease EP-B in vitro and in vivo as shown with rat

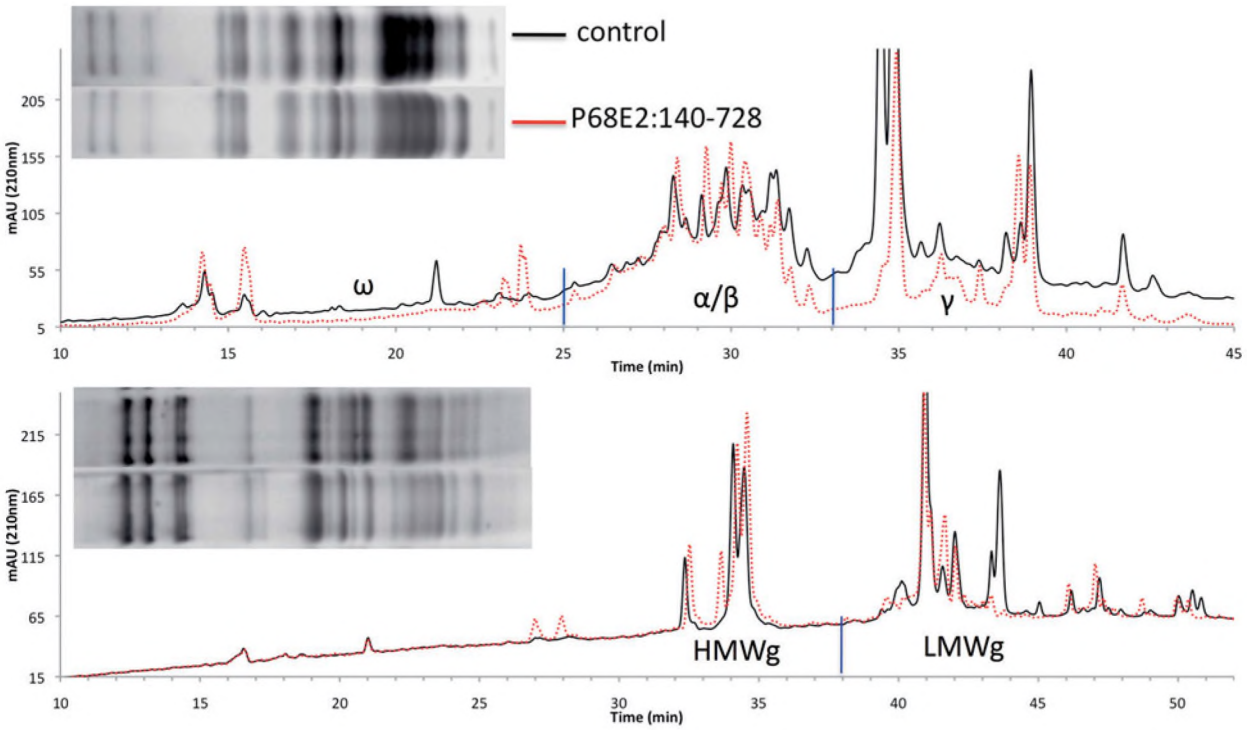



Figure 24.
RP-HPLC and SDS-PAGE gel of gliadins and glutenin fractions extracted from the $T_2$ grains of P68E2 (progeny of 140-728) and wild-type Brundage 96 showing specific eliminations and/or reductions in the amount of g-gliadins and LMW glutenins with a compensatory increase in the amount of ω-gliadins and HMW glutenins. [Source: Wen et al., 2012 Proc. Natl. Acad. Sci. U.S.A., Copyright (2012)]

intestinal perfusion experiments. We want to test, if it is possible to synthesize these enzymes in transgenic wheat during endosperm development, target the enzymes into the storage vacuoles and detoxify the prolamins during grain development. We have shown with specific monoclonal antibodies and a knock-out mutant of B-hordeins that the HMW glutenin (D hordein) is stored in a different compartment of the storage vacuole than the other prolamins. A codon-optimized microbial prolyl endopeptidase and the barley cysteine protease will be employed towards this end. The interesting biological challenge is to obtain detoxification by limited proteolysis of the gliadin and glutelin proteins without destroying the resilin/elastin-like structure of HMW glutenin. Proof of concept for this possibility has been obtained with the C hordein of barley. Expression of this prolamin in E. coli and its purification and folding into a native conformation permitted the determination of the cleavage sites for the cysteine endoprotease EP-B. A mutant C hordein lacking the primary cleavage sites for EP-B proved highly resistant to degradation by the enzyme. HMW glutenin can also be expressed in *E. coli* and folded into its native conformation. Thus producing a recombinant enzyme-resistant mutant HMW glutenin lacking the celiac epitopes seems feasible and its expression in transgenic wheat will be attempted.

## II. Results

The following paragraphs describe experiments performed to identify the best combination of enzymes from the so far characterized endoprotease and endo-

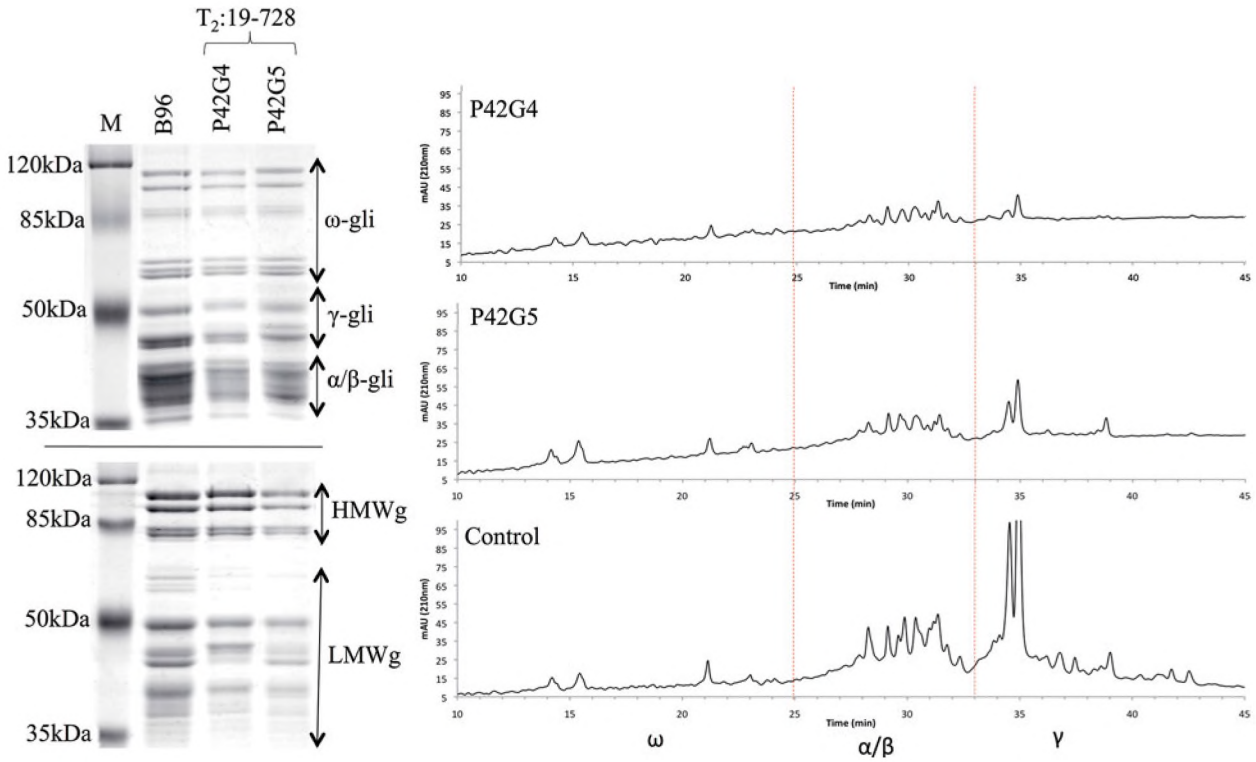



Figure 25.
RP-HPLC and SDS-PAGE gel of gliadins and glutenin fractions extracted from the $T_2$ grains of P42G4 and P42G5 (progeny of 19-728) and wild-type Brundage 96 showing significant reduction in the amount of all gliadins. [Source: Wen et al., 2012 Proc. Natl. Acad. Sci. U.S.A., Copyright (2012)]

peptidase by comparing the virtual digestion profiles of available prolamin sequences using each one of them under simulated gastric conditions. The enzymes selected based on the above analysis and published information, were expressed in wheat endosperm under the control of HMW glutenin promoter. In view of the risk of thermal denaturation of enzymes during the baking process the native proteases and peptidases were modified by site-directed mutagenesis to obtain enzymes with high thermal stability.

In the light of research conducted in the last four years the proposed experiments of engineering and expressing recombinant enzyme-resistant mutant HMW glutenin subunits appears unnecessary, as we now know that the barley endoprotease B2 is synthesized as a proenzyme. And the propeptide or prosegment serves as both inhibitor and chaperone to respectively facilitate spatiotemporal regulation of the proteolytic activity and proper folding of the protease. Both of these properties are of high significance for the current project because it will check degradation of the prolamins in the protein-bodies within the grains, and also in flour during the dough making process. The propeptide inhibits enzyme activity in basic, neutral and slightly acidic conditions and the acidic pH (≤4) is required for the removal of propeptide by an autocatalytic reaction. As the pH of mature grains in dry conditions (close to neutral) and of the dough (leavened with yeast ~6, sourdough ~5.5, or without a leavening agent ~6.5) is higher than the pH required for the auto-activation of enzyme no

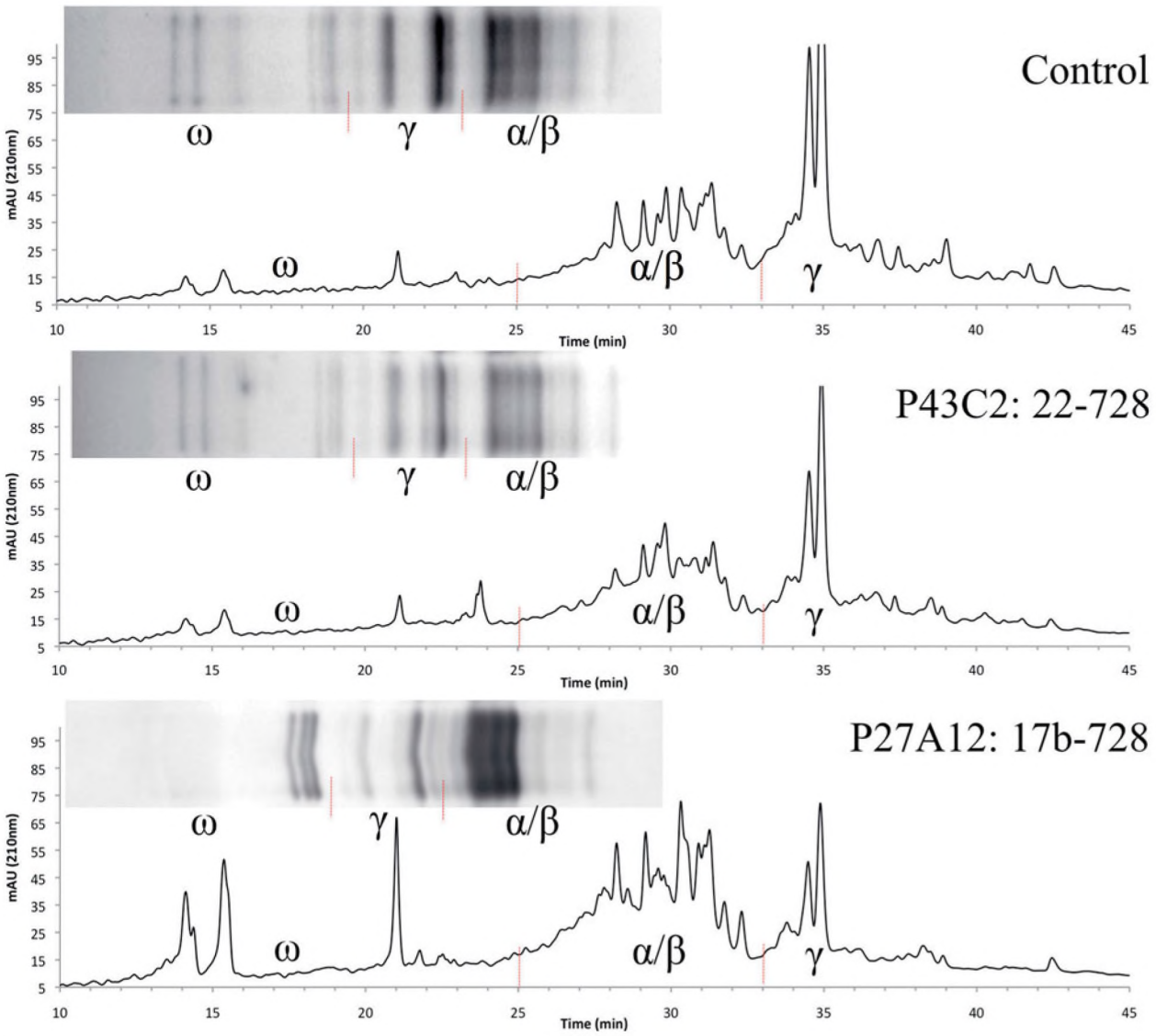



Figure 26.
RP-HPLC and SDS-PAGE gel of gliadins and glutenin fractions extracted from the $T_2$ grains of P43C2 (progeny of 22-728) and P27A12 (progeny of 17b-728), and wild-type Brundage 96 respectively showing significant reduction in the amount of α/β- and γ-gliadins and γ-gliadins alone with compensatory increase in amount of ω-gliadins. [Source: Wen et al., 2012 Proc. Natl. Acad. Sci. U.S.A., Copyright (2012)]

degradation of prolamins is expected. Similarly, the propeptide that serves as intramolecular chaperone is expected to refold the enzyme to its native form after thermal denaturation during the baking process, thus essentially no loss of enzyme activity is expected.

### *11.1. Virtual digestion of prolamins with endopeptidases and endoprotease under simulated gastro-intestinal conditions*

A total of 1336 prolamin sequences including wheat α/β- (151), g- (272) and ω- (13) gliadins, LMW- (457) and HMW- (318) glutenins, barley B- (26), C- (22), γ- (30) and D- hordeins (4), and rye g/w- (26) and

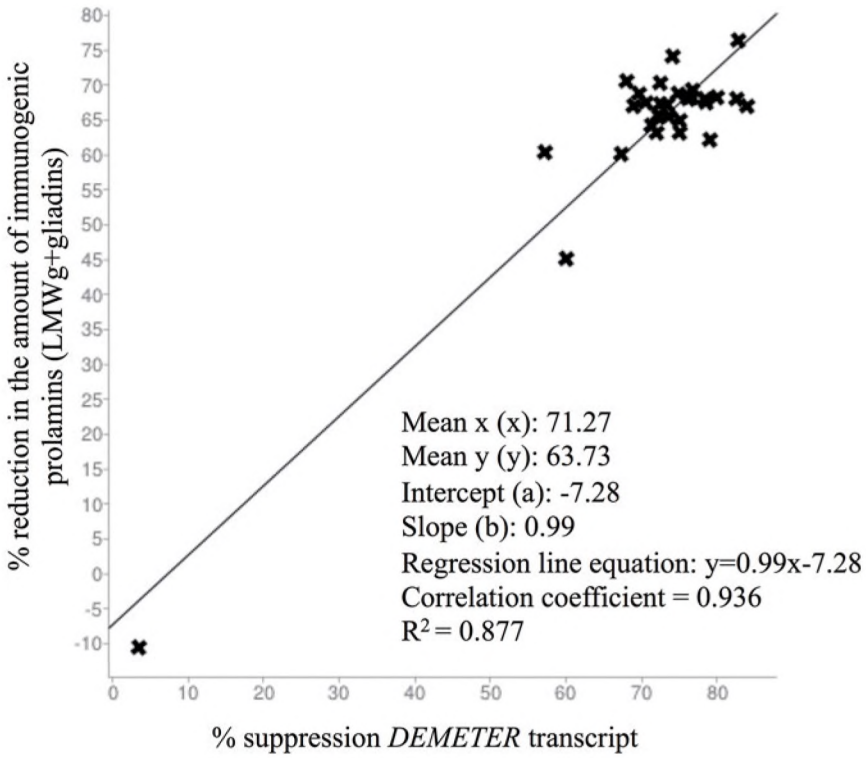



Figure 27.
Results of simple linear regression analysis performed using persent supression in *DEMETER* transctipt abundance as indipendent variable (x-axis) and persent reduction in the amount of immunogenic prolamins (all gliadins and LMW glutenins) as dependent variable (y-axis). [Source: Wen et al., 2012 Proc. Natl. Acad. Sci. U.S.A., Copyright (2012)]

HMW- (17) secalins were virtually digested under simulated gastric conditions with barely EP-B2 or a mixture of wheat endopeptidases followed by FM-PEP or AN-PEP treatment. Virtual digestion with pepsin, trypsin and chymotrypsin left significantly large number of peptides with $^{3}$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) (Fig. 32). 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 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.

It has been documented in literature that the optimal length of peptides stimulating T-cell response is 10-15 residues (Vader et al., 2002; Dewar et al., 2004). Thus, any peptide with 10 or more residues in length would potentially elicit immune response. The proteolytically resistant peptides when compared with 58 immunogenic peptides documented in literature (Appendix 3), encountered 413 cases showing similarity in γ-gliadins, 179 cases in α/β-gliadins, and 2 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 believe 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 great re-

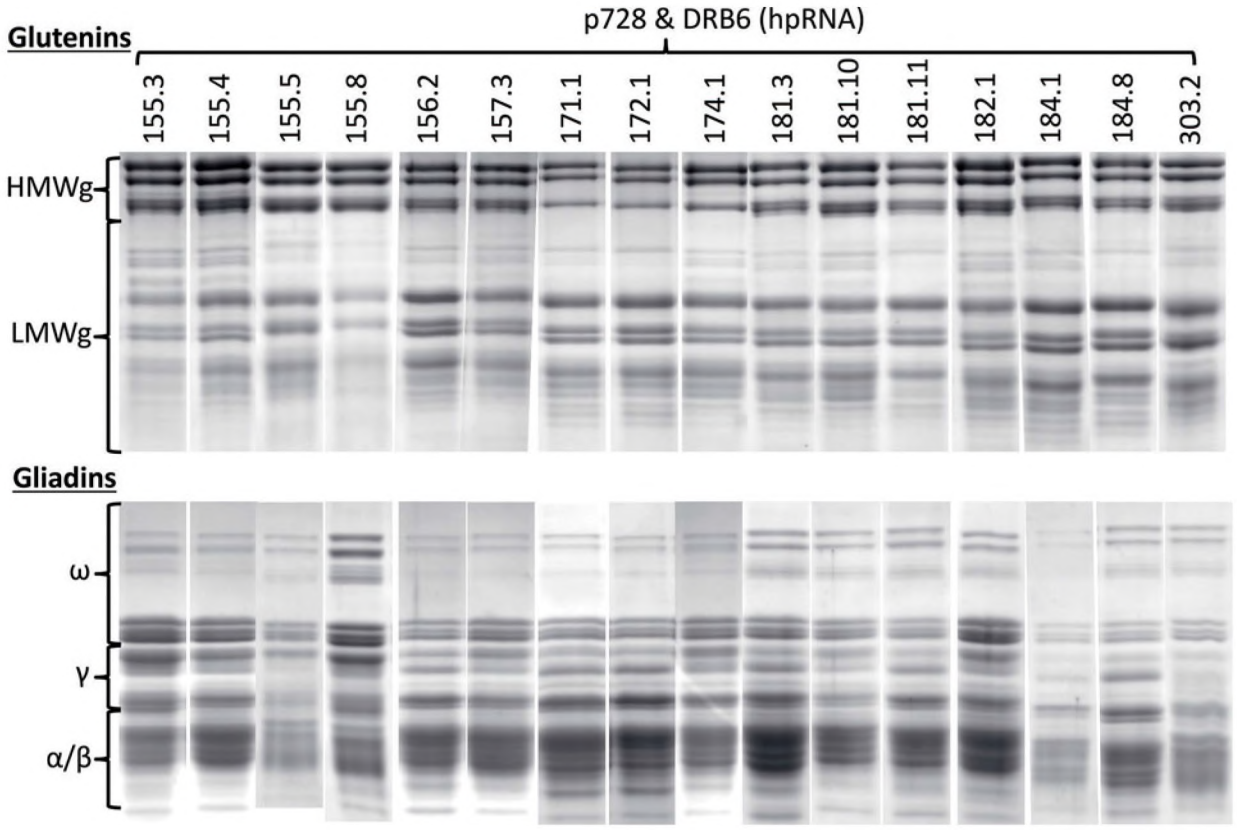



Figure 28. Part 1
Gliadin and glutenin profiles of selected $T_1$ transformants showing reduced accumulation of gliadins and LMW glutenins in their grains.

duction 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 (Fig. 33). Although wheat endoproteases work better on γ and ω-gliadins, but for the rest EP-B2 shows superior activity. This suggests that native enzymes work better on the respective storage proteins, which complies with the fact that these enzymes evolved specifically to degrade the storage proteins during germination. 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 (Siegel et al., 2006). Both *Flavobacterium meningosepticum* prolyl endopeptidase (FM-PEP) and *Aspergillus niger* prolyl endoprotease (AN-PEP) were equally active on the peptides obtained after EP-B2 or wheat endoproteases treatment under simulated gastric conditions, except for ω-gliadins where FM-PEP performed better than AN-PEP (Fig. 32). In view of the results of *in silico* analysis we undertook a transgenic approach to express barley EP-B2 and FM-PEP in large quantities in wheat endosperm to detoxify immunogenic gluten proteins.

## *11.2. Transformation of wheat scutellar calli*

### 11.2.1. Plasmid construction

In order to produce transgenic wheat expressing *F. meningosepticum* prolyl endopeptidase (FM-PEP) and barley EP-B2 two individual gene constructs were prepared. The first construct has FM-PEP cloned under the control of wheat *1Dy* high molecular weight glutenin (HMWg) promoter and *Ag. tumefaciens* nopa-

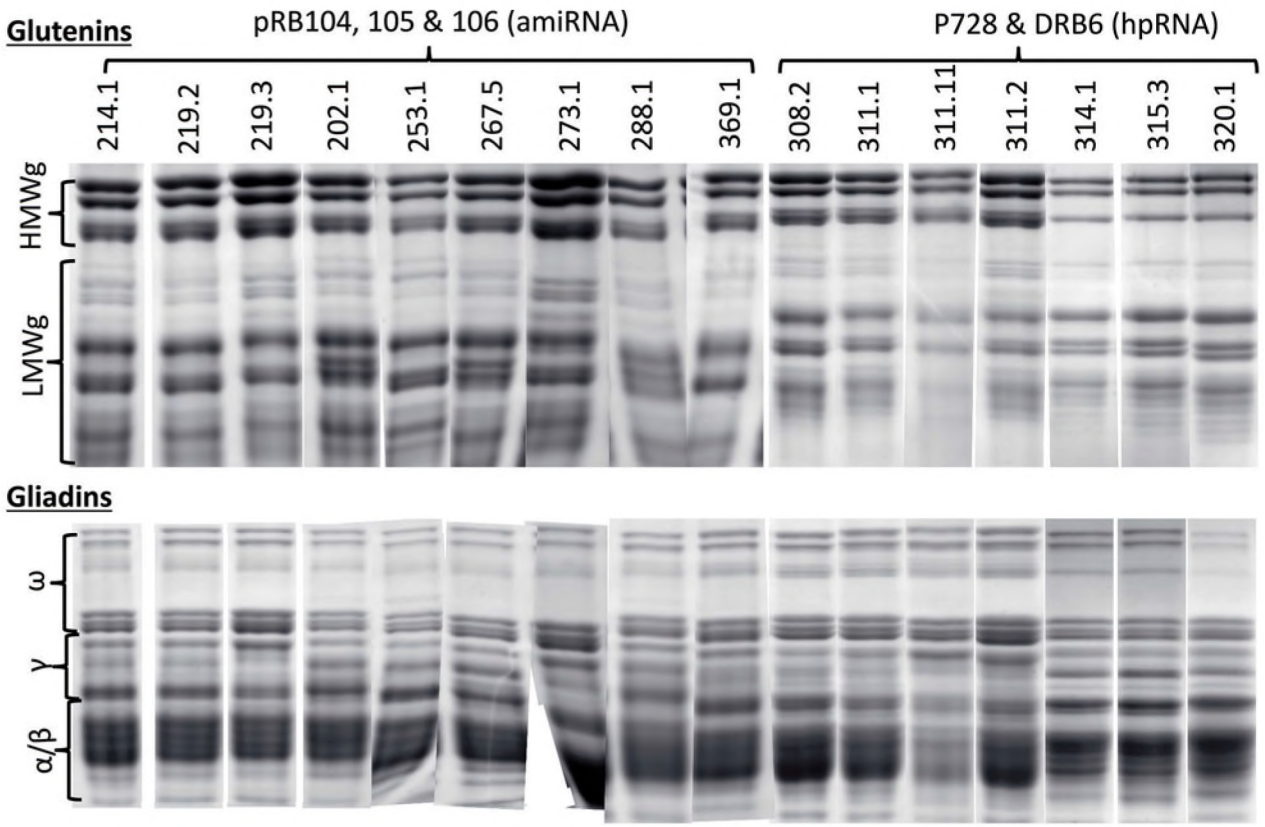



Figure 28. Part 2

line synthase (*nos*) terminator. In order to assure sequestering of the recombinant protein in the protein bodies, D-hordein signal peptide was synthesized in frame with the gene (Fig. 34a). The gene encoding for FM-PEP (Accession number M81461) was codon-optimized for codon usage in monocotyledonous plants preferring a GC content over 60%. The D-hordein signal peptide was added at the beginning of the gene, with restriction sites for *Apa*I and *Sac*I to assist its cloning. The gene was synthesized from GenScript (Piscataway, NJ), cloned into pUC57 between *EcoR*I and *Sal*I restriction sites, and cloned into DH5α cells. Plasmid DNA isolated from transformed cells was digested with *Apa*I/*Sac*I and the resulting fragment was ligated into plasmid pHMWg+nos, previously digested with the same restriction enzymes to achieve plasmid pHMWg+Fmen+nos, and transformed into DH5α cells. DNA was extracted from the transformed cells and the resulting plasmid was verified by restriction digestion with appropriate enzymes and sequencing at DNA Sequencing Core, WSU, Pullman.

The second construct that encodes for barley cysteine endoprotease EP-B2 (Accession number U19384) was cloned under the same promoter and terminator as before (Fig. 34b). Amplification of the gene was performed using genomic DNA extracted from cv. Golden Promise with following primers capable of introducing non-template specific restriction sites at the 5'(*Xho*I) and 3'(*Kpn*I) ends of the PCR product: 5'EP-B2 propeptide/*Xho*I GCGCTCGAGATCCCGATGGAGGACAAGGACC and 3'EP-B2/*Kpn*I GCGGGTACCCAGTGACTCCCTGGCTCCAAGGG (Bethune et al., 2006). Polymerase chain reaction (PCR) amplification was performed on thermocycler C1000 (BioRad, USA) using Ex-*Taq* DNA polymerase (Takara, Madison, WI), in a 25µl reaction

TABLE 9. Constructs used for transformation.

| Construct* | Promoter | Genes | Types |
|---|---|---|---|
| pDPG165** | *35S* | *Bar* | Circular |
| pHMWg+Fmen+nos | *1 Dy* high molecular weight glutenin | *F. meningosepticum* prolyl endopeptidase (FM-PEP) | Circular |
| pHMWg+EP-B2+nos | *1 Dy* high molecular weight glutenin | Barley endoprotease B2 (EP-B2) | Circular |
| pBSK+(HMWg+Fmen+nos/HMWg+EP-B2+nos) | *1 Dy* high molecular weight glutenin | FM-PEP + EP-B2 | Linear |

* plasmids pDPG165, pHMWg+Fmen+nos and pHMWg+EP-B2+nos were co-transformed in 1:2:2 molar ratio, and plasmid pDPG165 and pBSK+(HMWg+Fmen+nos/HMWg+EP-B2+nos) were co-transformed in 1:2 molar ratio.

** pDPG165 - expressing *Bar* gene conferring resistance for bialaphos was described in Gordon-Kamm et al. (1990)

TABLE 10. List of primers used for amplification of barley endoprotease B2 and to confirm transgene integration(s) in wheat genome.

| Primer | Sequence (5'-3') | Product size (bp) | Purpose |
|---|---|---|---|
| proEP-B2/*Xho*I* | F: GCGCTCGAGATCCCGATGGAGGACAAGGACC | 1047 | gene amplification |
| EP-B2/*Kpn*I | R: GCGGGTACCCAGTGACTCCCTGGCTCCAAGGG | | |
| EPB2 | F: GCATGGACGAGCTGTACAAGTAA<br>R: CTTGATGTACTCGAAGGCGTTGT | 594 | gene integration |
| Fmen | F: TGGGACCCCAAGTTCTC | 927 | gene integration |

* Bethune et al. (2006)

volume using manufacturer's instructions. Following PCR conditions were used for amplification: initial melting at 95°C for 3 min followed by 30 cycles at 95°C for 30 sec; 65°C for 30 sec and 72°C for 2 minutes with a final extension at 72°C for 5 minutes. A 5 µl aliquot of the PCR product was subjected to agarose gel electrophoresis (1% gel, 110V, 1h) to confirm amplification of expected size PCR product. Once the product was confirmed by gel electrophoresis the remaining 20µl of the product was purified with Geneclean III kit, (MP Biomedicals, Solon, OH) following manufacturer's instructions and the purified product was digested with *Xho*I and *Kpn*I. After the heat inactivation of enzymes, the product was ligated into plasmid pHMWg+nos, as described above to obtain plasmid pHMWg+EP-B2+nos, and was transformed into DH5α cells. Plasmids were extracted from the selected clones and verified by restriction digestion followed by sequencing. After confirmation, midi preps of both plasmids were obtained using NucleoBond® Xtra Midi/Midi Plus kit (Macherey-Nagel, Bethlehem, PA), following manufacturer's instructions. The DNA concentrations of these preps were adjusted at 1.5µg/µl and 12µl of each of the preps were used to transform scutellar calli of wheat cultivar Brundage 96, using particle bombardment. The co-transforma-

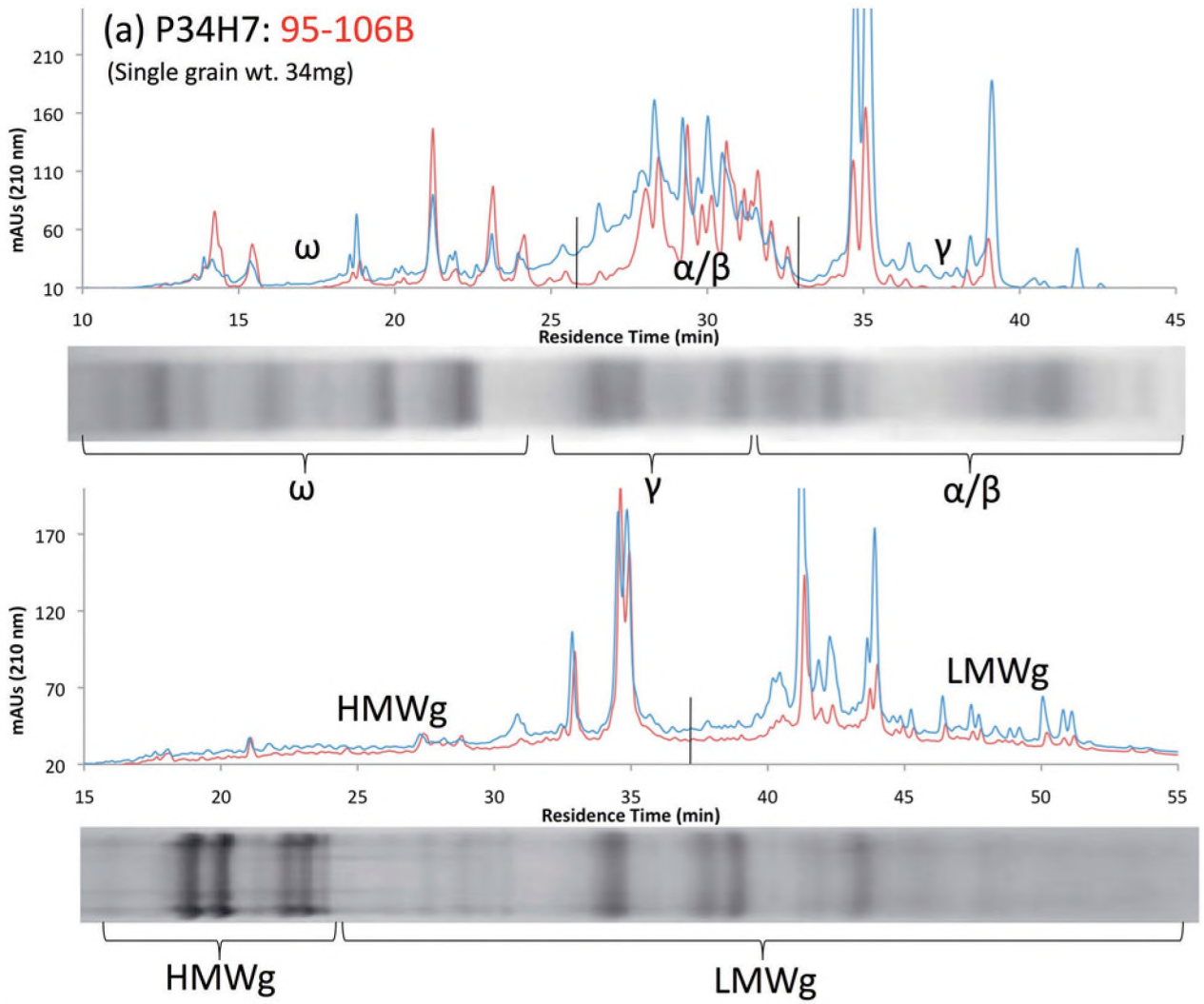
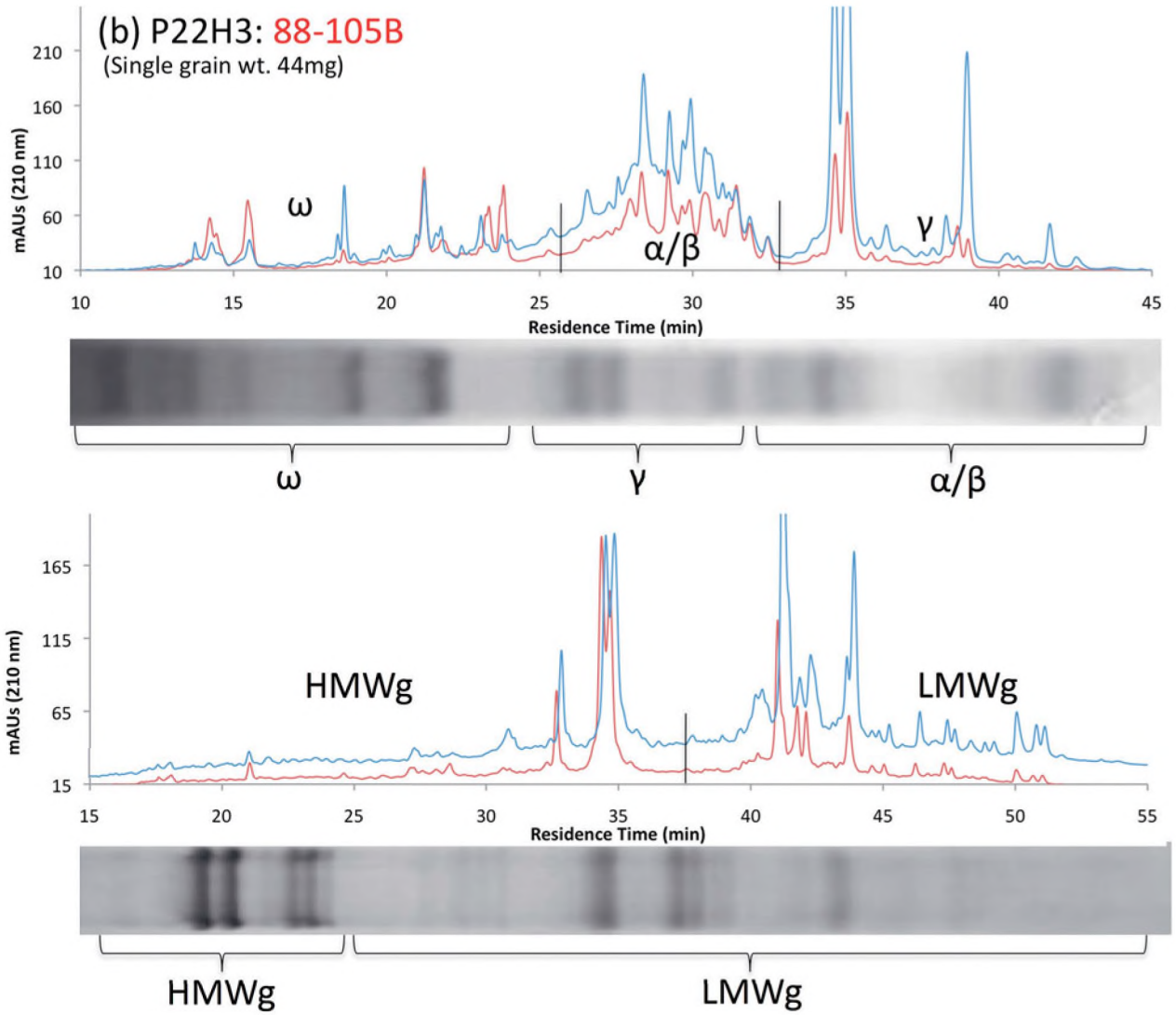



Figure 29a-h.
Gliadin and glutenin profiles of transformants ($T_2$ grains) selected for propagation in glasshouse. Wild type (Brundage 96) chromatograms are shown in blue.

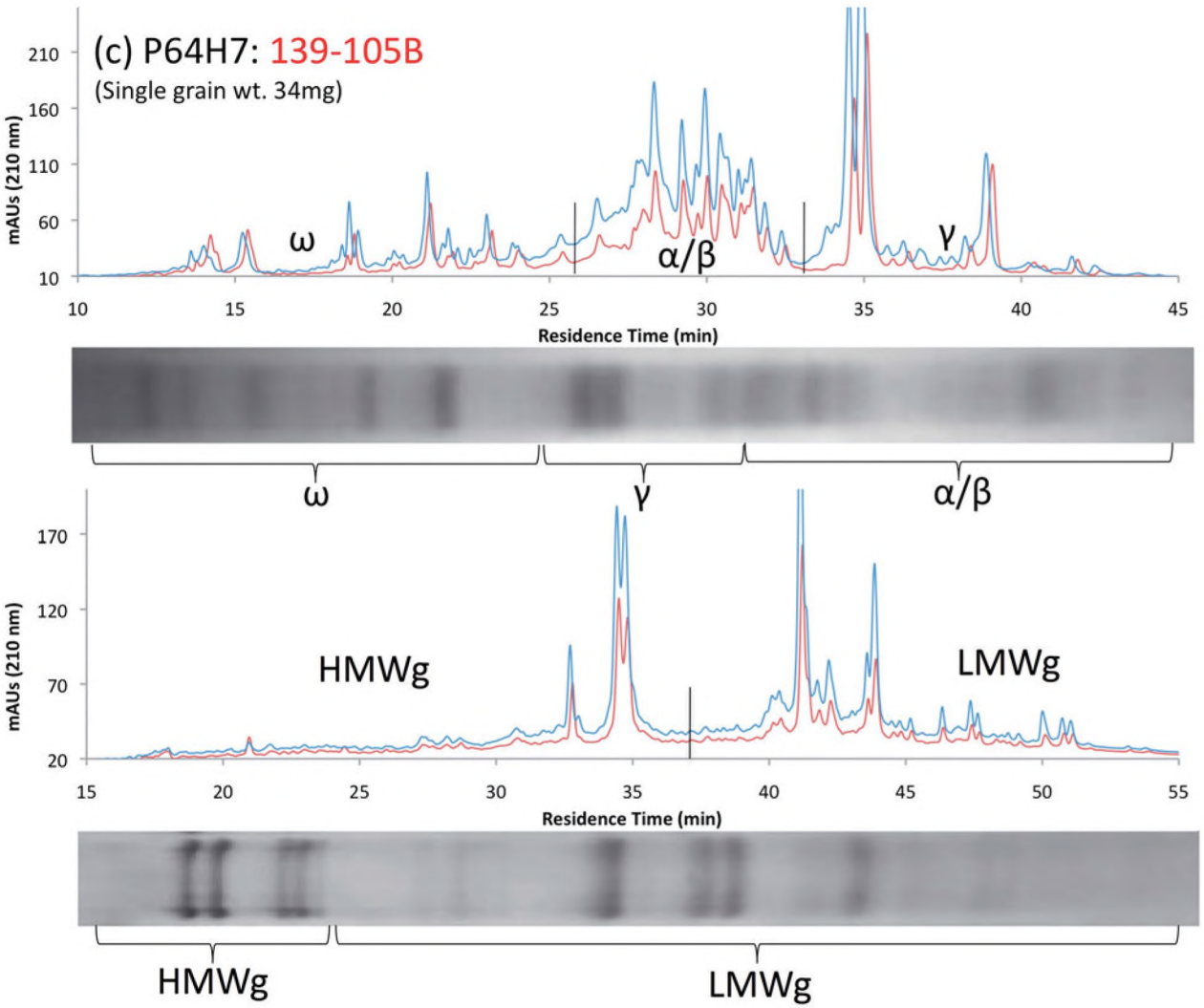
tions were performed using following plasmids pHMWg+Fmen+nos: pHMWg+EP-B2+nos: pDPG165 in a ratio of 2:2:1. The construct pDPG165 contains *Bar* gene confers resistance against bialaphos allowed selection of calli on bialaphos containing media.

### 11.2.2. Double gene construct

In order to avoid integration of plasmid backbone into the host genome, a third linear construct was prepared to transform calli. This construct has both genes with their corresponding promoters and terminators and is totally devoid of the plasmid backbone. Assemble of the above construct involves the following steps: i) Plasmid pHMWg+Fmen+nos was digested with *Hind*III/*Eco*RI and ligated overnight at 16°C with pBluescriptSK(+) backbone, and the resultant plasmid was used to transform DH5α cells followed by blue/white-screening. Selected white colonies were analyzed by PCR and restriction digestion; DNA from positive colonies was extracted as described earlier, and the resultant plasmid pBSK(+)HMWg+Fmen+nos was verified by sequencing. ii) The second




step involves production of pGEM(HMWg+EP-B2+nos) plasmid. The fragment was amplified from pHMWg+EP-B2+nos using specific primers in a 25ml reaction volume as described above. The PCR product was ligated into pGEM vector, using 50ng of backbone, 200ng of PCR product and 125 units of T4 DNA ligase. Reaction was incubated for 16 hours at 16°C, and 5 µl of the resultant plasmid was used to transform DH5α cells. Positive colonies carrying the plasmid pGEM(HMWg+EP-B2+nos) were selected on IPTG, x-GAL, ampicillin plates, DNA was extracted using Machinerey-Nagel miniprep kit and positive colonies were confirmed by PCR and by restriction digestion with suitable enzymes. The fragment containing HMWg+EP-B2+nos was excised from plasmid pGEM(HMWg+EPB2+nos) with *Eco*RI and ligated into vector pBSK(+)HMWg+Fmen+nos predigested with the same restriction enzymes, and 5 µl of the resultant plasmid was used to transform DH5α cells. Plasmids from positive colonies were extracted and confirmed by restriction analysis followed by sequencing (Fig. 34c). DNA of the confirmed plasmid pBSK(+)(HMWg+Fmen+nos/HMWg+EP-B2+nos) was digested with *Hind*III and *Not*I, and the product was subjected to agarose gel electrophoresis (1% gel, 1h, 110V). The corresponding 6kb band was excised



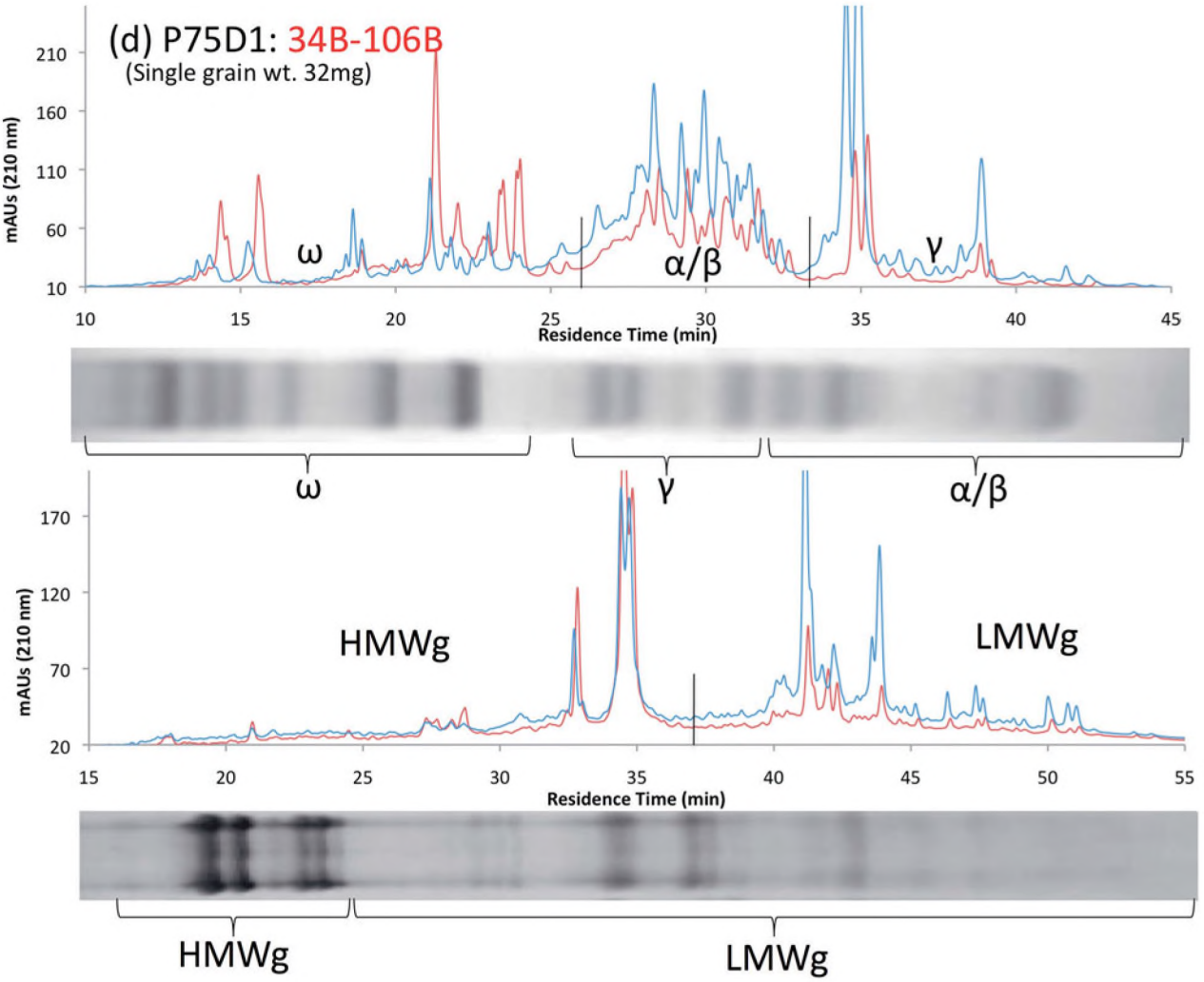

and purified using GenecleanIII kit. The concentration of DNA was adjusted at 1.5µg/µl with the help of freez drier, and 12µl of the above DNA was used to transform scutellar calli of wheat cv. Brundage 96.

### 11.2.3. Transformation procedure

The engineered plasmids were cotransformed in a 2:2:1 or 2:1 molar ratios (Table 9) into immature embryos of winter wheat variety Brundage 96 by microprojectile bombardment as described before (Okubara et al., 2002) except that both shoot regeneration media and rooting media contained 1.5 mg/liter bialaphos to select the transformants. The putative transformants thus obtained were later on transferred to selective media with increasing quantities of herbicide (bialaphos; up to 5 µg/ml) to reduce the number of false positives. The leaves of putative transformants at 4-5 leave stage were painted with a 2% Basta solution and evaluated for injury after 10-14 days on a 0-5 scale with 0 being no symptoms and 5 being extensive necrosis and/or dead.

### 11.2.4. PCR based confirmation of transformants

After four weeks on rooting media, the plants were transferred to soil, covered with a plastic lid for a week and kept under controlled conditions. After four weeks leaf samples were collected and DNA was extracted using Biosprint Plant DNA extraction kit



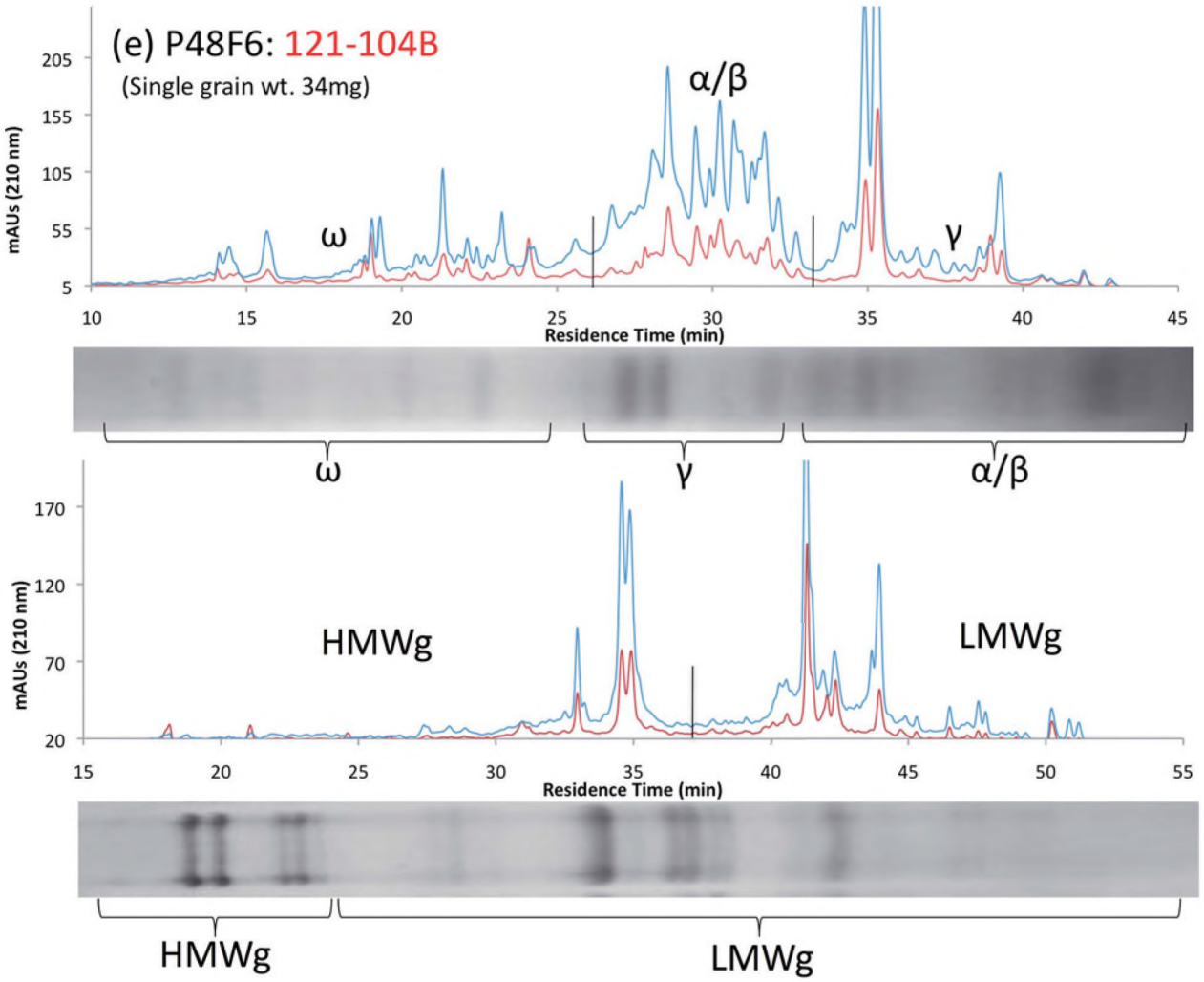

(Qiagen, Valencia, CA) following manufacturer's instructions. Candidate transformants were tested by PCR using gene-specific primers to confirm transgene integration(s) (Table 10). Positive plants identified from the assay were transferred to individual pots, and allowed to mature. As discussed earlier in view of the chimerism problem 10% of the grains from the plants showing transgene integration(s) were shown in glasshouse to assure transgene inheritance to next generation.

### 11.2.5. Sequencing of PCR products

Bands of expected sizes were excised from the agarose gel and DNA was eluted from the bands using Geneclean kit following manufacturer's instructions (MP Biomedicals). The eluted DNA was used as template for the sequencing reaction using either forward or reverse primers in separate reactions. Sequencing reactions were carried out in 10 µl reaction mixtures, each containing 100 ng template DNA, 0.35 µM of either forward or reverse primers, and BigDye® mixture (Applied Biosystems) using the following PCR profile: 96°C for 10 sec, 50°C for 15 sec, 60°C for 6 min with 24 iterations. Nucleotide sequences were edited using the DNAStar (Lasergene Software).

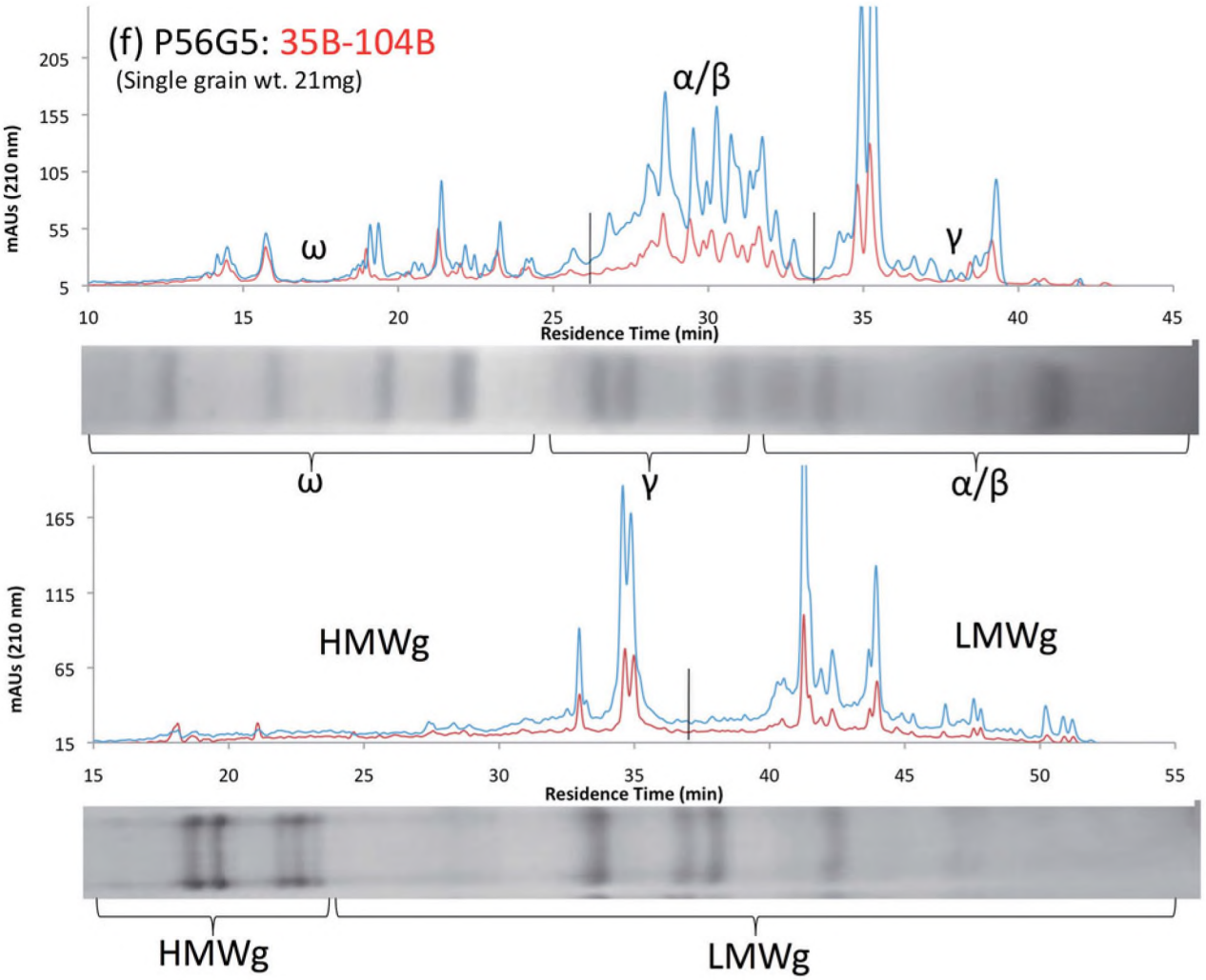
### 11.2.6. Results of transformation

Particle bombardment of wheat scutellar calli resulted in a total of 91 candidate transformants that survived bialaphos treatment. Out of the 91 candidate transfor-




mants 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 using gene specific primers (Table 10) to confirm transgene integration(s), 20 putative transformants showed integration(s) only for pHMWg+Fmen+nos, 4 showed integration(s) only for pHMWg+EP-B2+nos and 6 plants showed integrations for both (Fig. 35a,b). Out of the six plants showing integrations for both genes 2 plants were obtained using the double cassette linear construct and 4 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 similarity with the genes used for transformation, further confirming the transgene integration(s). These results suggested that the minimal gene cassettes lacking the vector backbone, functions as efficiently as the conventional vectors for wheat transformation, as was earlier documented for rice by Thi Loc et al. (2002).

Lines showing transgene integration(s) were raised to maturity, and the $T_1$ grains were collected. Ten percent of $T_1$ grains per $T_0$ plant were propagated in glasshouse and inheritance of transgene was studied. So far $T_1$ progeny of 4 $T_0$ transformants was screened for the inheritance of Fm-PEP integration(s), where 32.6% to 92.8% the progeny plants were found to show faithful



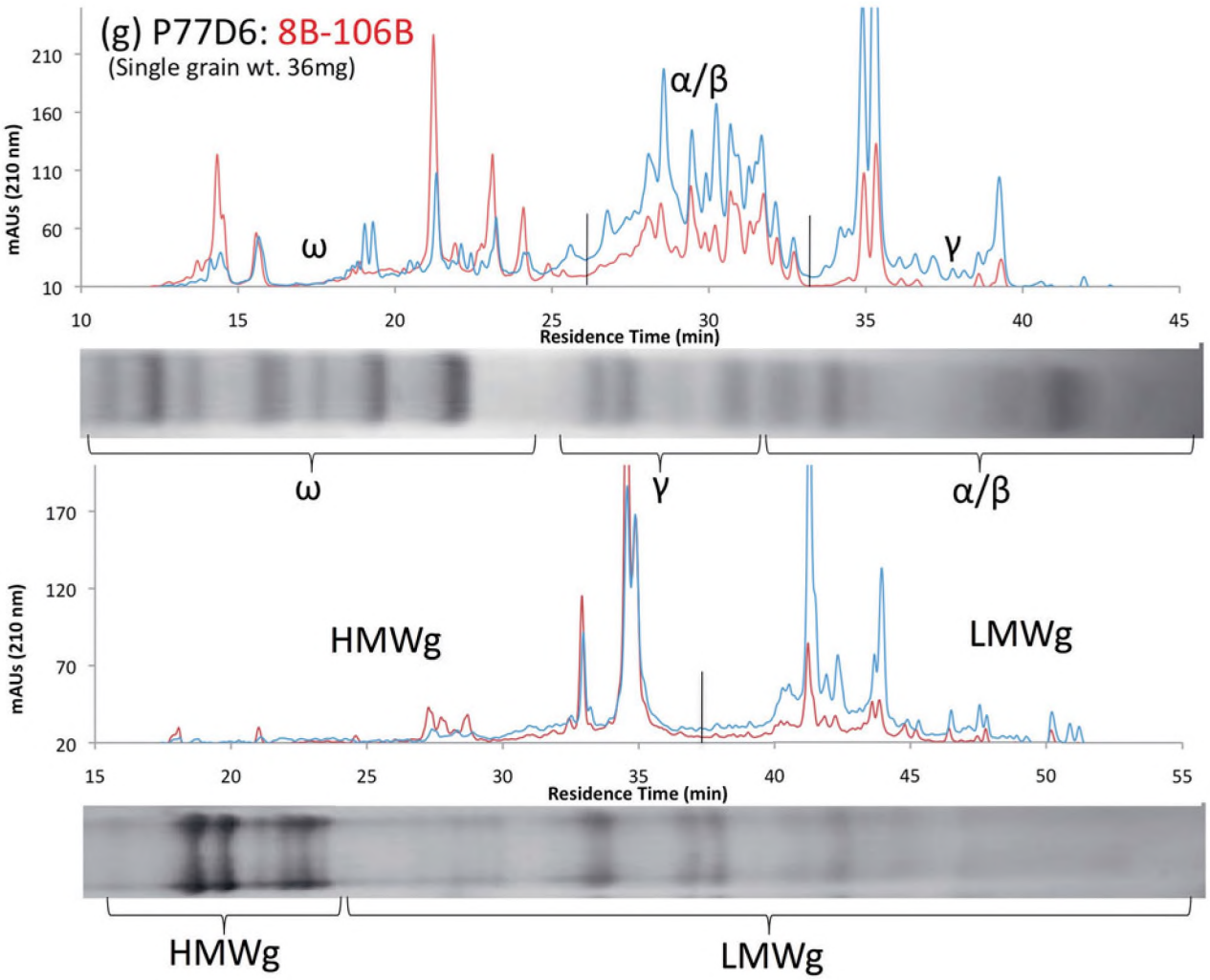

inheritance of the transgene. Sequencing of PCR product from 10 randomly selected $T_1$s also confirmed the inheritance to FmPEP in these wheat transformants. The $T_1$ plants showing faithful inheritance of the Fm-PEP will be screened for the inheritance of EP-B2 and will be raised to maturity to obtain $T_2$ grains, which will be assayed for the enzyme activity.

### *11.3. Site-directed mutagenesis of 'glutenases'*

#### 11.3.1. Flavobacterium meningosepticum prolyl endopeptidase (Fm-PEP)

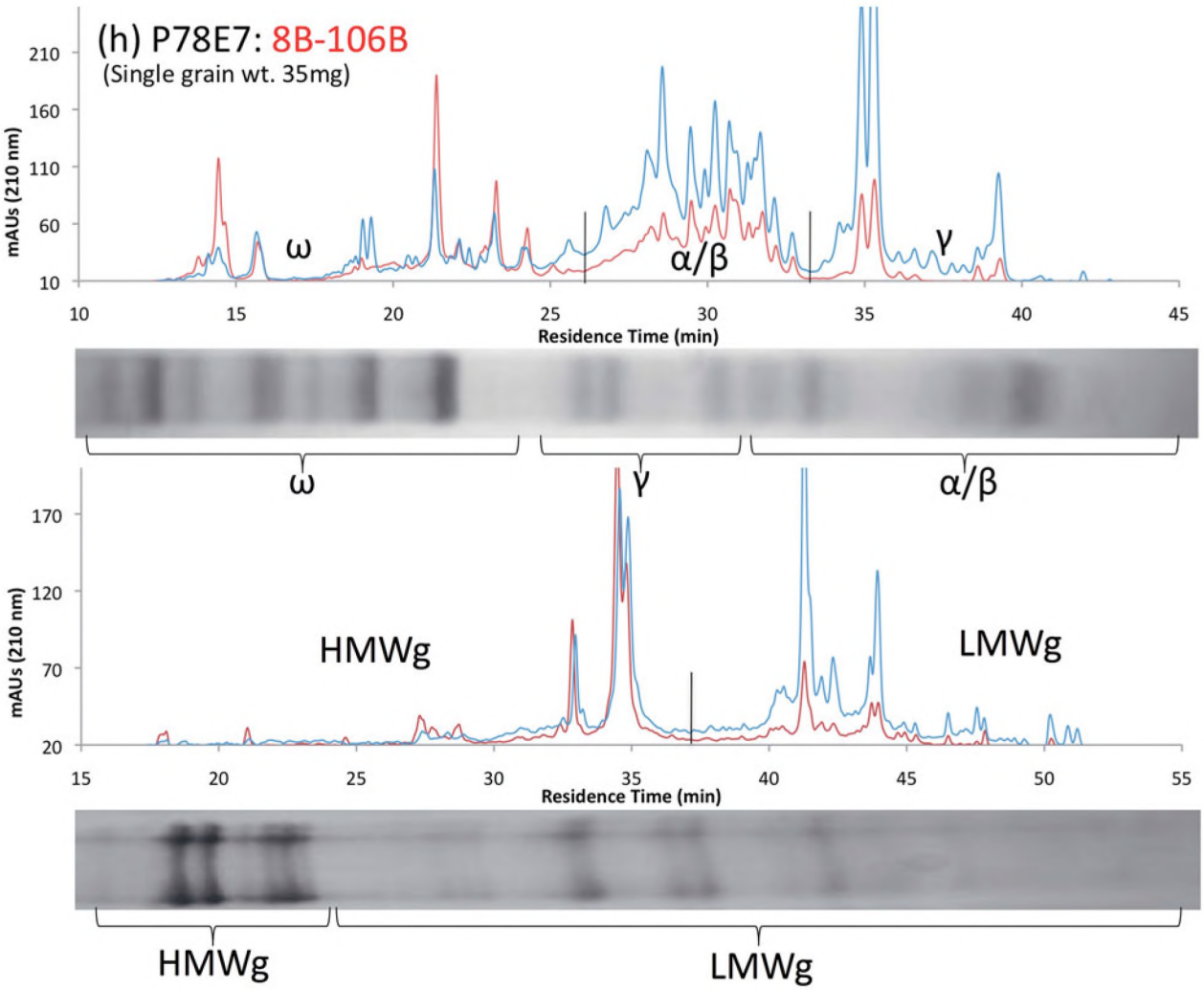
Due to the availability of prolyl endopeptidase (PEP) sequences from a number of thermophilic archaea and bacteria a sequence-based approach was followed to improve thermostability of the Fm-PEP. The sequence-based approach relies on conservation of sequence among related enzymes, and identification of motifs/domains contributing to the functional similarity among these enzymes. Multiple sequence alignment and phylogenetic analysis (dendrogram construction) was used to explore amino acid conservation and ancestral relationships among the group of homologous protein sequences.

The enzymes from different sources were identified by the BLAST P (protein blast) searches against NCBI nr (non-redundant) protein database using *Pyrococcus furiosus* (a thermophilic archaea) PEP sequence as query. The aim of this search was to find all possible candidate PEPs from thermophilic organisms to




compare with Fm-PEP sequence, and to find out residues showing differences among Fm-PEP and the PEPs derived from thermophilic organisms. Later, sequence conservation among allegedly thermostable PEPs at the variable residues identified from the above comparison was also studied from the same sequence alignment.

From our previous experience in the engineering of the *Bacillus* (1,3;1,4)-β-glucanase it is evident that thermostability can be achieved by increasing the amount of hydrogen bonding between the C- and N-terminal domains of the enzyme (von Wettstein, 2007). Similarly conclusions were reached in another study by structural analysis of a thermostable variant (stable up to 60°C) of *F. meningosepticum* prolyl endopeptidase, where the amount of hydrophobic forces was shown to determine the structural stability of the enzyme (Hamamatsu et al., 2005). Thus it is likely to increase thermostability of Fm-PEP by increasing the number of hydrogen bonds between the first and last (seventh) blade of the β propeller domain. Based on the above hypotheses comparison between Fm-PEP and Pfu-PEP was performed, and the amino acid residues showing large entropy differences within the first and seventh blade of the β propeller domain were selected for site-directed mutagenesis. By using the sequence information available for the thermophilic organisms at these sites for the first round of mutagenesis. With hope that substituting these amino acids with the ones present in thermophilic organisms will




improve the stability of enzyme by increasing number of hydrogen bonds. Primers were designed to create a total of 9 substitutions in the beta-propeller domain of Fm-PEP, 4 in the first blade and 5 in the seventh blade. Two mutations were introduced at a time using these primers, and the fragments were assembled by SOE-PCR. The fragments obtained from splicing by overlap extension (SOE)-PCR were digested with the appropriate set of restriction enzymes and ligated into the corresponding sites in the expression vector. Various combinations of these mutants were developed giving rise to a library of 81 variants, which are currently being tested for their thermostability and fidelity. The clones were combined in such a way that allows the occurrence of at least two mutations in each case, allowing different mutant combinations to be tested. Transformants were selected and cultured into 2 ml LB media containing 50µg/ml of kanamycin in 15ml culture tubes at 37°C until OD600=0.6 has reached, at this point isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce expression of the desired protein at a final concentration of 0.25mM. Cultures were shaken at 200rpm at 37°C for 20 hours. The transformants were harvested by centrifugation at 14,000g for 5 minutes, supernatant discarded. The soluble fraction and inclusion bodies were extracted with BugBuster™ protein extraction reagent (Novagen) following manufacturer's recommendations. Protein size was determined by 12% SDS-PAGE gel. Solubilization of inclusion bodies was performed by

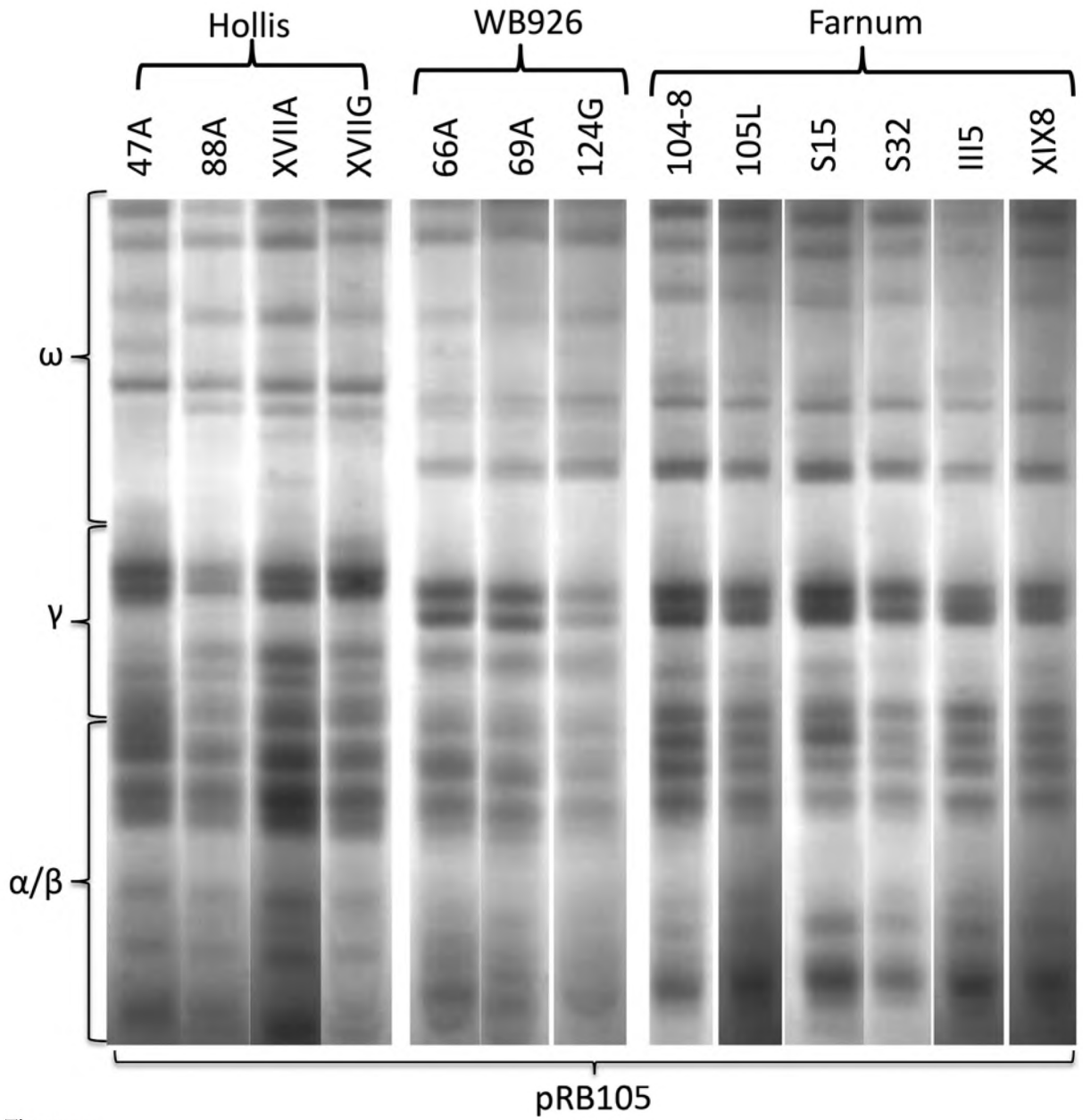



Figure 30.
Gliadin profiles (on lactic acid-PAGE) of $T_1$ grains derived from selected microspore-derived transformants showing reduced accumulation of gliadins.

addition of 500mL of 2M urea and 100 mM Tris-HCl (pH 12.5), followed by centrifugation at 14,000 rpm for 30 minutes at 4°C. For refolding of the protein, the eluted fraction was diluted into the refolding buffer (50mM Tris-HCl, pH 8.0; 0.5mM EDTA; 2M urea; 10% v/v glycerol; 5% sucrose; 1mM PMSF) (Singh and Panda 2005) to a concentration of 30ug/ml, followed by overnight incubation at 4°C with gently agitation. After the incubation the fraction was concentrated by acetone precipitation, followed by determination of its concentration by Bradford assay, and proper folding by SDS-PAGE gel analysis. The Fm-PEP activity was determined by the assay described in Uchiyama et al. (2000) and modi-

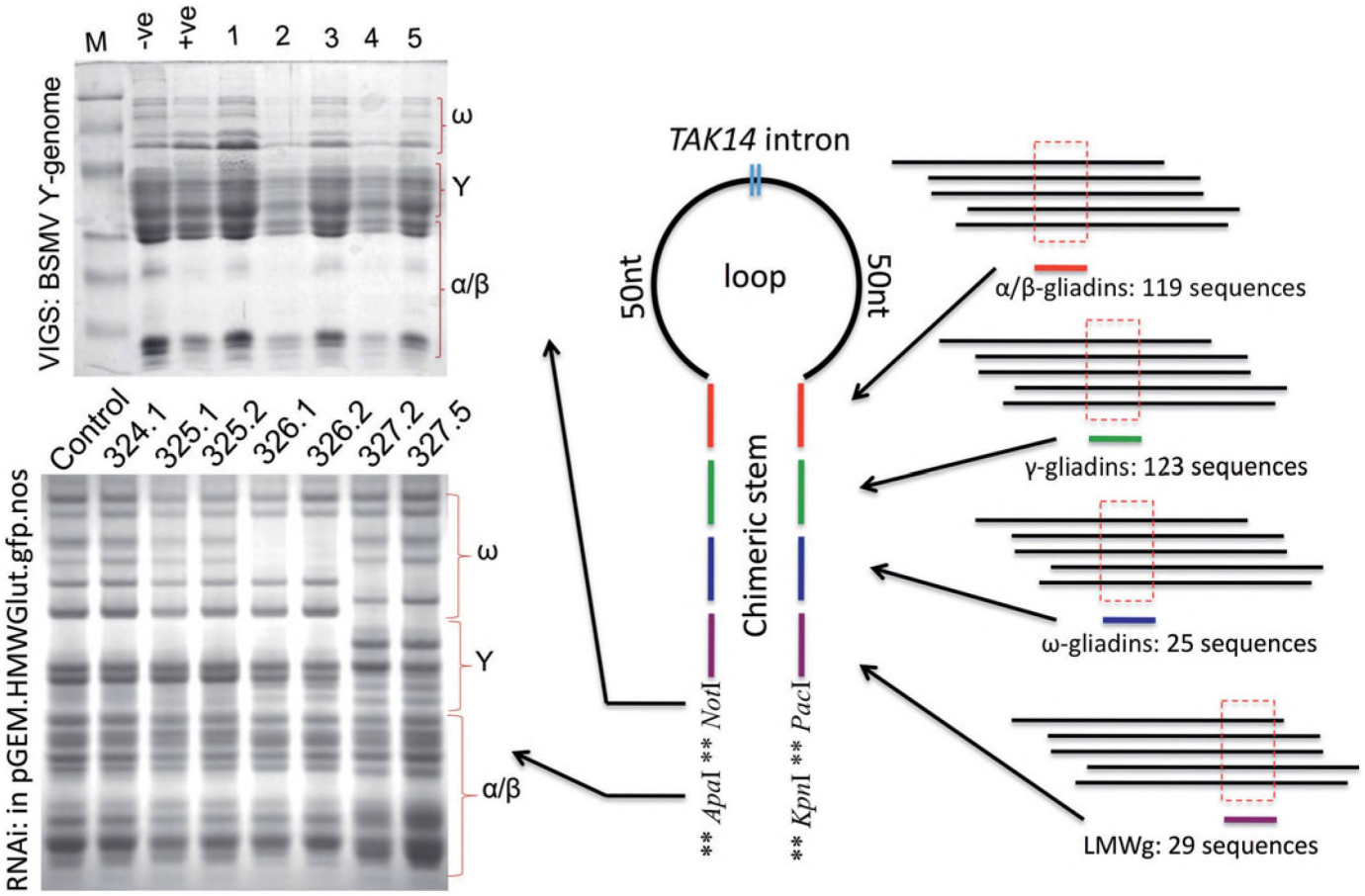



Figure 31.
Diagrammatic representation of the chimeric hairpin construct derived from short conserved stretches of DNA sequences identified from the sequence alignment of individual prolamin families (right hand). The chimeric hairpin was tested using virus induced gene silencing (upper left hand). The results showed reduced accumulation of gliadins in virus infested plants. The chimeric hairpin was also introduced in wheat genome using biolistic and microspore electroporation based approaches. The preliminary analysis of the $T_1$ grains showed elimination/reduced accumulation of different gliadins (lower left hand).

fied by Hamamatsu et al. (2005). Briefly, 100 ml of the extracted protein was initially incubated for 10 min at different temperatures ranging from 60–90°C with 10° intervals. After the heat shock each sample was mixed with 50 µl of 4.0 mM Z-Gly-Pro-2-NNap in 40% aqueous 1,4-dioxane and 940mL of 0.1M potassium phosphate buffer containing 1mM dithiothreitol (DTT) and 100mg/ml bovine serum albumin (BSA), and incubated at 30°C for 10 min. After incubation absorbance of the resulting diazo dye was measured at 410 nm. One unit of the enzyme activity is defined as the amount of activity shown by 1 pmol of P-naphthylamine/min at 30°C. The preliminary screen for the 12 variants having 1 to 7 substitutions showed thermostability up to 90°C for 10 minutes in one of the 11 variants carrying 2 substitutions at the adjoining sites (T412R and I413L) in the seventh blade (Fig. 36). The structural analysis of the variant based on the crystal structure of the Fm-PEP as template showed introduction of new hydrogen-bonds, which might have contributed to the enzyme stability (Fig. 36).

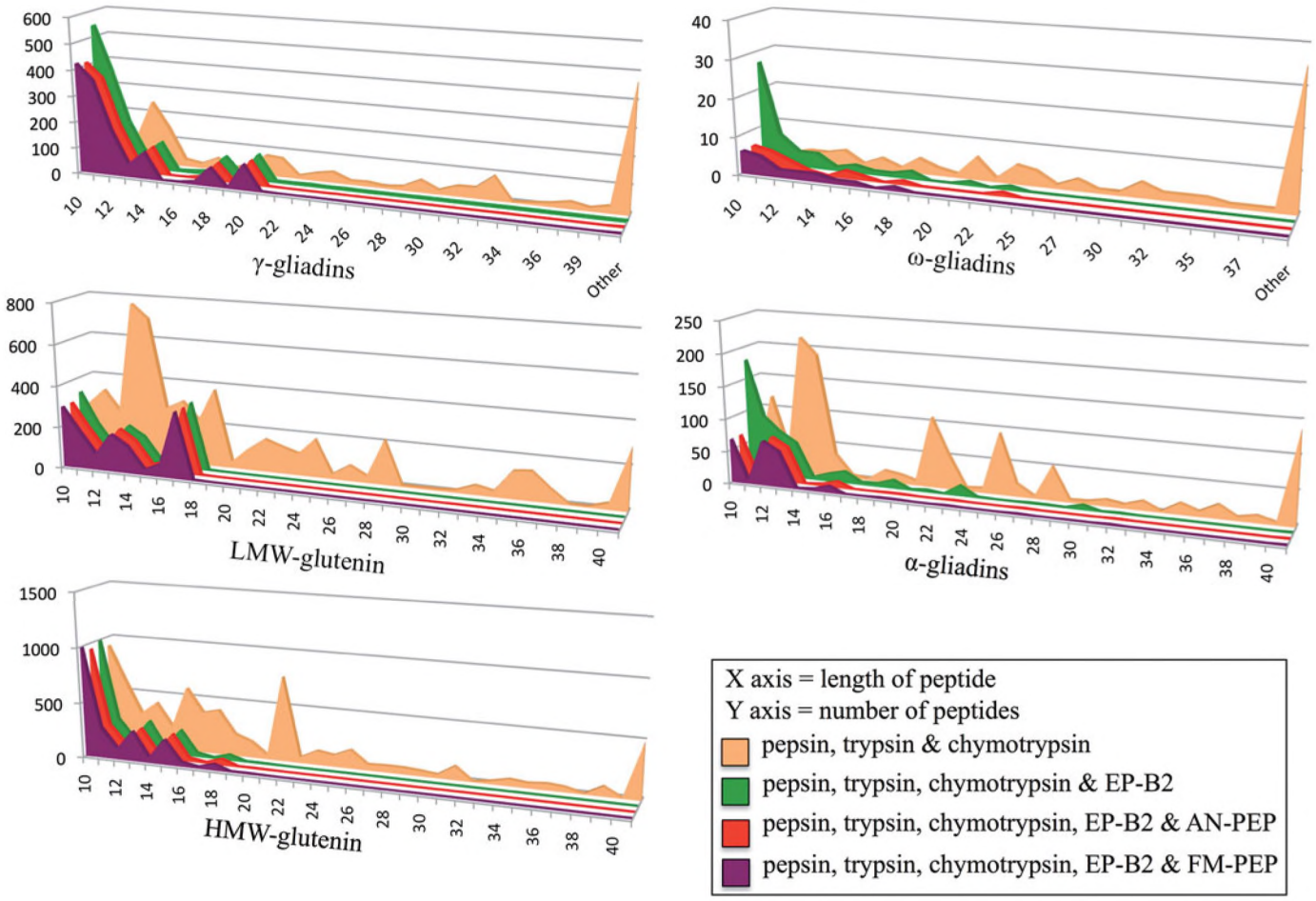



Figure 32.
Results of virtual digestion of wheat prolamins with endopeptidases and endoprotease under simulated gastro-intestinal conditions. [Modified from Osorio et al., 2012 Funct. Integr. Genomics]

### 11.3.2. Barley endoprotease B2 (EP-B2)

Due to high sequence and structural similarity between Ervatimin C and EP-B2, but differences in thermostabilities of the two enzymes a structure based-approach is used in this case. In 2004, Dattagupta and coworkers deducted that in Ervatamin C, a cysteine endoprotease, three amino acids, Ser32, Ser36, and Arg172 constitute an intricate network of hydrogen bonds between the two domains of the protein, as a result they are involved in both protein-protein and water mediated inter domain contacts (Guha Thakurta et al., 2004). The NH2, NH1, and NE atoms of Arg172 interact directly with residues Thr14, Pro15, and Ser32 of the left domain and provide stability to the protein.

Later, the same group used this information to introduce mutations in papain (a cysteine protease). Therefore the three corresponding residues Val32, Gly36 and Lys174 in the interdomain region were mutated to Ser, Ser and Arg, respectively, as found in Erv C (Choudhury et al., 2010).

After effective mutagenesis, the effect of temperature on activity of papain mutants was determined by incubating purified pro-enzyme samples (50–100 mg) in 100 mM Tris-HCl (pH 8.6) for 10 min at different temperatures ranging from 40–80°C with 5° intervals.

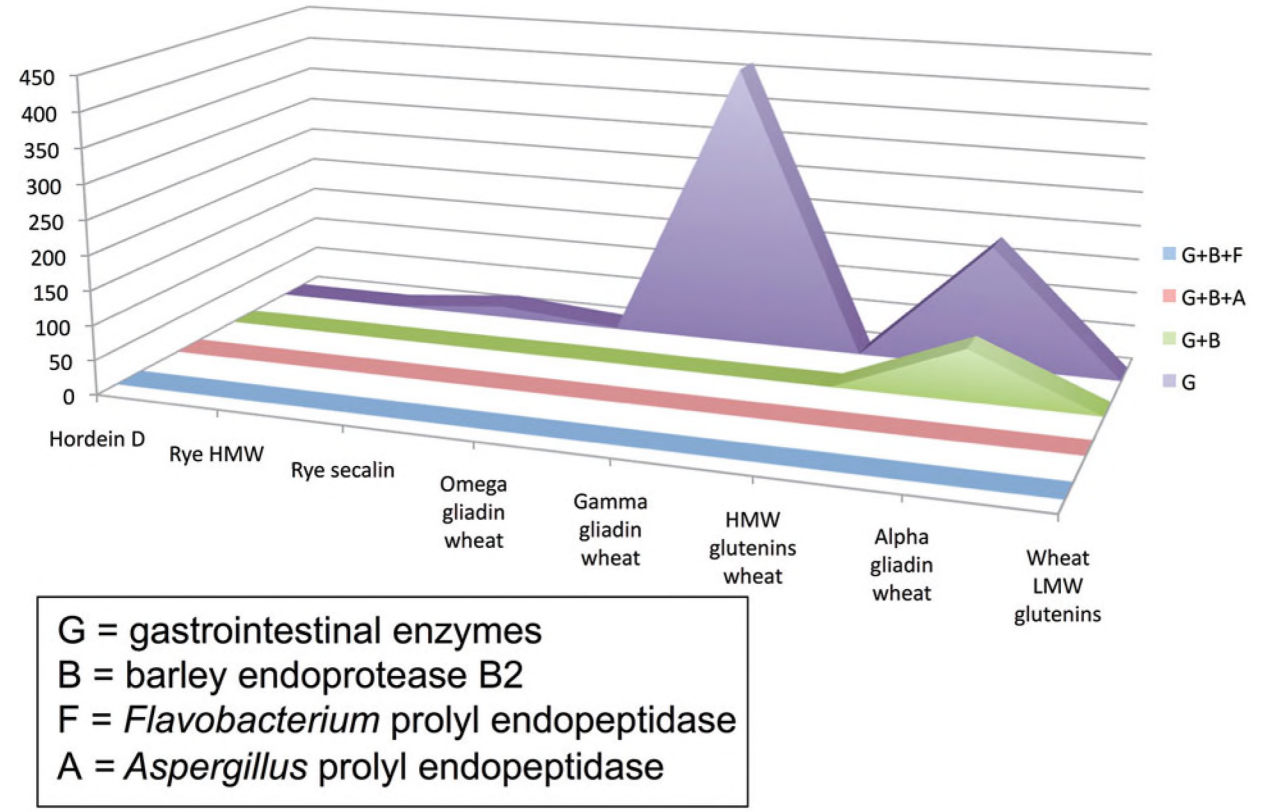



Figure 33.
Immunogenic peptides left undigested after different treatments.

After the thermal treatment protein samples were cooled immediately in an ice bath followed by activation of the pro-proteins to their respective mature forms. The initial and residual enzyme activities (at each temperature) were determined using azocasein assay at 37°C, and are expressed as percentages of the maximum activities. Results of the analysis suggested that the three mutations introduced in papain significantly increased its thermostability from 50 to 67°C.

In view of the structural and functional similarities between Ervatamin C (ERVC) and barley cysteine endoprotease B2 we used ERVC as a template to make alterations in EP-B2, with an anticipated change in the enzyme activity (Fig. 37a,b). Primers were designed to introduce mutations at the Val32, Gly36 and Lys174 to Ser, Ser and Arg. These three substitutions were already introduced in the EP-B2 gene by specifically designed primers and assembled in one fragment by splicing by overlap extension (SOE) PCR, and are currently being tested for its thermostability.

## 12. Conclusion

The best combination of 'glutenases' was identified as a result of a virtual screen based on the relative performance of different enzymes under simulated gastric conditions and literature survey. Genes encoding these glutenases were introduced in the wheat genome by biolistic approach, and their integration in genome and faithful inheritance was confirmed. The expression of desired enzymes will be soon verified at transcript level using qRT-PCR and their activities will be measured using assays previously described by Chevallier et al. (1992) for FM-PEP and Bethune et al. (2006) for EP-B2. The grains from the transformants

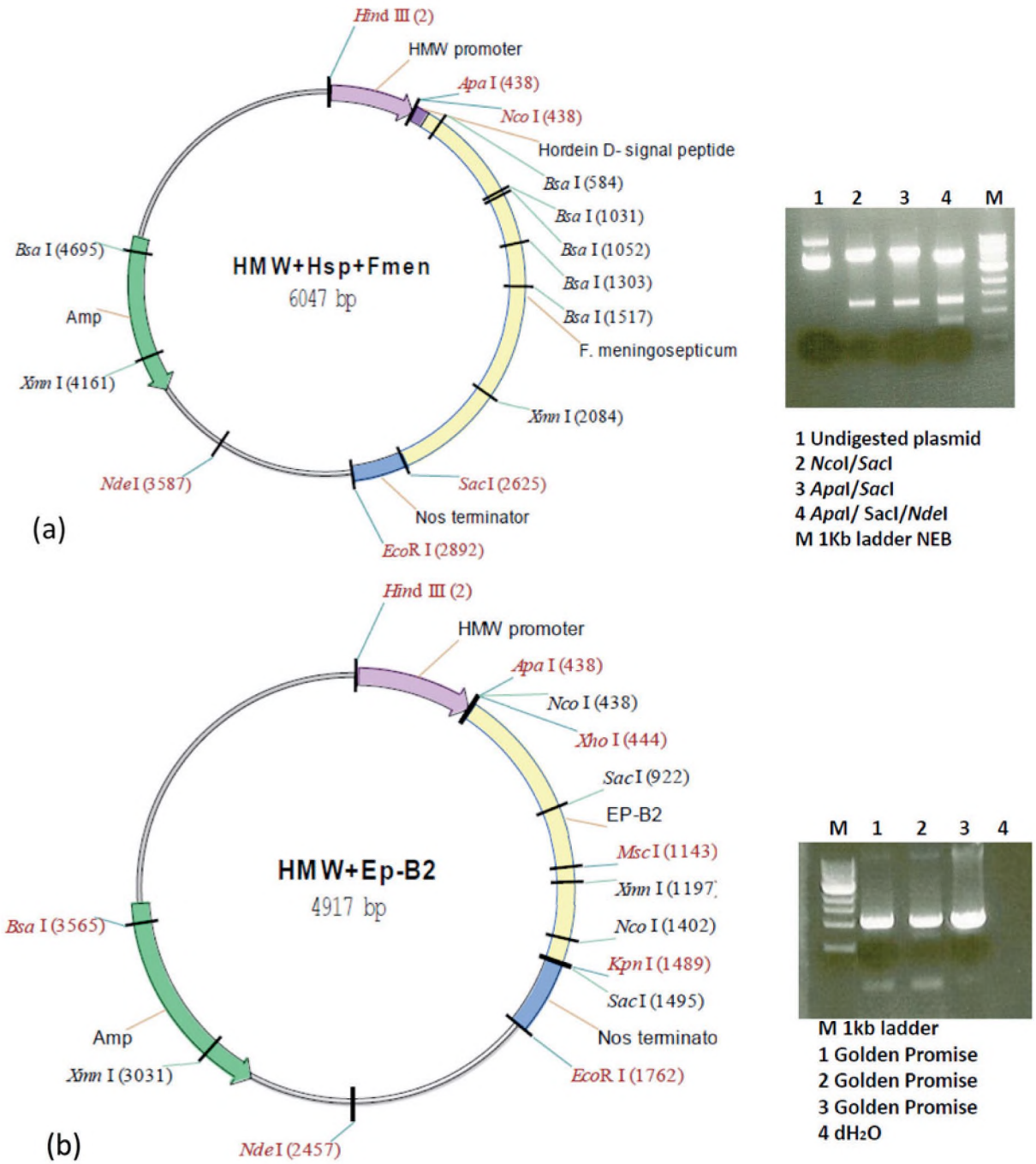
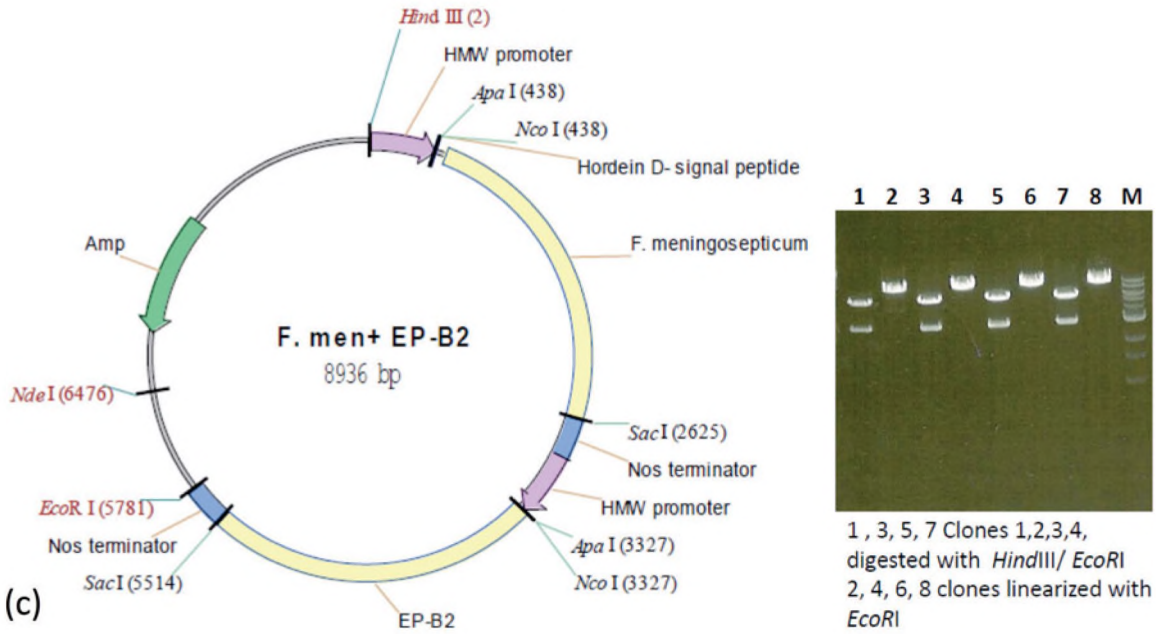



Figure 34.
Constructs developed for co-transformation of wheat scutellar calli. (A) Fragment encoding codon optimized version of F. meningosepticum prolyl endopeptidase (FM-PEP) under the control of wheat 1Dy high molecular weight glutenin (HMWg) promoter and nos terminator was cloned in the plasmid backbone of pGEM3zf (Promega, USA). (B) Fragment encoding for barley cysteine endoprotease B2 (EP-B2) under the control of wheat 1Dy HMWg promoter and nos terminator was cloned in the plasmid backbone of pGEM3zf. (c) Fragment encoding FM-PEP and EP-B2 both under the control of wheat 1Dy HMW promoter and nos terminator.




expressing high amounts of the two enzymes will be tested in whole grain breads prepared by the addition of whole/cracked transgenic grains to dough (prepared from normal wheat flour) just prior to baking process to avoid hydration and thermal denaturation of the enzymes. Deposition of glutenases in the prolamin storage protein bodies is also expected to protect them from degradation during the programmed cell death of the endosperm in the final stages of grain maturation and also from thermal denaturation during baking. Based in our previous experience with the expression of (1,3;1,4)-β-glucanase in barley endosperm, we know that it is possible to identify transformants expressing large quantities of foreign enzyme(s). In this situation only a small proportion of the transgenic grains containing large amount of 'glutenases' will be sufficient to detoxify the consumed gluten, and will prove as a perfect therapeutic alternative for celiac disease.

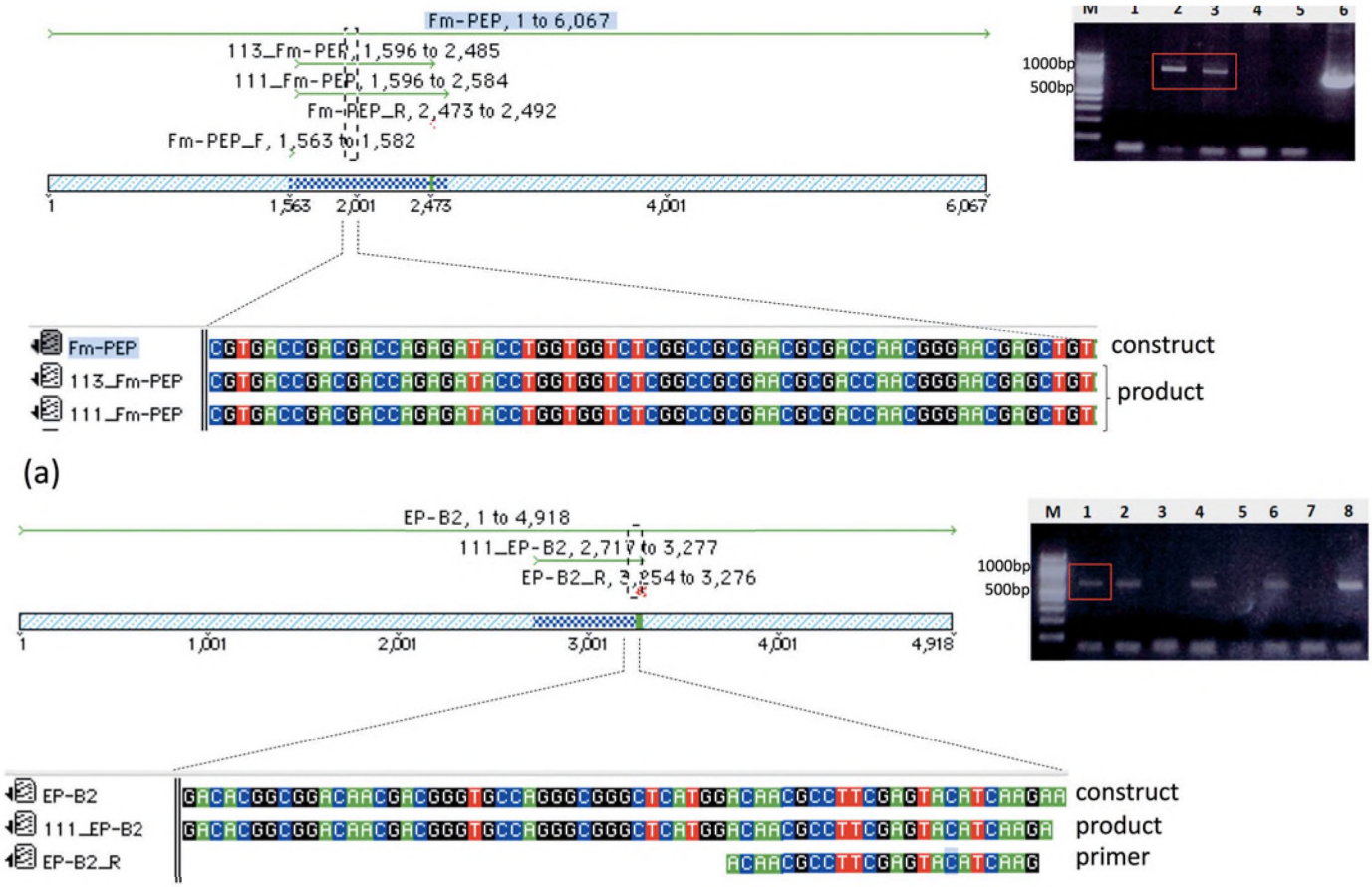



Figure 35.
PCR analysis of $T_0$ transformants using (a) *F. meningosepticum* prolyl endopeptidase specific primers. Lane number: 2 = 111, 3 = 113. (b) barley cysteine endoprotease B2 specific primers. Lane number: 1 = 111; 2 = 113. DNA was eluted from selected bands (highlighted by red rectangle) and sequenced using gene specific primer to confirm integration. M = 100 bp ladder (New England BioLabs Inc. Cat # N3231S).

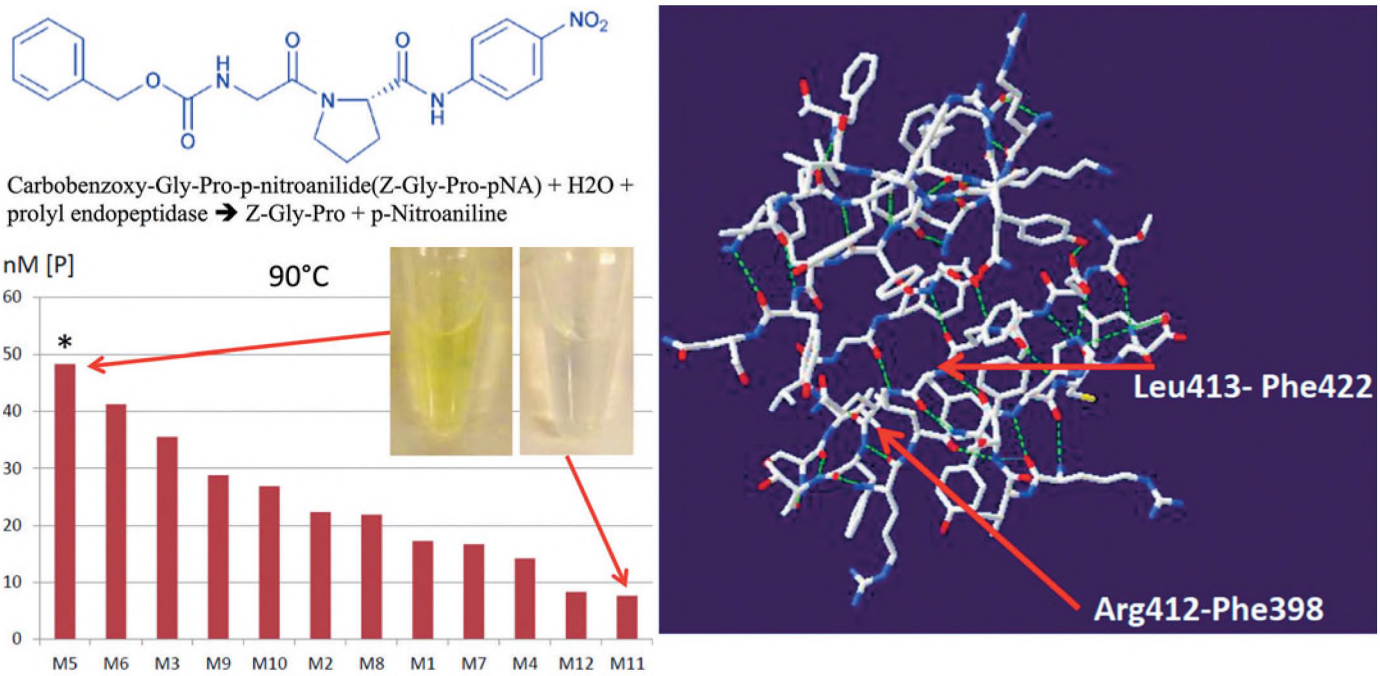



Figure 36.
Screening for thermostability of 11 (M2-12) variants of Fm-PEP engineered by combining different mutations introduced by directed mutagenesis in 1st and 7th blades of the β-propeller domain. The four amino acid substitutions are introduced in the 1st blade (R123I, D125W, P136E and N137L) and 5 aa substitutions are introduced in the 7th blade (N406S, T412R, I413L, F414Y and K415E), and various mutant combinations are produced by splicing by overlap extension PCR. A colorimetric assay was used to test the thermostability of Fm-PEP variants at 90°C for 10 min (upper and lower left). A variant M5 carrying two substitutions (T412R and I413L) in the 7th blade of the β-propeller domain showed high thermostability probably due to the introduction of additional hydrogen bonds between Leu413 and Phe422 and Arg412 and Phe398 (right). M1 = Fm-PEP (control); M2 = 123-125; M3 = (123-125)+(136-137); M4 = 406; M5 = 412-413; M6 = 412-415; M7 = 406 (412-415); M8 = (123-125)+(136-137)+(412-413); M9 = 406 (414-415); M10 = (123-125)+(136-137)+406 (414-415); M11 = (123-125)+(136-137)+406 (412-415) clone 1; 12 = (123-125)+(136-137)+406 (412-415) clone 2.

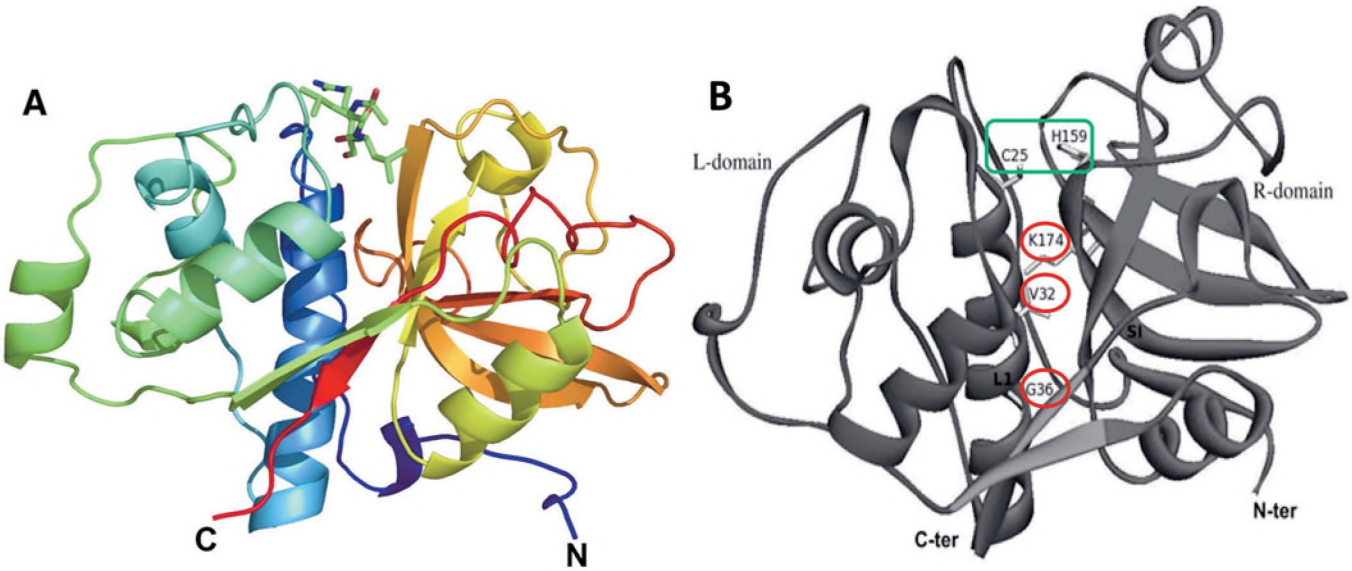



Figure 37.
Crystal structures of (a) EP-B2 and (b) papain showing structural similarities between the two enzymes.

# Acknowledgements

The authors would like to thank P. Reisenauer, J. Hansen, E. Mackenzie, S. Rynearson, M. Claar, Drs. C.G. Kannangara, X. Lu, and R. Brueggeman for field and laboratory assistance. Financial support by NIH grants GM080749-01A2 and 2R42DK072721-02, LSDS grant 3143956, Mercator Professorship to D.v.W. from German Research Foundation and the Programme of Introducing Talents of Discipline to Universities project B07017 is gratefully acknowledged.

# References


Arentz-Hansen, H., Fleckenstein, B., Molberg, Ø., Scott, H., Koning, F., Jung, G., Roepstorff, P., Lundin, K. & L. Sollid, 2004: The molecular basis for oat intolerance in patients with celiac disease. - PLoS Med., 1:e1.

Arentz-Hansen, H., Körner, R., Molberg, Ø., Quarsten, H., Vader, W., Kooy, Y., Lundin, K., Koning, F., Roepstorff, P., Sollid, L. & S. McAdam, 2000: The intestinal T cell response to a-gliadin in adult celiac disease is focused on a single deamidated glutamine targeted by tissue transglutaminase. - J. Exp. Med., 191:603-612.

Arentz-Hansen, H., McAdam, S.N., Molberg, Ø., Fleckenstein, B., Lundin, K.E.A., Jørgensen, T.J.D., Jung, G., Roepstorff, P. & L.M. Sollid, 2002: Celiac lesion T cells recognize epitopes that cluster in regions of gliadins rich in proline residues. - Gastroenterology, 123:803-809.

Bethune, M.T. & C. Khosla, 2012: Oral enzyme therapy for celiac sprue. - Methods Enzymol., 502:241-271.

Bethune, M.T., Strop, P., Tang, Y., Sollid, L.M. & C. Khosla, 2006: Heterologous expression, purification, refolding, and structural-functional characterization of EPB2, a self-activating barley cysteine endoprotease. - Chem. Biol., 13:637-647.

Boavida, L.C. & S. McCormick, 2007: Temperature as a determinant factor for increased and reproducible *in vitro* pollen germination in *Arabidopsis thaliana*. - Plant J., 52:570-582.

Bodd, M., Kim, C.Y., Lundin, K.E. & L.M. Sollid, 2012: Characterization of the T-cell reponse to gluten in patients with celiac disease expressing HLA-DQ2.2 but not any other HLA-DQ risk molecules. - Gastroenterology, 142:552-561.

Chevallier, S., Goeltz, P., Thibault, P., Banville, D. & J. Gagnon, 1992: Characterization of a prolyl endopeptidase from *Flavobacterium meningosepticum*. Complete sequence and localization of the active-site serine. - J. Biol. Chem., 267:8192-8199.

Choudhury, D., Biswas, S., Roy, S. & J.K. Dattagupta, 2010: Improving thermostability of papain through structure-based protein engineering. - Protein Eng. Des. Sel., 23:457-467.

Dewar, D., Pereira, S.P. & P.J. Ciclitira, 2004: The pathogenesis of coeliac disease. - Int. J. Biochem. Cell Biol., 36:17-24.

Druka, A., Franckowiak, J., Lundqvist, U., Bonar, N., Alexander, J., Houston, K., Radovic, S., Shahinnia, F., Vendramin, V., Morgante, M., Stein, N. & R. Waugh, 2011: Genetic dissection of barley morphology and development. - Plant Physiol., 155:617-627.

Ellis, H., Pollock, E., Engel, W., Fraser, J., Rosen-Bronson, S., Wieser, H. & P. Ciclitira, 2003: Investigation of the putative immunodominant T cell epitopes in coeliac disease. - Gut, 52:212-217.

Gianfrani, C., Troncone, R., Mugione, P., Cosentini, E., De Pascale, M., Faruolo, C., Senger, S., Terrazzano, G., Southwood, S., Auricchio, S. & A. Sette, 2003: Celiac disease association with CD8 T cell responses: identification of a novel gliadin-derived HLA-A2-restricted epitope1. - J. Immunol., 170:2719-2726.

Gil-Humanes, J., Piston, F., Tollefsen, S., Sollid, L.M. & F. Barro, 2010: Effective shutdown in the expression of celiac disease-related wheat gliadin T-cell epitopes by RNA interference. -Proc. Nat. Acad. Sci. U.S.A., 107:17023-17028.

Gordon-Kamm, W.J., Spencer, T.M., Mangano, M.L., Adams, T.R., Daines, R.J., Start, W.G., O'Brien, J.V., Chambers, S.A., Adams, W.R., Willetts, N.G., Rice, T.B., Mackey, C.J., Krueger, R.W., Kausch, A.P. & P.G. Lemaux, 1990: Transformation of maize cells and regeneration of fertile transgenic plants. - Plant Cell, 2:603-618.

Guha Thakurta, P., Biswas, S., Chakrabarti, C., Sundd, M., Jagannadham, M.V. & J.K. Dattagupta, 2004: Structural basis of the unusual stability and substrate specificity of ervatamin C, a plant cysteine protease from *Ervatamia coronaria*. - Biochemistry, 43:1532-1540.

Hamamatsu, N., Aita, T., Nomiya, Y., Uchiyama, H., Nakajima, M., Husimi, Y. & Y. Shibanaka, 2005: Biased mutation-assembling: an efficient method for rapid directed evolution through simultaneous mutation accumulation. - Protein Eng. Des. Sel., 18:265-271.

Jensen, 1979: Location of a high-lysine gene and the DDT-resistance gene on barley chromosome 7. - Euphytica, 28:47-56.

Kooy-Winkelaar, Y., van Lummel, M., Moustakas, A.K., Schweizer, J., Mearin, M.L., Mulder, C.J., Roep, B.O., Drijfhout, J.W., Papadopoulos, G., van Bergen, J. & F. Koning, 2011: Gluten-specific T cells crossreact

between HLA-DQ8 and the HLA-DQ2α/DQβ transdimer. - J. Immunol., 187:5123-5129.

Lundin, K.E.A., Scott, H., Hansen, T., Paulsen, G., Halstensen, T.S., Fausa, O., Thorsby, E. & L.M. Sollid, 1993: Gliadin-specific, HLA-DQ(crl*0501,-I*0201) restricted T cells isolated from the small intestinal mucosa of celiac disease patients. - J. Exp. Med., 178:187-196.

Mazzarella, G., Maglio, M., Paparo, F., Nardone, G., Stefanile, R., Greco, L., van de Wal, Y., Kooy, Y., Koning, F., Auricchio, S. & R. Troncone, 2003: An immunodominant DQ8 restricted gliadin peptide activates small intestinal immune response in in vitro cultured mucosa from HLA-DQ8 positive but not HLA-DQ8 negative coeliac patients. - Gut, 52:57-62.

Mok, Y.G., Uzawa, R., Lee, J., Weiner, G.M., Eichman, B.F., Fischer, R.L. & J.H. Huh, 2010: Domain structure of the DEMETER 5-methylcytosine DNA glycosylase. - Proc. Natl. Acad. Sci. U.S.A., 107:19225-19230.

Okubara, P.A., Blechl, A.E., McCormick, S.P., Alexander, N.J., Dill-Macky, R. & T.M. Hohn, 2002: Engineering deoxynivalenol metabolism in wheat through the expression of a fungal trichothecene acetyltransferase gene. - Theor. Appl. Genet., 106:74-83.

Qiao, S.W., Bergseng, E., Molberg, O., Jung, G., Fleckenstein, B. & L.M. Sollid, 2005: Refining the rules of gliadin T cell epitope binding to the disease-associated DQ2 molecule in celiac disease: Importance of proline spacing and glutamine deamidation. - J. Immunol., 175:254-261.

Qiao, S.W., Bergseng, E., Molberg, O., Jung, G., Fleckenstein, B. & L.M. Sollid, 2005: Refining the rules of gliadin T cell epitope binding to the disease-associated DQ2 molecule in celiac disease: importance of proline spacing and glutamine deamidation. - J. Immunol., 175:254-261.

Siegel, M., Bethune, M.T., Gass, J., Ehren, J., Xia, J., Johannsen, A., Stuge, T.B., Gray, G.M., Lee, P.P. & C. Khosla, 2006: Rational design of combination enzyme therapy for celiac sprue. - Chem. Biol., 1:649-658.

Singh, S.M. & A.K. Panda, 2005: Solubilization and refolding of bacterial inclusion body proteins. - J. Biosci. Bioeng., 99:303-310.

Sjostrom, H., Lundin, K.E.A., Molberg, O., Korner, R., McAdam, S.N., Anthonsen, D., Quarsten, H., Noren, O., Roepstorff, P., Thorsby, E. & L.M. Sollid, 1998: Identification of a gliadin T-cell epitope in coeliac disease: General importance of gliadin deamidation for intestinal T-cell recognition. - Scand. J. Immunol., 48:111-115.

Thi Loc, N., Tinjuangjun, P., Gatehouse, A.M.R., Christou, P. & J.A. Gatehouse, 2002: Linear transgene constructs lacking vector backbone sequences generate transgenic rice plants which accumulate higher levels of proteins conferring insect resistance. - Mol. Breed., 9:231-244.

Tollefsen, S., Arentz-Hansen, H., Fleckenstein, B., Molberg, R.M., Kwok, W.W., Jung, G., Lundin, K.E. & L.M. Sollid, 2006: HLA-DQ2 and -DQ8 signatures of gluten T cell epitopes in celiac disease. - J. Clin. Invest., 116:2226-2236.

Tye-Din, J.A., Stewart, J.A., Dromey, J.A., Beissbarth, T., van Heel, D.A., Tatham, A., Henderson, K., Mannering, S.I., Gianfrani, C., Jewell, D.P., Hill, A.V., McCluskey, J., Rossjohn, J. & R.P. Anderson, 2010: Comprehensive, quantitative mapping of T cell epitopes in gluten in celiac disease. - Sci. Transl. Med., 2:41ra51.

Uchiyama, H., Inaoka, T., Ohkuma-Soyejima, T., Togame, H., Shibanaka, Y., Yoshimoto, T. & T. Kokubo, 2000: Directed evolution to improve the thermostability of prolyl endopeptidase. - J. Biochem., 128:441-447.

Vader, L.W., de Ru, A., van der Wal, Y., Kooy, Y.M.C., Benckhuijsen, W., Mearin, M.L., Drijfhout, J.W., van Veelen, P. & F. Koning, 2002: Specificity of tissue transglutaminase explains cereal toxicity in celiac disease. - J. Exp. Med., 195:643-649.

Vader, W., 2002: The gluten response in children with celiac disease is directed toward multiple gliadin and glutenin peptides. - Gastroenterology, 122:1729-1737.

Vader, W., Stepniak, D., Kooy, Y., Mearin, L., Thompson, A., van Rood, J.J., Spaenij, L. & F. Koning, 2003: The HLA-DQ2 gene dose effect in celiac disease is directly related to the magnitude and breadth of gluten-specific T cell responses. - Proc. Natl. Acad. Sci. U.S.A., 100:12390-12395.

van de Wal, Y., Kooy, Y., Van Veelen, P., Pena, S., Mearin, L., Molberg, O., Lundin, K., Sollid, L., Mutis, T., Benckhuijsen, W., Drijfhout, J.W. & F. Koning, 1998b: T cells from the small intestinal mucosa of coeliac disease patients recognize unique peptide sequence of gliadin. -Gastroenterology, 114:A1105.

van de Wal, Y., Kooy, Y., van Veelen, P., Pena, S., Mearin, L., Papadopoulos, G. & F. Koning, 1998a: Cutting edge: Selective deamidation by tissue transglutami-

nase strongly enhances gliadin-specific T cell reactivity. – J. Immunol. 161:1585-1588.

van de Wal, Y., Kooy, Y.M.C., Drijfhout, J.W., Amons, R. & F. Koning, 1996: Peptide binding characteristics of the coeliac disease-associated DQ(alpha 1*0501, beta 1*0201) molecule. – Immunogenetics, 44:246-253.

van de Wal, Y., Kooy, Y.M.C., van Veelen, P., Vader, W., August, S.A., Drijfhout, J.W., Pena, S.A. & F. Koning, 1999: Glutenin is involved in the gluten-driven mucosal T cell response. – Eur. J. Immunol., 29:3133-3139.

von Wettstein, D., 2007: From analysis of mutants to genetic engineering. – Annu. Rev. Plant Biol., 58:1-19.

Wieser, H., Antes, S. & W. Seilmeier, 1998: Quantitative determination of gluten protein types in wheat flour by reversed-phase high-performance liquid chromatography. – Cereal Chem., 75:644-650.

# List of project related publications


von Wettstein, D., 2009: Mutants pave the way to wheat and barley for celiac patients and dietary health. - In "Induced Plant Mutations in the Genomics Era" (ed. Q.Y. Shu), pp. 187-190. Food and Agriculture Organization of the United Nations, Rome.

von Wettstein, D., Rustgi, S., Kannangara, C.G., Ankrah, N., Wen, S., Brew-Appiah, R.A.T., Wen, N., Gemini, R., Brueggeman, R., Reisenauer. P., Gill, K.S., Liu, B., Pang, J., Wang, X., Claar, M., Langen, G. & K.H. Kogel, 2010: A multipronged approach to develop nutritionally improved, celiac safe, wheat cultivars. - Ann. Wheat Newslet., 56:261-264.

Rustgi, S., Brew-Appiah, R.A.T., Wen, N., Ankrah, N., Wen, S., Gemini, R., Brueggeman, R., Kannangara, C.G., Claar, M., Langen, G., Kogel, K.-H., Pang, J., Liu, B. & D. von Wettstein, 2010: Epigenetics for the elimination of celiac-causing epitopes from wheat grains. - Plant Molecular Biology, Montreal, Canada, July 31 to August 4, Abstract # P08015.

Brew-Appiah, R.A.T., Rustgi, S., Claar, M., Langen, G., Kogel, K.-H., Weigel, D. & D. von Wettstein, 2010: Artificial microRNAs for silencing wheat proteins causing celiac disease. - Plant Molecular Biology, Montreal, Canada, July 31 to August 4, Abstract # P08017.

Wen, N., Rustgi, S., Wen, S., Claar, M., Langen, G., Pang, J. & D. von Wettstein, 2010: Molecular cloning and chromosomal localization of wheat *DEMETER* genes. - Plant Molecular Biology, Montreal, Canada, July 31 to August 4, Abstract # P08013.

Wen, S., Rustgi, S., Kannangara, G., Claar, M., Langen, G., Pang, J. & D. von Wettstein, 2010: Epigenetic regulation of wheat gliadin and low molecular weight glutenins. - Plant Molecular Biology, Montreal, Canada, July 31 to August 4, Abstract # P08019.

von Wettstein, D., 2010: Epigenetic elimination of celiac-epitopes from wheat grains. - In "4th International Workshop Rauischholzhausen on Novel molecular targets for improvement of crop resistance as a measure against famine", September 30-October 2, 2010, Justus Liebig University, Giessen.

von Wettstein, D. & S. Rustgi, 2010: Wheat for celiac patients and improved disease prevention. - In "Washington Biotechnology & Biomedical Assoc (WBBA)/ Life Sciences Discovery Fund (LSDF) Open House", Seattle, WA 98102 United States, August 12, 2010.

von Wettstein, D. 2011: Wheat for celiac patients and improved disease prevention. - In "The International Conference on Gene Targeting", February 9-Februrary 12, 2011, Vienna, Austria.

Langen, G., Kogel, K.H. & D. von Wettstein, 2011: Gluten free wheat - New hope for celiac patients. Mirror of Research University of Giessen, No. 1, May 2011.

Wen, S., Wen, N., Pang, J., Langen, G., Brew-Appiah, R.A.T., Mejias, J.H., Osorio, C., Yang, M.M., Gemini, R., Moehs, C.P., Zemetra, R.S., Kogel, K.H., Liu, B., Wang, X., von Wettstein. D. & S. Rustgi, 2012: The structural genes of wheat and barley 5-methylcytosine DNA glycosylases and their potential applications for human health. - Proc. Natl. Acad. Sci. U.S.A., 109:20543-20548.

[Note: This paper was selected as the National Human Genome Research Institute's (NHGRI) Genomic Advances of the Month (http://www.genome.gov/27551968)]

Osorio, C., Wen, N., Gemini, R., Zemetra, R., von Wettstein, D. & S. Rustgi, 2012. Targeted modification of wheat grain protein to reduce the content of celiac causing epitopes. - Funct. Integr. Genomics, 12:417-438.

Rustgi, S., von Wettstein, D., Ankrah, N., Brew-Appiah, R.A.T., Wen, S., Wen, N., Osorio, C., Gemini, R., Reisenauer, P., Lu X. & J.H. Mejias, 2012: Engineering wheat for celiac patients. - Ann. Wheat Newslet., 58:248-253.

Rustgi, S., von Wettstein, D., Ankrah, N., Mejías, J., Brew-Appiah, R., Wen, S., Wen, N., Osorio, C., Gemini, R., Reisenauer, P., Mohan, J. & J. Brabb II, 2013: Multipronged approach to develop nutritionally improved, celiac safe, wheat cultivars. - In "2013 Dryland Field Day Abstracts: Highlights of Research Progress", Technical Report 13-1.

Brew-Appiah, R.A.T., Ankrah, N., Rustgi, S. & D. von Wettstein, 2013: Microspore electroporation based transformation for the production of homozygous wheat lines deficient in immunogenic prolamins. - in "Annual Meeting of American Society of Plant Biologists", July 20-24, 2013, Rhode Islands, USA, P06039.

Rustgi, S., von Wettstein, D., Ankrah, N., Mejias, J.H., Brew-Appiah, R.A.T., Wen, S., Wen, N., Osorio, C., Gemini, R., Reisenauer, P., Mohan, J. & I. Brabb, 2013: A natural dietary-therapy for gluten intolerance, sensitivity and allergenicity. - Ann. Wheat Newslet. 59:151-155.

# Appendices

APPENDIX 1. CpG-islands detected in prolamin sequences.

| Accession# | Size (bp) | locus | Allele | Cultivar | Type | 5' UTR | CpG-island*** | Size | Location |
|---|---|---|---|---|---|---|---|---|---|
| High molecular weight glutenins (HMWgs) | | | | | | | | | |
| DQ533690 | 4648 | Glu-A1 | GluA1-2 | 211.10214 | - | 2173bp | Junction of 5'UTR & TR | 224 | 2048-2445 |
| X61009 | 2885 | Glu-A1 | Glu-1Ax1 | Hope | 1Ax1 | 264bp | Junction of 5'UTR & TR | 408 | 133-540 |
| JN172932 | 2601 | Glu-A1 | Glu-1Ax1.1 | Barbela | 1Ax1.1 | 0bp | After TSS | 228 | 49-276 |
| EF055262 | 2448 | Glu-A1 | Glu-1Ax-2*B | Bankuti 1201 | 1Ax | 0bp | After TSS | 224 | 49-272 |
| HQ846968 | 1800 | Glu-A1 | pseudogene | Saumur | 1Ay | 0bp | After TSS | 300 | 49-348 |
| AF145590 | 2537 | Glu-A1 | Glu-A1-x-null | Pane 247 | - | 19bp | After TSS | 247 | 49-295 |
| EU287439 | 2355 | Glu-B1 | Glu-B1-1 | - | - | 0bp | After TSS | 227 | 49-275 |
| DQ478571 | 2373 | Glu-B1 | - | Jinan177 | 1Bx | 0bp | After TSS | 227 | 49-275 |
| BK006773 | 2388 | Glu-B1 | Glu-B1-1a | Chinese Spring | Bx7 | 0bp | After TSS | 227 | 49-275 |
| FM955452 | 2391 | Glu-B1 | - | - | 1Bx13 | 0bp | After TSS | 217 | 49-265 |
| EF540764 | 2391 | Glu-B1 | - | - | 1Bx13 | 0bp | After TSS | 217 | 49-265 |
| AB263219 | 2244 | Glu-B1 | Glu-B1i | Haruyutaka | Bx17 | 0bp | After TSS | 227 | 49-275 |
| EU360771 & AY367771 | 5252 | Glu-B1 | Glu-1Bx14 | Xiaoyan 6 | 1Bx14 | 2378bp | Junction of 5'UTR & TR | 458 | 2211-2668 |
| AY553933 | 3426 | Glu-B1 | - | - | 1Bx23 | 1038bp | Junction of 5'UTR & TR | 443 | 871-1313 |
| DQ086215 | 2418 | Glu-B1 | - | - | 1By15 | 55bp | Junction of 5'UTR & TR | 321 | 51-371 |
| EF540765 | 2220 | Glu-B1 | - | - | 1By16 | 0bp | After TSS | 235 | 80-314 |
| EF381742 | 1979 | Glu-B1 | - | subsp. Tibeticum | 1By9 | 0bp | After TSS | 241 | 49-289 |
| EF381741 | 1979 | Glu-B1 | - | subsp. Yunnanense | 1By9 | 0bp | After TSS | 233 | 49-281 |
| EU137874 | 3578 | Glu-B1 | - | Xiaoyan 54 | 1By15 | 1421bp | After TSS | 237 | 1501-1737 |

| Accession# | Size (bp) | locus | Allele | Cultivar | Type | 5' UTR | CpG-island*** | Size | Location |
|---|---|---|---|---|---|---|---|---|---|
| BK006458 | 2547 | Glu-D1 | | Glenlea | Dx5 | 0bp | After TSS | 201 | 118-318 |
| GQ241722 | 2487 | Glu-D1 | - | - | 1Dx1.5* | 0bp | n/d | - | - |
| DQ478570 | 2523 | Glu-D1 | - | Jinan177 | 1Dx | 0bp | n/d | - | - |
| BK006460 | 2508 | Glu-D1 | Glu-D1-1a | Chinese Spring | Dx2 | 0bp | n/d | - | - |
| AB481100 | 2262 | Glu-D1 | - | KU7510 | - | 0bp | n/d | - | - |
| X12929[b] | 6462 | Glu-D1 | Glu-1D-2b | Cheyenne | 10 | 2937bp | n/d | - | - |
| DQ211819 | 1470 | Glu-D1 | - | PBW 343 | 1Dy10 | 17bp | n/d | - | - |
| EU266533 | 2022 | Glu-D1 | - | Trebisovka | 1Dy12* | 0bp | n/d | - | - |
| EU287437 | 1950 | Glu-D1 | Glu-D1-2-Dy10 | - | Dy10 | 0bp | n/d | - | - |
| FJ226583 | 1980 | Glu-D1 | - | - | 1Dy12.2* | 0bp | After TSS | 241 | 49-289 |
| EU495302 | 1980 | Glu-D1 | - | yn11 | 1Dy12* | 0bp | After TSS | 241 | 49-289 |
| EU528008 | 1917 | Glu-D1 | - | - | 1Dy11 | 0bp | After TSS | 245 | 49-293 |
| AY695379 | 1971 | Glu-D1 | - | Xinjiang Rice | 1Dy10.1 | 0bp | After TSS | 241 | 49-289 |
| BK006459 | 1977 | Glu-D1 | - | Chinese Spring | Dy12 | 0bp | After TSS | 241 | 49-289 |
| X03041* | 3095 | Glu-D1 | - | - | 1Dy | 425bp | Junction of 5'UTR & TR | 335 | 380-714 |
| X12928 | 8463 | Glu-D1 | Glu-1D-1b | Cheyenne | 5 | 3898bp | 2 in 5'UTR, 1 after TSS | 753, 253, 201 | 699-1451, 1652-1904, 4016-4216 |
| EU287438 | 2523 | Glu-D1 | Glu-D1-1 | - | - | 0bp | After TSS | 201 | 118-318 |
| AJ314772 | 2490 | Glu-R1 | - | S-149 [40+1R(1D)] | X | 139bp | Junction of 5'UTR & TR | 325 | 110-434 |
| AF216868 | 2490 | Glu-R1 | Glu-R1x | 7841 | X | 139bp | Junction of 5'UTR & TR | 325 | 110-434 |
| AJ314784 | 2625 | Glu-R1 | - | CS 1R addition | X | 139bp | At TSS | 280 | 140-419 |
| AF216869 | 2331 | Glu-R1 | Glu-R1y | 7841 | Y | 139bp | After TSS | 301 | 179-479 |
| AJ314783 | 2331 | Glu-R1 | - | S-149 [40+1R(1D)] | Y | 139bp | After TSS | 301 | 179-479 |
| AJ314785 | 2330 | Glu-R1 | - | CS 1R addition | - | 139bp | After TSS | 237 | 219-455 |

| Accession# | Size (bp) | locus | Allele | Cultivar | Type | 5' UTR | CpG-island*** | Size | Location |
|---|---|---|---|---|---|---|---|---|---|
| AY899823 | 2391 | - | pseudogene | 4072 | X | 0bp | After TSS | 251 | 49-299 |
| JN408502 | 2250 | - | - | CS/Ae.longissima | Y | 0bp | After TSS | 254 | 49-302 |
| EU863823 | 1977 | - | - | recital | - | 0bp | After TSS | 241 | 49-289 |
| DQ000161 | 1980 | - | - | Jinan 177 | - | 0bp | After TSS | 233 | 49-281 |
| AY249141** | 2297 | - | - | Shanda No.9 | - | 0bp | n/d | - | - |
| Barley D-hordein | | | | | | | | | |
| JQ867076.1 | 2369 | Hor3 | - | Golden Promise | - | 2bp | After TSS | 341 | 50-390 |
| JQ867091 | 2368 | Hor3 | - | Barke | - | 2bp | After TSS | 341 | 50-390 |
| JQ867077 | 2309 | Hor3 | - | Golden Promise | - | 2bp | After TSS | 341 | 50-390 |
| AY998008 | 698 | Hor3 | - | *H. chilense* (H65) | - | 59bp | Junction of 5'UTR & TR | 401 | 50-450 |
| AY998006 | 699 | Hor3 | - | *H. chilense* (H65) | - | 59bp | Junction of 5'UTR & TR | 402 | 50-451 |
| AY998009 | 1210 | Hor3 | - | *H. chilense* (H1) | - | 873bp | Junction of 5'UTR & TR | 448 | 707-1154 |
| AY998007 | 699 | Hor3 | - | *H. chilense* (H65) | - | 59bp | Junction of 5'UTR & TR | 402 | 50-451 |
| AY998005 | 695 | Hor3 | - | *H. chilense* (H65) | - | 56bp | Junction of 5'UTR & TR | 398 | 50-447 |
| EF417988 | 4305 | Hor3 | - | *H. chilense* (H1) | - | 826bp | Junction of 5'UTR & TR | 558 | 660-1218 |
| EF417989 | 4227 | Hor3 | - | *H. chilense* (H7) | - | 826bp | Junction of 5'UTR & TR | 558 | 660..1218 |
| D82941.1 | 2296 | Hor3 | - | Haruna Nijo | - | 36bp | After TSS | 375 | 50-424 |
| X84368 | 1859 | Hor3 | - | Bomi | - | 434bp | Junction of 5'UTR & TR | 448 | 375-822 |
| Low molecular weight glutenins (LMWgs) | | | | | | | | | |
| EU871816 | 1292 | Glu-A3 | Glu-A3-d | COOK | - | 197bp | n/d | - | - |

| Accession# | Size (bp) | locus | Allele | Cultivar | Type | 5' UTR | CpG-island*** | Size | Location |
|---|---|---|---|---|---|---|---|---|---|
| EU189087 | 2269 | Glu-A3 | - | Glenlea | LMW-ig (1594F5-I) | 796bp | n/d | - | - |
| FJ876819 | 1406 | Glu-A3 | - | Shanyou225 | - | 197bp | n/d | - | - |
| FJ876821 | 1379 | Glu-A3 | - | Chinese Spring | - | 197bp | n/d | - | - |
| EU189089 | 1878 | Glu-B3 | - | Glenlea | LMW-mg (1557N24-M) | 515bp | n/d | - | - |
| EU189088 | 2218 | Glu-B3 | - | Glenlea | LMW-sg (0154F22-S) | 807bp | n/d | - | - |
| FJ972196 | 1386 | Glu-B3 | - | COOK | - | 405bp | n/d | - | - |
| FJ876824 | 1449 | Glu-B3 | - | Shanyou225 | - | 396bp | n/d | - | - |
| FJ876825 | 1458 | Glu-B3 | - | Xiaoyan 6 | - | 405bp | n/d | - | - |
| X13306 | 3165 | GluD1 | - | - | LMWG-1D1 | 983bp | n/d | - | - |
| EU189097 | 1965 | Glu-D3 | - | Glenlea | LMW-mg (0877L13-M) | 495bp | n/d | - | - |
| EU189095 | 1723 | Glu-D3 | - | Glenlea | LMW-sg (0359D24-S) | 295bp | n/d | - | - |
| EU189093 | 1958 | Glu-D3 | - | Glenlea | LMW-mg (0275P20-M) | 465bp | n/d | - | - |
| EU189091 | 1763 | Glu-D3 | - | Glenlea | LMW-mg (0099E23-M) | 433bp | n/d | - | - |
| EU189098 | 2755 | Glu-D3 | - | Glenlea | LMW-mg (1238L16-M) | 1325bp | n/d | - | - |
| EU189096 | 1934 | Glu-D3 | - | Glenlea | LMW-mg (0703A9-M) | 494bp | n/d | - | - |
| EU189094 | 2092 | Glu-D3 | - | Glenlea | LMW-mg (0359D24-M) | 809bp | n/d | - | - |
| EU189092 | 1677 | Glu-D3 | - | Glenlea | LMW-mg (0154A5-M) | 439bp | n/d | - | - |
| EU189090 | 1445 | Glu-D3 | - | Glenlea | LMW-mg (0072P19-M) | 125bp | n/d | - | - |

| Accession# | Size (bp) | locus | Allele | Cultivar | Type | 5' UTR | CpG-island*** | Size | Location |
|---|---|---|---|---|---|---|---|---|---|
| FJ615309 | 1603 | Glu-D3 | - | COOK | - | 605bp | n/d | - | - |
| FJ615310 | 1603 | Glu-D3 | - | Suneca | - | 605bp | n/d | - | - |
| FJ615311 | 1603 | Glu-D3 | - | Chinese Spring | - | 605bp | n/d | - | - |
| DQ457416 | 1645 | GluD3-4 | GluD3-41 | Aroona | LMWg P3-41 | 385bp | n/d | - | - |
| DQ457417 | 1642 | GluD3-4 | GluD3-42 | Chinese Spring | LMWg P3-42 | 385bp | n/d | - | - |
| DQ457418 | 1642 | GluD3-4 | GluD3-43 | Tasman | LMWg P3-43 | 385bp | n/d | - | - |
| DQ457420 | 1300 | GluD3-6 | - | Tasman | LMWg P3-6 | 174bp | n/d | - | - |
| AY831865 | 1383 | - | - | Glenlea | - | 17bp | n/d | - | - |
| DQ681079 | 1137 | - | - | Guanfeng 2 | GF-1 | 0bp | n/d | - | - |
| FM212916 | 1077 | - | - | Prinqual | D-type | 0bp | n/d | - | - |
| X07747 | 1827 | - | - | Yamhill | - | 550bp | n/d | - | - |
| AY831780 | 1374 | - | - | Glenlea | - | 32bp | n/d | - | - |
| Gliadins | | | | | | | | | |
| GQ891684 | 1018 | - | - | Shaan253 | Alpha | 70bp | n/d | - | - |
| K02068.1 | 1152 | - | - | Cheyenne | Alpha | 33bp | n/d | - | - |
| X54688 | 3573 | - | Pseudogene | Cheyenne | Alpha | 400bp | n/d | - | - |
| X54517 | 3581 | - | pseudogene | Cheyenne | Alpha | 401bp | n/d | - | - |
| X54689 | 3566 | - | pseudogene | Cheyenne | Alpha | 397bp | n/d | - | - |
| X01130 | 2347 | - | - | - | Alpha | 771bp | n/d | - | - |
| AY293730[a] | 1335 | - | - | Grebe | Alpha | 1335bp | in 5'-UTR | 264 | 48-311 |
| TAU08287 | 6115 | | - | Cheyenne | Alpha | 2833bp | in 5'-UTR | 606 | 1083-1688 |
| U51308.1 | 1799 | - | pseudogene | Cheyenne | Alpha | 593bp | n/d | - | - |
| K03074.1 | 3043 | - | - | - | alpha/beta | 593bp | n/d | - | - |
| M11073.1 | 1156 | - | - | - | alpha-/beta | 33bp | n/d | - | - |
| K03075.1 | 3310 | - | - | - | alpha/beta | 921bp | n/d | - | - |
| AF234646 | 1329 | - | - | Cheyenne | Gamma | 342bp | n/d | - | - |

| Accession# | Size (bp) | locus | Allele | Cultivar | Type | 5' UTR | CpG-island*** | Size | Location |
|---|---|---|---|---|---|---|---|---|---|
| AF234644 | 1227 | - | - | Cheyenne | Gamma | 51bp | n/d | - | - |
| AF234650 | 1178 | - | - | Cheyenne | Gamma | 38bp | n/d | - | - |
| AF234647 | 5718 | - | - | Cheyenne | Gamma | 2769bp | n/d | - | - |
| M11336.1 | 1130 | - | - | - | Gamma | 0bp | n/d | - | - |
| M11077.1 | 1348 | - | - | Cheyenne | Gamma | 48bp | n/d | - | - |
| M13712.1 | 2450 | - | pseudogene | - | Gamma | 512bp | n/d | - | - |
| M13713.1 | 2450 | - | - | - | Gamma | 503bp | n/d | - | - |
| GQ871772 | 1468 | - | - | Shaan253 | C2 gamma | 389bp | n/d | - | - |
| GQ871770 | 1290 | - | - | Shaan253 | Gamma | 399bp | n/d | - | - |
| FJ598073 | 1082 | - | - | Jinan177 | Omega | 0bp | n/d | - | - |
| FJ598069 | 1038 | - | - | Jinan177 | Omega | 0bp | n/d | - | - |
| FJ598070 | 1146 | - | - | Jinan177 | Omega | 0bp | n/d | - | - |
| GQ871775 | 1064 | - | - | Shaan253 | Gamma | 27bp | n/d | - | - |
| GQ871773 | 1067 | - | - | Shaan253 | Gamma | 3bp | n/d | - | - |
| AF280605 | 3789 | - | - | - | Omega | 2463bp | n/d | - | - |
| AB059812 | 1858 | - | Pseudogene | Chinese Spring | Omega | 324bp | n/d | - | - |
| AB181301 | 1275 | - | Pseudogene | Norin 61 | omega-5 | 29bp | n/d | - | - |
| AF280606 | 3925 | - | pseudogene | - | omegaG3 | 2476bp | n/d | - | - |
| AJ937839 | 1399 | gli | - | Neepawa | Omega | 0bp | n/d | - | - |

*sequence homologous to 5' end of a-gliadin gene

**Locus unknown

***TR = transcribed region; TSS = transcription start site; UTR = untranslated region; n/d = not detected

[a]only 5'UTR

[b]showed similarity with wheat methylation-filtered sequence CZ889805 derived from leaf genomic DNA - confirmed its hypomethylated status

[Source: Wen et al., 2012 Proc. Natl. Acad. Sci. U.S.A., Copyright (2012)]

APPENDIX 2. Data on transcriptional supression of *DEMETER* homoeologues and elimination of immunogenic prolamins using immature and mature $T_2$ grains.

| $T_0$ | $T_1$ | % supression qRT-PCR | Gliadins | | | % cumulative reduction gliadin | Glutenins | | % Reduction LMW+ gliadins |
|---|---|---|---|---|---|---|---|---|---|
| | | | Omega (%) | Alpha (%) | Gamma (%) | | LMW | HMW | |
| 10-728 | P32F2 | 68.9 | 115.0 | 56.2 | 21.8 | 56.3 | 22.1 | 143.5 | 67.0 |
| | P31D3 | 57.2 | 40.9 | 50.4 | 29.2 | 65.4 | 89.0 | 101.9 | 60.4 |
| | P32E9 | 73.4 | 59.5 | 33.3 | 18.4 | 74.8 | 89.9 | 111.1 | 67.2 |
| | P32G8 | 76.3 | 36.7 | 35.0 | 26.1 | 71.1 | 46.3 | 89.5 | 68.0 |
| | P31D12 | 72.5 | 60.3 | 37.6 | 20.7 | 67.9 | 34.8 | 117.6 | 67.2 |
| | P31A10 | 74.6 | n/a | n/a | n/a | n/a | 71.9 | 136.2 | n/a |
| | P32F1 | 73.7 | 59.4 | 38.0 | 29.4 | 63.3 | n/a | n/a | n/a |
| | P33A8 | 69.3 | 24.5 | 9.4 | 48.6 | 66.5 | n/a | n/a | n/a |
| | P31C8 | 85.6 | 26.7 | 44.1 | 24.7 | 68.8 | n/a | n/a | n/a |
| | P31D6 | 66.7 | 47.5 | 37.9 | 29.7 | 65.0 | n/a | n/a | n/a |
| | P33A7 | 79.0 | 32.4 | 35.4 | 29.8 | 68.3 | n/a | n/a | n/a |
| | P32G11 | 70.2 | 205.3 | 24.8 | 15.3 | 57.0 | n/a | n/a | n/a |
| | P31E1 | 70.8 | 80.3 | 38.0 | 22.0 | 68.2 | n/a | n/a | n/a |
| 38-728 | P45A5 | 3.5 | 170.7 | 74.5 | 116.4 | -10.5 | 110.5 | 149.0 | -10.5 |
| 46-728 | P21C4 | 81.7 | 48.6 | 33.9 | 11.0 | 76.0 | n/a | n/a | n/a |
| 22-728 | P43C2 | 75.0 | 35.2 | 28.1 | 34.9 | 67.2 | 43.9 | 85.3 | 63.1 |
| | P43A6 | 76.6 | 18.7 | 25.2 | 22.0 | 77.5 | 58.6 | 92.2 | 68.2 |
| | P43D6 | 69.7 | 114.6 | 29.3 | 19.2 | 69.1 | 31.9 | 88.8 | 68.8 |
| | P43A12 | 72.3 | 74.4 | 13.1 | 25.8 | 73.0 | 43.3 | 89.8 | 65.8 |
| | P42H3 | 76.7 | 27.8 | 31.2 | 21.3 | 74.5 | 39.7 | 89.1 | 69.3 |
| | P43C5 | 70.6 | 58.0 | 54.1 | 14.4 | 73.4 | 48.7 | 96.1 | 67.5 |
| | P42H12 | 74.7 | 45.7 | 38.1 | 29.3 | 66.1 | 29.5 | 84.0 | 68.8 |
| 19-728 | P42F1 | 78.6 | 37.3 | 31.8 | 23.0 | 72.7 | 40.9 | 88.1 | 67.6 |
| | P32G7 | 72.5 | 15.2 | 46.9 | 54.7 | 57.9 | 24.5 | 73.5 | 70.2 |

| $T_0$ | $T_1$ | % supression qRT-PCR | Gliadins | | | % cumulative reduction gliadin | Glutenins | | % Reduction LMW+ gliadins |
|---|---|---|---|---|---|---|---|---|---|
| | | | Omega (%) | Alpha (%) | Gamma (%) | | LMW | HMW | |
| | P42E9 | 72.3 | 22.4 | 34.9 | 39.3 | 64.2 | 33.0 | 83.8 | 65.6 |
| | P42E7 | 67.7 | 118.9 | 51.7 | 33.7 | 54.9 | n/a | n/a | n/a |
| | P42F11 | 83.9 | 31.8 | 26.1 | 21.8 | 76.4 | 58.9 | 85.3 | 67.0 |
| | P41B5 | 82.7 | 12.2 | 33.3 | 91.4 | 43.1 | 40.7 | 26.6 | 76.4 |
| | P42G5 | 78.9 | 78.4 | 41.2 | 10.4 | 72.0 | 85.7 | 94.3 | 62.1 |
| | P42G4 | 79.9 | 150.9 | 46.5 | 17.3 | 64.6 | 3.5 | 79.6 | 68.4 |
| | P41D4 | 78.2 | 87.7 | 52.8 | 27.9 | 61.6 | 58.7 | 90.8 | 56.2 |
| | P41D8 | n/a | 51.8 | 25.9 | 41.4 | 62.0 | 62.9 | 89.4 | 34.6 |
| | P41C9 | 74.5 | 54.6 | 31.7 | 24.9 | 68.5 | n/a | n/a | n/a |
| 29b-728 | P27C8 | 74.7 | 57.8 | 57.0 | 22.4 | 67.2 | n/a | n/a | n/a |
| | P27B8 | 74.5 | 45.5 | 45.1 | 21.5 | 71.4 | n/a | n/a | n/a |
| 140-728 | P67C2 | 82.6 | 77.5 | 56.4 | 13.9 | 71.5 | 41.6 | 96.8 | 68.0 |
| | P67B10 | 59.9 | 35.7 | 85.2 | 91.5 | 25.3 | 40.7 | 86.1 | 45.2 |
| | P67D1 | 74.1 | 21.9 | 28.6 | 43.6 | 65.7 | 19.9 | 76.8 | 74.1 |
| | P67C7 | 67.2 | 42.9 | 31.1 | 30.9 | 68.0 | 61.5 | 82.8 | 60.1 |
| | P68E2 | 78.0 | n/a | n/a | n/a | n/a | 61.4 | 108.9 | n/a |
| | P67C9 | 66.7 | n/a | n/a | n/a | n/a | 27.8 | 70.0 | n/a |
| 17-728 | P62E4 | 78.4 | n/a | n/a | n/a | n/a | 10.4 | 16.3 | n/a |
| | P61E1 | n/a | 16.5 | 87.6 | 133.0 | 9.1 | 67.0 | 83.9 | 25.7 |
| 17b-728 | P27B3 | 68.0 | 17.4 | 20.2 | 21.8 | 79.4 | 70.5 | 95.5 | 70.6 |
| | P27A12 | 82.7 | 109.4 | 49.5 | 7.9 | 74.3 | n/a | n/a | n/a |
| 34-728 | P13A6 | 75.1 | 69.5 | 43.7 | 24.4 | 67.5 | 42.1 | 90.9 | 64.9 |
| 25-728 | P53A12 | 73.5 | 69.0 | 36.6 | 37.3 | 59.9 | 27.1 | 80.5 | 65.6 |
| | P53B9 | n/a | 83.6 | 45.4 | 48.8 | 47.1 | 58.4 | 106.5 | 45.0 |
| 18-728 | P52F3 | 71.9 | 87.3 | 40.9 | 30.3 | 61.5 | 34.8 | 89.9 | 63.1 |

| $T_0$ | $T_1$ | % supression qRT-PCR | Gliadins | | | % cumulative reduction gliadin | Glutenins | | % Reduction LMW+ gliadins |
|---|---|---|---|---|---|---|---|---|---|
| | | | Omega (%) | Alpha (%) | Gamma (%) | | LMW | HMW | |
| | P51B8 | n/a | 65.3 | 38.9 | 38.6 | 57.3 | 66.5 | 109.8 | 51.2 |
| | P51F5 | n/a | 88.7 | 58.1 | 31.8 | 55.8 | 45.6 | 90.7 | 55.4 |
| 28-728 | P44E9 | 78.2 | 37.1 | 27.4 | 31.7 | 68.8 | 33.5 | 85.3 | 68.0 |
| 29-728 | P53D7 | 71.3 | 38.0 | 33.2 | 30.7 | 67.6 | 41.8 | 86.7 | 64.1 |
| Control | - | - | 100 | 100 | 100 | - | 100 | 100 | - |

[Source: Wen et al., 2012 Proc. Natl. Acad. Sci. U.S.A., Copyright (2012)]

APPENDIX 3. Immunogenic peptide epitopes from gluten involved in celiac disease.

| Amino acid sequence | Position | References |
|---|---|---|
| VRVPVPQLQPQNPSQQQPQ | α gliadin 1-19 | (Lundin et al., 1993) |
| QNPSQQQPQEQVPLVQQQ | α gliadin 11- 28 | |
| QVPLVQQQQFLGQQQPFPPQ | α gliadin 21-40 | |
| LGQQQPFPPQQPYPQPQPF | α gliadin 31-49 | |
| FPGQQQPFPPQQPYPQPQPF | α gliadin 30-49 | |
| QPYPQPQPFPSQQPYLQL | α gliadin 41- 58 | |
| PQPFPSQQPYLQLQPFPQ | α gliadin 46- 63 | (van de Wal et al., 1996) |
| PFPQPQLPY | α-9 (60–68) E65 | (Arentz-Hansen et al., 2000) |
| PQPQLPYPQ | α-2 (62–70) E65 | |
| PYPQPQLPY | α-2 (67–75) E72 | (Arentz–Hansen et al., 2002) |
| PSGQGSFQPSQQ | α gliadin 205- 216 (deamidated) | (van de Wal et al., 1998b; van de Wal et al., 1998a) |
| SGQGSFQPSQQN | α gliadin 206- 217 (deamidated) | |
| QGSFQPSQQN | α gliadin 208- 217 (deamidated) | |
| QQPQQQYPSGQGSFQPSQQNPQAQG | α gliadin 198- 222 | (van de Wal et al., 1998b) |
| LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF | α gliadin 57- 89 | (Arentz-Hansen et al., 2000) |
| QLQPFPQPQLPY | α gliadin 58-68 | (Arentz-Hansen et al., 2000) |
| QYPLGQGSFRPSQQNPQA | α gliadin 203-220 (deamidated) | (Mazzarella et al., 2003) |
| LIFCMDVVLQ | α gliadin 123- 132 | (Gianfrani et al., 2003) |
| PFRPQQPYPQPQPQ | α gliadin 93-106 (deamidated) | (van de Wal et al., 1999; Vader, 2002) |
| PQQSFPQQQ | γ-5 (115–123) E121 | (Sjostrom et al., 1998) |
| IIQPQQPAQL | γ-5 (222–236) E232 (deamidated) | (Vader, 2002) |
| FPQQPQQPYPQQP | γ-5 (66–78) E68, E71 | (Arentz–Hansen et al., 2002) |

| Amino acid sequence | Position | References |
|---|---|---|
| FSQPQQQFPQPQ | γ-5 (102–113) E106, E108 | |
| LQPQQPFPQQPQQPYPQQPQ | γ-5 (60–79) | |
| OQPQQSFPQQQ | γ gliadin 134- 153 (deamidated) | (Sjostrom et al., 1998) |
| QQQQPPFSQQQQSPFSQQQQPFSQQQQSPF | glutenin-156 (40-59) (deamidated) | (Vader, 2002) |
| QQPPFSQQQQQPLPQ PFSQQQQQ | glutenin 17 (46-60) (deamidated) | |
| SGQGQQRPGQWLQPGQGQQGYYPTSPQQS-GQGQQLGQ | glutenin 707- 742 (deamidated) | (van de Wal et al., 1999) |
| PGQWLQPGQGQQGYYPTSPQQSGQ | glutenin 719- 736 | |
| GYYPTSPQQSGQGQQLGQ | glutenin 725- 742 | |
| GYYPTSPQQSG | glutenin 725- 735 | |
| QGYYPTSPQQS | glutenin 724- 734 | |
| QQGYYPTSPQQSG | glutenin 723- 735 | |
| GQQGYYPTSPQQSG | glutenin 722- 735 | |
| GQQGYYPTSPQQS | glutenin 723- 734 | |
| PQPELPYPQPQLPY | α-2 gliadin, 62–75, E65 | (Ellis et al., 2003) |
| LQLQPFPQPELPY | α-9 gliadin, 56–68, E65 | |
| LQLQPFPQPQLPYPQPQLPY | α-2 gliadin, 56–75 | |
| LQLQPFPQPELPYPQPQLPY | α-2 gliadin, 56–75, E65 | |
| LQPQQPFPQQPQQPYPQQPQ | γ 6 gliadin | (Qiao et al., 2005) |
| PQQPFPQPQQQFPQPQQPQQ | γ 7 gliadin | |
| PYPEQQEPF | Avenin I | (Arentz-Hansen et al., 2004) |
| PYPEQQQPF | Avenin II | |
| PYPEQPEQP | γ-2 gliadin | (Vader et al., 2003) |
| PQPEQPFPQ | sec a2 and hor | |
| IQPEQPAQL | γ gliadin | (Qiao et al., 2005) |

| Amino acid sequence | Position | References |
|---|---|---|
| PQPEQPFCQ | γ gliadin | (Qiao unpublished) |
| PFPQPEQPF | ω gliadin | (Tye-Din et al., 2010) |
| PQPEQPFPW | ω gliadin | |
| PFPQPEQPF | hor 9 | |
| PIPEQPQPY | Hor | |
| PFPQPEQPF | sec a9 | |
| PFSEQEQPV | Glutenin | (Bodd et al., 2012) |
| EQPQQPFPQ | γ gliadin | (Tollefsen et al., 2006) |
| EQPQQPYPE | γ gliadin | |
| EGSFQPSQE | γ gliadin | (Kooy-Winkelaar et al., 2011) |
| PQQSFPEQE | α gliadin | |
| QGYYPTSPQ | HMW glutenin | |

[Modified from Osorio et al., 2012 Funct. Integr. Genomics]

## General guidelines

The Academy invites original papers that contribute significantly to research carried on in Denmark. Foreign contributions are accepted from temporary residents in Denmark, participants in a joint project involving Danish researchers, or those in discussion with Danish contributors..

## Instructions to authors

Please make sure that you use the stylesheet on our homepage www.royalacademy.dk. All manuscripts will be refereed. Authors of papers accepted for publication will receive digital proofs; these should be returned promptly to the editor. Corrections other than of printer's errors will be charged to the author(s) insofar as the costs exceed 15% of the cost of typesetting.

Authors receive a total of 50 free copies. Authors are invited to provide addresses of up to 20 journals to which review copies could profitably be sent.

Manuscripts can be returned, but only upon request made before publication of the paper. Original photos and artwork are returned upon request.

## Manuscript

*General*

Book manuscripts and illustrations must comply with the guidelines given below. The digital manuscript and illustrations plus one clear printed copy of both should be sent to the editor of the series. Digital manuscripts should be submitted in a commonly used document format (contact the editor if you are in doubt), and the illustrations should be sent as separate files. Please do not embed illustrations within text files.

A manuscript should not contain less than 48 printed pages. This also applies to the Sci.Dan.M where contributions to the history of science are welcome.

*Language*

Manuscripts in Danish, English, German and French are accepted; in special cases other languages too. Linguistic revision may be made a condition of final acceptance.

*Title*

Titles should be kept as short as possible, preferring words useful for indexing and information retrieval.

*Abstract, Summary*

An abstract in English is required. It should be of 10-15 lines, outline main features, stress novel information and conclusions, and end with the author's name, title, and institutional and/or private postal address. - Papers in Danish must be provided with a summary in another language as agreed between author and editor.

*Manuscript*

Page 1 should contain title, author's name and the name of the Academy. Page 2: Abstract, author's name and address. Page 3: Table of contents if necessary. Consult a recent issue of the series for general layout. Indicate the position of illustrations and tables. A printout must accompany manuscripts submitted electronically.

*Figures*

All illustrations submitted must be marked with the author's name. It is important that the illustrations are of the highest possible quality. Foldout figures and tables should be avoided.

*References*

In general, the editor expects all references to be formally consistent and in accordance with accepted practice within the particular field of research. Bibliographical references should be given in a way that avoids ambiguity.

Det Kongelige Danske Videnskabernes Selskab
The Royal Danish Academy of Sciences and Letters

## Biologiske Skrifter, BS

Biol.Skr.Dan.Vid.Selsk.

VOL DKK

53 Jørgen Olesen:
*External morphology and larval development of Derocheilocaris remanei Delamare-Deboutteville & Chappuis, 1951 (Crustacea, Mystacocarida) with a comparison of crustacean segmentation and tagmosis patterns*. 2000. 60 pp. 40 fig. 100.-

54 *Biodiversity Research in the Horn of Africa Region*. Proceedings of the Third International Symposium on the Flora of Ethiopia and Eritrea at the Carlsberg Academy, Copenhagen, August 25-27, 1999. Edited by Ib Friis and Olof Ryding. 2001. 439 pp. Lavishly illustrated. 500.-

55 *Plant Diversity and Complexity Patterns. Local, Regional and Global Dimensions*. Proceedings of an international symposium held at the Royal Danish Academy of Sciences and Letters in Copenhagen, Denmark, 25-28 May, 2003.
Edited by Ib Friis and Henrik Balslev. 2005. 603 pp. 174 fig. 600.-

56 *Poly-unsaturated Fatty Acids Neural Function & Mental Health*. Proceedings of an International and Interdisciplinary symposium. The Royal Danish Academy of Sciences and Letters, August 9, 2007. Edited by Ole G. Mouritsen. 2007. 88 pp. 23 fig. 100.-

57 Philippe Provençal:
*The Arabic Plant Names of Peter Forsskål's flora Aegyptiaco-Arabica*. 2010. 160 pp. 200.-

58 Ib Friis, Sebsebe Demissew and Paulo van Breugel:
*Atlas of the Potential Vegetation of Ethiopia*. 2010. 308 pp. Lavishly illustrated. 400.-

## Scientia Danica. Series B, Biologica

Sci.Dan.B

VOL DKK

1 Inge Bødker Enghoff:
*Regionality and biotope exploitation in Danish Ertebølle and adjoining periods*. 2011. 394 pp. Lavishly illustrated. 320.-

2 Jesper Guldberg Hansen, Reinhardt Møbjerg Kristensen & Aslak Jørgensen:
*The armoured marine tardigrades (Arthrotardigrada, Tardigrada)*. 2012. 92 pp. Lavishly illustrated. 150.-

3 Sachin Rustgi, Nuan Wen, Claudia Osorio, Rhoda A.T. Brew-Appiah, Shanshan Wen, Richa Gemini, Jaime H. Mejias, Nii Ankrah, Charles P. Moehs & Diter von Wettstein:
*Natural dietary therapies for the 'gluten syndrome'*. 2014. 87 pp. Lavishly illustrated. 150.-

Priser ekskl. moms / Prices excl. VAT

Printed in Denmark by Specialtrykkeriet Viborg.
ISSN 1904-5484 · ISBN 978-87-7304-376-9