Published in final edited form as:

Funct Integr Genomics. 2019 January ; 19(1): 123–136. doi:10.1007/s10142-018-0632-x.

Development of wheat genotypes expressing a glutamine-specific endoprotease from barley and a prolyl endopeptidase from *Flavobacterium meningosepticum* or *Pyrococcus furiosus* as a potential remedy to celiac disease

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Abstract

Ubiquitous nature of prolamin proteins dubbed gluten from wheat and allied cereals imposes a major challenge in the treatment of celiac disease, an autoimmune disorder with no known treatment other than abstinence diet. Administration of hydrolytic glutenases as food supplement is an alternative to deliver the therapeutic agents directly to the small intestine, where sensitization of immune system and downstream reactions takes place. The aim of the present research was to evaluate the capacity of wheat grain to express and store hydrolytic enzymes capable of gluten detoxification. For this purpose, wheat scutellar calli were biolistically transformed to generate plants expressing a combination of glutenase genes for prolamin detoxification. Digestion of prolamins with barley endoprotease B2 (EP-HvB2) combined with *Flavobacterium meningosepticum* prolyl endopeptidase (PE-FmPep) or *Pyrococcus furiosus* prolyl endopeptidase (PE-PfuPep), significantly reduced (up to 67%) the amount of the indigestible gluten peptides of all prolamin families tested. Seven of the 168 generated lines showed inheritance of transgene to the T₂ generation. Reversed phase high performance liquid chromatography of gluten extracts under simulated gastrointestinal conditions allowed the identification of five T₂ lines that

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Author contributions: S. Rustgi, D.v.W., S. Reinbothe, and B.L. designed research; C.E.O., N.W., J.H.M., and S. Rustgi performed research; C.E.O., N.W., J.H.M., D.v.W., and S. Rustgi analyzed data; and S. Rustgi, C.E.O., B.L. and S. Reinbothe wrote the paper.

Conflict of Interest: The authors declare no conflict of interest.

contained significantly reduced amounts of immunogenic, celiac disease-provoking gliadin peptides. These findings were complemented by the R5 ELISA test results where up to 72% reduction was observed in the content of immunogenic peptides. The developed wheat genotypes open new horizons for treating celiac disease by an intraluminal enzyme therapy without compromising their agronomical performance.

Keywords

Celiac disease; Gluten; Glutenase; Combined-enzyme therapy; Wheat

Introduction

Wheat is known to the mankind for more than 10,000 years (Shewry 2018). Due to its wide adaptability, nutritive value, unique end-use quality and affordability, wheat, in fact, become one of the most fundamental sources of human nutrition over time. Wheat grains are good sources of carbohydrates, proteins and dietary fibers (Shewry and Tatham 2016). In fact, 8–15% of the dry matter in common wheat grains is comprised of seed storage proteins of which 80% is represented by a protein complex dubbed gluten (Shewry and Tatham 2016). This protein complex is classified in various ways in the literature; however, in the present communication we used the word prolamins to describe both the gliadin and glutenin fractions of gluten. It is a complex mixture of polypeptides that confer wheat its characteristic end-use quality. However, the prolamins fraction of the wheat grain storage proteins is also recognized as the causal agent of celiac disease. The glutamine (Gln or Q, 35%) and proline (Pro or P, 15%) repeat tracts (Stern et al. 2001) in the gluten proteins make them highly resistant to digestion by gastric and pancreatic proteases. The partial digestion of gluten proteins results in the production of large number of long Pro/Gln-rich peptides (Shan et al. 2002) in both healthy and gluten intolerant individuals. The healthy individuals excrete out the indigestible gluten peptides (Fasano 2009). By contrast, celiac patients suffering from gluten-intolerance absorb these peptides during passage through the epithelial lining due to leaky tight junctions, early exposure to viral infections or changes in the gut microbiota (Verdu and Caminero 2017). After passing through the intestinal epithelium and reaching the lamina propria these peptides get deamidated by the tissue transglutaminase 2 (tTG2), which increases their affinity to the human leukocyte antigen (HLA)-DQ2 or HLA-DQ8 (Molberg et al. 1998; Quarsten et al. 2001) and trigger celiac disease.

A large number of disease-provoking epitopes (about 190) are already recognized from the gluten peptides, but the mapping is incomplete and the number of distinct epitopes a matter of ongoing discussion (Barro et al. 2016). Both the amino acid sequence as well as deamidation of glutamine to glutamic acid residues make a peptide an epitope. Specifically, deamidated gluten peptide-DQ2 complex enhances the inflammatory gut mucosa response, elicits an increase in CD4⁺ T-helper 1 (Th 1) cells and ultimately causes destruction of the intestinal microvilli (Alaeldini and Green 2005).

In view of developing a possible dietary therapy for celiac disease, we assumed that degradation of indigestible gluten proteins before triggering immune responses in the

intestinal lamina propria could allow consumption of wheat and derived products by gluten intolerant individuals. If successful, this dietary therapy would drastically improve the life quality of celiac patients, as it would allow them to resume a normal diet (Løvik et al. 2017). It has been demonstrated by *in vitro* studies, for example, that detoxification of gluten proteins via endoproteases is a possible option for dietary therapy (Salden et al. 2015). However, the enzymes to be used to this end must meet the following criteria: i) they must represent endoprotease/peptidase that cleaves at glutamine and/or proline residues; ii) they must retain their activity under natural conditions in the gastrointestinal; iii) they must be highly temperatureresistant and stay active during baking process (Rustgi et al. 2014). Based on the current understanding of the biological activity of different proteases and peptidases (Gorokhovets et al. 2017; König et al. 2017; Tack et al. 2013), we hypothesized that a combination of enzymes with complementary specificities would be capable of degrading the immunogenic gluten proteins to near completion and serve as a dietary agent (cf. Bethune and Khosla 2012). Conceptually, we thus chose barley cysteine endoprotease B2 (EP-HvB2) and prolyl endopeptidase from *Flavobacterium meningosepticum* (PE-FmPep) or *Pyrococcus furiosus* as dietary agents for detoxification of immunogenic gluten proteins and peptides.

In fact, previous work performed by our group that were based on *in silico* digestion of wheat, barley and rye gluten sequences available in the public domain, with digestive enzymes in combination with different ‘glutenases’, including EP-HvB2, wheat cysteine endoproteases, and prolyl endopeptidase from *F. meningosepticum* or *Aspergillus niger* (Osorio et al. 2012), had revealed the utility of the chosen approach. Since, gluten proteins upon hydration forms a contiguous protein network that gives rise to the characteristic wheat dough, it is anticipated to reduce accessibility of these proteins to the digestive enzymes. Therefore, to test the efficacy of glutenases in degrading gluten proteins to non-immunogenic peptides, Mitea et al. (2007) processed a slice of bread in a dynamic system that closely resembles the human gastrointestinal in presence/absence of PE-AnPep. In this connection, samples of the digesting bread were collected from the stomach, duodenum, jejunum and ileum compartments at different timepoints and the levels of immunogenic peptides in different samples were determined using immunological assays. Interestingly, PE-AnPep accelerated gluten degradation in the stomach compartment to the level that hardly any gluten reached the duodenum compartment. Collectively, this study suggested that the gluten proteins remain accessible to the proteinases in the food matrix and endorsed the idea that the ‘combination enzyme therapy’ is a viable approach of celiac disease treatment.

In the present work, transgenic wheat plants were generated stably expressing a combination of EP-HvB2 and PE-FmPep in wheat grain endosperm, as a new therapy for celiac disease. Metabolic studies under simulated gastrointestinal conditions showed that gluten derived from such plants contain significantly less immunogenic peptides than control plants. Together, our results propose a new way of treating celiac disease and thus underscore the commercial viability of the combination enzyme therapy (Kaukinen and Lindfors 2015).

Materials and Methods

Gene synthesis and amplification

In order to match the codon usage for monocots, genes encoding *F. meningosepticum* prolyl endopeptidase (PE-FmPep; Accession number M81461) and *P. furiosus* prolyl endopeptidase (PE-PfuPep; Accession number PF0825) were codon-optimized at a GC content of >60%. The gene fragments were synthesized from GeneScript (Piscataway, NJ) and provided with a barley D-hordein signal peptide sequence at their 5'-ends for targeting of the recombinant protein to the protein storage bodies in the developing endosperm (Cameron-Mills and Ingversen, 1978). Restriction sites for *Apa* I and *Sac* I were introduced on the either ends of each gene fragment to assist its cloning in pUC57 plasmid, and the resulting constructs were named pUC57-FmPep and pUC57-PfuPep.

Gene encoding the barley cysteine endoprotease B2 (EP-HvB2; Accession number U19384) with the N-terminal propeptide sequence was amplified by PCR using genomic DNA of barley cv. Golden Promise with the following primers: 5' Ep-HvB2/*Xho* I GCGCTCGAGATCCCGATGGAGGACAAGGACC and 5' Ep-HvB2/*Kpn* I GCGGGTACCCAGTGACTCCCTGGCTCCAAGGG (Bethune et al. 2006). The forward and reverse primers were respectively supplemented with the non-template specific restriction sites of *Xho* I and *Kpn* I to facilitate cloning of the PCR product. PCR amplification was performed on thermocycler, C1000 (BioRad, USA) using *Taq* DNA polymerase (Takara, Madison, WI), in a 25 µL reaction volume using manufacturer's instructions. The following PCR conditions were used for amplification: Initial melting at 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 30 sec; annealing at 65°C for 30 sec and extension at 72°C for 2 minutes with a final extension at 72°C for 5 minutes. PCR product were resolved on 1% agarose gel (w/v) and validated by DNA sequencing at the Genomics Core Facility in WSU, Pullman.

Construction of transformation vectors

Five constructs were developed using standard DNA cloning procedure in the *E. coli* strain DH5α (Sambrook et al. 1989) (Fig. 1). In brief, the following cloning strategy was applied to plasmids pUC57-FmPep and pUC57-PfuPep: i) the DH5α cells were genetically transformed with each plasmid; ii) plasmids were extracted using the Nucleobond miniprep kit from Macherey-Nagel (Bethlehem, PA) following manufacturer's recommendations; iii) digestion of plasmids with *Apa* I/*Sac* I, and iv) ligation of released gene fragment with the pHMWg::nos plasmid preprocessed with same restriction enzymes to yield plasmids, pHMWg-FmPep-nos and pHMWg-PfuPep-nos. The pHMWg::nos plasmid contains the endosperm specific wheat *IDy* high molecular wheat glutenin promoter (pHMWg) and *Agrobacterium* nopaline synthase (*nos*) terminator. Similarly, to develop plasmid carrying the barley EP-HvB2 gene, purified PCR product was digested with enzymes *Xho* I and *Kpn* I, and ligated with pHMWg::nos to yield pHMWg-EPHvB2-nos. These plasmids were cloned in DH5α cells and sequence validated at the Genomics Core Facility of WSU, Pullman.

In order to avoid integration of plasmid backbone in the host genome, two double cassette liner constructs were developed. These constructs consisted of two expression cassettes one each for prolyl endopeptidase and EP-HvB2, each expression cassette comprised a HMWg promoter and a *nos* terminator, and both constructs were devoid of the plasmid backbone (Fig. 1). Assembly of the above construct involved the following steps: i) Plasmid pHMWg-FmPep-nos and pHMWg-PfuPep-nos were digested with *Hind* III and *Eco* R I combination and individually ligated with pBluescriptSK(+) overnight at 16°C. The resultant plasmids pBSK(+):pHMWg-FmPep-nos and pBSK(+):pHMWg-PfuPep-nos were used to transform DH5α cells followed by blue/white-screening. Selected white colonies were analyzed by PCR; plasmids were extracted from the positive colonies and verified by DNA sequencing. ii) The second step involved production of plasmid pGEM::pHMWg-EPHvB2-nos. A fragment containing the wheat HMW promoter, EP-HvB2 and *nos* terminator was amplified from pHMWg-EP-HvB2-nos using specific primers 5' Hor AAGCTTACCGCAGATTTGCAAAAGC and 5' nos CCGATCTAGTAACATAGATGACACC in a 25 µL reaction volume using the above mentioned PCR conditions and purified using standard procedure (Sambrook et al. 1989). The PCR product was ligated into the pGEM vector, using 50 ng of vector DNA, 200 ng of PCR product and 125 U of T4 DNA ligase (Promega). 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::pHMWg-EPHvB2-nos were selected by blue-white screening, plasmid from positive colonies was extracted using Machinerey-Nagel miniprep kit, and validated by restriction digestion with *Apa* I and *Eco* R I.

The fragment containing pHMWg-EPHvB2-nos was excised from plasmid pGEM::pHMWg-EPHvB2-nos with *Eco* R I and ligated with vectors pBSK(+):pHMWg-FmPep-nos and pBSK(+):pHMWg-PfuPep-nos in separate reactions. 5 µl each of the resultant plasmids were used to transform DH5α cells. The positive colonies were selected, and the derived plasmids were confirmed by restriction digestion followed by DNA sequencing. DNA of the confirmed plasmid pBSK(+):pHMWg-FmPep-nos::pHMWg-EPHvB2-nos and pBSK(+):pHMWg-PfuPep-nos::pHMWg-EPHvB2-nos was digested with *Hind* III and *Not* I enzyme combination for 2 h, and the product was subjected to agarose gel electrophoresis (1% gel at 110V for 1 h). The corresponding 5.7 kb band was excised and purified using GeneClean® III kit (MP Biomedical, USA).

Wheat genetic transformation

For genetic transformation, the pure plasmids were obtained using the NucleoBond®Xtra Midi/Midi Plus kit (Macherey-Nagel, Bethlehem, PA). After extraction, the plasmid DNA concentrations were adjusted to 1.5 µg/µL. 12 µL of each plasmid was used for genetic transformation of the scutellar calli of common wheat cultivar Brundage 96 by biolistic approach (Okubara et al. 2002). Plasmid pDPG165 carrying the *bialaphos resistance* (*bar*) gene was used for co-transformation to allow selection of the transformed calli in culture media. The co-transformations were performed using the expression constructs of PE-FmPep or PE-PfuPep with EP-HvB2 and the selectable marker gene in molar ratios listed in Table 1. The root and shoot generation media were supplemented with bialaphos at a concentration of 1.5 µg/mL for initial selection of the transformants. The survivors were

later transferred to the selective media with increased quantity of herbicide (up to 5 µg/mL) to reduce the number of false positives.

PCR analysis

After four weeks in rooting media, the plants were transferred to soil, covered with a plastic dome for a week and kept under controlled condition. At this stage, genomic DNA was extracted from the candidate transformants and used for PCR with gene-specific primers (Table S1), followed by DNA sequencing of the PCR products. Briefly, the total genomic DNA was extracted from the young leaves following the modified cetyltrimethylammonium bromide (CTAB) method and purified using phenol extraction (Spaenij-Dekking et al. 2005). After extraction, concentrations of each DNA sample were adjusted to 50 ng/µL. Subsequently, the target region was amplified using gene-specific primers, and the amplified product was resolved on 2% agarose gel. 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 DNA sequencing reaction using gene-specific primers in separate reactions. Sequencing reactions were carried out in 10 µL reaction volume, each containing 100 ng template DNA, 0.5 µM primer and 3 µL BigDye mixture (Applied Biosystems). The following PCR protocol was used for the sequencing reaction: 96°C for 10 sec, 50°C for 15 sec, 60°C for 6 min with 24 iterations. Nucleotide sequences were analyzed using Sequencher 4.9 (Gene Codes Corporation).

Plant growth conditions and leaf assay for the *Bar* gene expression

Regenerated plants were vernalized at 4°C for eight weeks in a plant growth chamber; thereafter they were transferred to glasshouse and grown under controlled conditions until maturity. Grains were harvested and the positive transformants were analyzed further. Seeds were counted, weighed and 10% of the total T₁ grains were sown to obtain T₁ plants and T₂ grains.

In order to eliminate false positive plants, bialaphos assay was conducted, where a single leaf from each putative transformant was painted with 2% Basta™ solution. Results of the assay in terms of injury was recorded on a scale of 0 to 5, where 0 being highly resistant (no injury) and 5 being susceptible (dead tissue) (Fig. S1). PCR analysis was performed as mentioned earlier to validate the faithful inheritance of the transgenes.

Expression analysis

For expression analysis of *EP-HvB2*, *PE-FmPep* and *PE-PfuPep*, the immature spikes (17 days post anthesis ±3 days) of selected transformants (in the T₂ generation) were collected in liquid nitrogen and stored at -80°C until further use. About 200–300 mg of the developing grains were grinded to fine powder, and total RNA was extracted using 1 mL of TRIzol reagent (Invitrogen) as per the manufacturer's recommendations. RNA was quantified by absorbance at 260 nm on a SmartSpec 3000 spectrophotometer (Bio-Rad), and converted to cDNA with oligo-dT primer using the Reverse Transcription System (catalog no. A3500; Promega) following the manufacturer's instructions. The qRT-PCR analysis was performed on the LightCycler 480 Real-Time PCR System (Roche Diagnostics) using the

DNA Master SYBR Green 1 chemistry using gene-specific primers. The native *Actin* gene was used as internal control and expression data was analyzed following Wen et al. (2012).

Extraction of total seed protein, enzyme activity assay and liquid chromatography

The protocol of total seed protein extraction was as in (Altenbach and Allen 2011) with minor modification. Briefly, about 4 to 5 wheat grains were ground to get 200 mg flour. The flour was transferred to a 2 mL tube and suspended in 600 μ L extraction buffer (2% SDS, 10% glycerol, 62.5 mM Tris-HCl pH 8.5 and 1% DTT). The mixture was vortexed for 20 sec and another 600 μ L extraction buffer was added. The mixture was shaken at 200 rpm at room temperature for 30 min and centrifuged at 20,800 $\times$ g for 10 min at 4°C. Supernatant containing the total protein was transferred to a new tube. Protein extracts were resolved on 12% sodium dodecyl sulfate polyacrylamide gel following Mejías et al. (2014).

To activate EP-HvB2, 100 μ L sodium citrate buffer (500 mM, pH 3.0) was added to the 100 μ L of the protein extract from the previous step and incubated at room temperature for 30 min (Bethune et al. 2006). Following EP-HvB2 activation, pepsin was added to the reaction mixture (pH 3.0) at a final concentration of 0.375 mg/mL and incubated for 60 min at 37°C. Subsequently, pH of the reaction mixture was adjusted to 6.0 by sodium phosphate buffer (500 mM, pH 6.0), and trypsin as well as chymotrypsin each at a final concentration of 0.375 mg/mL and elastase as well as carboxypeptidase A each at the final concentration of 0.075 mg/mL were added to the reaction mixture (cf. Comino et al. 2012; Siegel et al. 2006). The reaction mixture was then incubated at 37°C for another 60 min with gentle shaking.

Gluten peptides were extracted from the reaction mixture (200 μ L total volume) by adding equal volume of absolute ethanol (E7023, Sigma-Aldrich), followed by centrifugation at 10,000 $\times$ g at 4°C for 20 min (Kim 2011). The supernatant was filtered through 0.45 μ m Durapore® membrane filters and analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC) using a C18 reversed-phase analytical column (Eclipse Plus C18, 5 μ m particle size, 150 mm length and 4.6 mm inner diameter Agilent Technologies) and 1200 Series Quaternary HPLC-System (Agilent Technologies), with a diode array UV-V detector. Column temperature was kept at 60°C. A linear elution gradient was implemented using two mobile phases, the polar solvent A consisting of 0.1% trifluoroacetic acid (TFA) (vol/vol) in type I ultrapure water (18 MOhm-cm specific resistance), and the non-polar solvent B containing 0.1% TFA (vol/vol) in acetonitrile (ACN). Absorbance was monitored at a detection wavelength of 210 nm, and the flow rate was maintained at 0.5 mL min⁻¹. The elution gradient conditions were 0% to 50% B in 30 min. After each run, the column was cleared by linearly decreasing solvent B to 0% in 4 min, and then the column was equilibrated with the starting B concentration for 10 min. Injection volume was 10 μ L. For peptide semi-quantification, standard curves were developed on RPHPLC using increasing concentrations of bovine serum albumin (Mejías et al. 2014). HPLC peaks were integrated and the obtained peak area in milli-absorbance unit (mAU) was regressed against the concentrations of BSA loaded onto the HPLC column to obtain a standard curve (cf. Mejías et al. 2014). Two biological and three technical replicates were used, and the values were averaged for presentation.

Immunogenic detection by ELISA

After pretreatment with gastric and pancreatic enzymes the protein extracts of the control and select transformants in the T₂ generation were used in an ELISA assay using the Veratox gliadin R5 kit (8510, Neogen Corporation) according to the manufacturer's specifications. Two biological and three technical replicates were used and the values were averaged for presentation.

Results

***In-silico* degradation of gluten by barley endoprotease B2 (EP-HvB2) and prolyl endopeptidases from *F. meningosepticum* (PE-FmPep)**

A large number of prolamin sequences from wheat and allied cereals were digested *in-silico* to identify the best combination of glutenases for expression in wheat grain endosperm. For this purpose, sequences of wheat α/β -, γ - and ω -gliadins, low- and high-molecular weight glutenin subunits, barley B-, C-, γ - and D-hordeins, and rye γ -, ω - and high molecular weight secalins were retrieved from the National Center for Biotechnology Information (NCBI). A Visual Basic (Version 6)-based computer program (Macro) was developed for each enzyme, the cleavage rules for digestive enzymes pepsin, trypsin and chymotrypsin were deduced from specificity data summarized by (Keil 1992), and for EP-HvB2 and wheat (*Triticum aestivum* L.) cysteine proteases from (Davy et al. 2000; Hartmann et al. 2006). In brief, sequences were virtually digested with i) digestive enzymes alone; ii) with digestive enzymes followed by wheat or barley cysteine endoprotease; and iii) with digestive enzymes, wheat or barley cysteine endoprotease followed by *F. meningosepticum* or *Aspergillus niger* endopeptidase, PE-FmPep or PE-AnPep. Products of digestion were checked for the presence of 58 different immunogenic peptides documented in the literature via sequence comparisons (Rustgi et al. 2014). Results showed that the size and amount of Pro/Gln-rich immunogenic peptides are significantly reduced after digestion with EP-HvB2 or wheat cysteine endoprotease combined with PE-FmPep (Fig. 3). EP-HvB2 is a glutamine specific endoprotease. It cleaves proteins after glutamine residues, preferentially with prolines at P2 or P2' positions, which overlaps with tTG2-mediated deamidation sites (Bethune et al. 2006; Fleckenstein et al. 2002) (Fig. 2). The pro-EP-HvB2 is activated rapidly in the acidic environment of the stomach with pepsin digesting the pro-peptide region or via autocatalysis. The enzyme maintains activity in the stomach and its activity increases with the increase in pH (up to pH 7). It has been demonstrated in the rat that EP-HvB2 is effective in decreasing concentration of the highly immunogenic 33-mer gliadin peptide in stomach by 50-fold within 90 min with no signs of toxicity (Gass et al. 2006). However, the mature EP-HvB2 enzyme is susceptible to physiological concentrations of trypsin in the small intestine with a half-life of 15 min (Bethune et al. 2006).

Prolyl endopeptidases (PE-Pep), classified under the serine proteases, are generally capable of cleaving peptides within 30 residues length at the carboxyl side of the proline residue (Szeltner and Polgar 2008; Gass and Khosla 2007) (Fig. 2). PE-Peps were found in archeal, bacterial and eukaryotic species and exhibited similar activities against the reference substrates (Savvateeva et al. 2015; Venalainen et al. 2004). PE-Pep from *F. meningosepticum* has been well studied for its activity, specificity and stability under

gastrointestinal conditions (Shan et al. 2004). Where it showed resistance to pancreatic proteases and membrane peptidases of the small intestinal mucosa, retained 30–90% of its original activity between pH 1.6–3.9, and exhibited optimal activity at a pH between 6–7. PE-Pep from *P. furiosus*, on the other hand showed survival under extreme temperatures, making it a good candidate to survive the baking process, while retaining its catalytic properties (Venalainen et al. 2004). Despite the three-dimensional model of PEPfuPep was established and detailed kinetic studies under various pH and temperature regimes were undertaken, less remained known about its target site and cleavage pattern (Harris et al. 2001). Since, the cleavage rules for PE-PfuPep were not fully known it was not included in the *in-silico* analysis. However, the former biochemical studies on *P. furiosus* endopeptidase established it beyond any doubt as a post-proline cleaving enzyme (Harris et al. 2001).

Based on the results of the *in-silico* analysis and the former biochemical studies a combination of barley EP-HvB2 with PE-FmPep or PE-PfuPep was expressed in wheat grains. Results of this experiment are discussed in the following paragraphs.

Generation of transgenic wheat lines using different vectors

Transgenic wheat lines were generated expressing combinations of genes encoding EP-HvB2 and PE-FmPep or PE-PfuPep. Biolistic transformation of wheat scutellar calli was performed using five different construct combinations. These efforts resulted in a total of 168 candidate transformants that survived bialaphos treatment. Out of the 168 candidate transformants, 91 were transformed with a linear construct consisting of either PE-FmPep and EP-HvB2 or PE-PfuPep and EP-HvB2 and 77 were transformed with different combinations of circular constructs (Fig. S2). When screened using gene specific primers (Table S1), 47 out of 168 candidate transformants amplified a product of expected size for both or either of the genes. For 35 putative transformants obtained using circular constructs a desired PCR product could be amplified for EP-HvB2 in 8 cases, PE-PfuPep in 2 cases, PE-FmPep in 20 cases, PE-FmPep and EP-HvB2 combination in 4 cases, and PE-PfuPep and EP-HvB2 combination in a single case. Similarly, out of 12 putative transformants obtained using linear constructs 2 genotypes amplified a product each for PE-FmPep and EP-HvB2, and 10 genotypes amplified a product each for PE-PfuPep and EP-HvB2 (Fig. S2).

Transgenic origin of the PCR products was confirmed by DNA sequencing. All sequences from PCR products showed identity with the constructs used for genetic transformation (Fig. S3). These results showed a success rate of 11.67% for circular constructs and 4.0% for the linear construct (Table 2), which is significantly higher than the usual frequency (0.9% to 2%) of transformants reported for the *Agrobacterium*-mediated stable genetic transformation method in wheat (Cheng et al. 1997). Our results are nevertheless in accordance to the results of Wang et al. (2016) who generated backbone free transgenic wheat lines using *Agrobacterium* mediated genetic transformation method. For the linear constructs, the efficiency of genetic transformation is about one-third of that reported before for the circular constructs. However, when plants having both transgenes were considered alone, the linear constructs performed twice as better than the circular constructs, and the transformation frequency also remained high in comparison to the *Agrobacterium*-based method. Use of a double cassette linear construct might have contributed to the observed high frequency of the

double transformants. Integration of a single transgene or truncated copies of the transgenes is possible albeit none of the transformants with single transgene integrations were identified with the use of linear constructs.

Collectively, the results showed that the minimal gene construct, which was a linear DNA fragment lacking vector sequence, functioned as efficiently as whole plasmids in wheat genetic transformation. These results were consistent with the previous results in rice (Loc et al. 2002). Since the linear constructs used in the present study lack any marker or vector DNA sequence, it serves as an example of the “clean” DNA technology, which allows the production of plants expressing desired traits by introducing minimum alteration to their genomes.

Analysis of T₁ seeds and plants

Twenty transformants were chosen for further analysis under green-house conditions. To avoid the chimerism problem associated with the biolistic method used in the present study (Kumlehn and Hensel 2009), 10% of the total T₁ grains from 20 selected T₀ transformants were sown and treated with a 2% Basta™ solution to test for bialaphos resistance. Application of the herbicide three days after the start of vernalization cycle resulted in on average 60% mortality, as wild type wheat plants are known to be highly susceptible to bialaphos at the three to four leaves stage. Additionally, metabolism-based resistance of plants to herbicide is known to be temperature sensitive. For instance, atrazine tolerant corn could be only damaged, when atrazine is applied at low temperature because of reduced herbicide detoxification by decreased glutathione activity at the low temperatures (Thompson et al. 1970). Similarly, in the case of glufosinate resistant Liberty-Link soybeans, enzyme encoded by the *pat* gene exhibited very low activity at 10 °C and a gradual increase in enzyme activity was witnessed up to 45°C (Botterman et al. 1991).

Plants that survived bialaphos treatment, were subjected to DNA analysis. Two genomic DNA samples were collected from the leaf material of putative transformants, one before the application of herbicide and the other from the survivors of the herbicide treatment at the Feekes developmental stage 10.0 (Large 1954). Each sample was used as template for PCR reaction. The objective of this sampling strategy was to check for the chimeric origin of plants, and to ascertain transgene integration in the genome. Of the plants that attained maturity, seven gave positive results when analyzed by PCR followed by DNA sequencing. Out of these 7 transformants 2 plants were transformed with circular constructs where one plant was positive for PE-FmPep (Fme108) and the other for both PE-FmPep and EP-HvB2 (Fme113), and the remaining five plants were transformed with linear construct and all of them were positive for both genes either PE-FmPep and EP-HvB2 combination (Fme145 and Fme162) (Fig. S4) or PEPfuPep and EP-HvB2 combination (Pfu2, Pfu4 and PfuC11) (Table 3). The transformants were grown to maturity and allowed to self-pollinate. Seeds from these plants were morphologically indistinguishable from wild type seeds and were fully fertile.

T₂ seeds analysis

RNA was extracted from the developing grains (17 days after anthesis) of seven wheat transformants, namely Fme108, Fme113, Fme145, Fme162, Pfu2, Pfu4 and PfuC11, and analyzed for the level of transgene (*EP-HvB2*, *PE-FmPep* or *PE-PfuPep*) expression in relation to the native *Actin* gene (Wen et al. 2012). In relation to *Actin*, the selected transformants exhibited 2–18 fold higher expression of PE-PfuPep, 20–80 fold higher expression of PE-FmPep, and 17–100 fold higher expression of EP-HvB2. The elevated expression levels of transgenes in the wheat grains suggested high accumulation of glutenases in the wheat endosperm.

Total grain protein was also extracted from the selected wheat transformants, and analyzed for the degradation of immunogenic prolamins under simulated gastro-intestinal conditions. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the total seed proteins from transformants and the untransformed control showed no significant differences at either qualitative or quantitative levels (Fig. S5). In this case, the seeming lack of recombinant protein(s) could be explained by the large number of proteins accumulating in wheat grains or to the insufficient accumulation of these proteins to form protein bodies (Saberianfar and Menassa 2017). In addition, the limited resolution of Coomassie blue stained SDS-PAGE gel prevents recognition of proteins with expression levels at or below 1% of the total soluble proteins (Desai et al. 2010; Gao et al. 2010; Rasheed et al. 2014).

The activity of EP-HvB2 and PE-FmPep/PE-PfuPep was assessed under the conditions similar to those in the gastrointestinal (cf. Materials and Methods). After digestion, peptides were extracted from the reaction mixture with absolute ethanol and analyzed by RP-HPLC using a C18 column. Conceptually, RP-HPLC separates peptides according to their size and hydrophobicity. Whereas shorter, hydrophilic peptides elute earlier, larger, hydrophobic peptides elute later on the C18 column (Gass et al. 2005, 2006). If applied to our study, owing to their hydrophobic nature immunogenic peptides should have longer retention times under increasingly hydrophobic column conditions than their respective degradation products (Matysiak-Budnik et al. 2005). In addition, it should be kept in mind that PE-Pep largely processes peptides produced by EP-HvB2 and further reduces their length by cutting after the underlying proline residues, which upon HPLC of the peptide mixture appear as a peak in the beginning of the chromatogram or remain undetected if the initial peaks were not integrated. Therefore, only reductions in the length and area of different peaks, rather than major changes in the pattern of peaks is expected on a chromatogram (see Fig. S6). To permit relative-quantification, the amounts of proteins/peptides in a given sample were determined based on curve area under a chromatogram and a standard curve developed using BSA as reference (cf. Materials and Methods). Protein quantification for the digested samples of wheat transformants expressing PE-PfuPep and EPHvB2 exhibited 32.30% to 66.43% reduction in amount of indigestible gluten peptides for Pfu2 and PfuC11, respectively (Fig. 4; Table 3). Since prolyl endopeptidase preferentially cleaves peptides of about 13–33 amino acids in length and exhibit negligible activity against longer peptides and proteins, significant reductions in amount of gluten proteins ascertained expression of both EP-HvB2 and PE-PfuPep in these transgenic lines. Moreover, the background activities of the germination proteases and peptidases were accounted for by comparing peptide profiles

of transgenic lines with that of wild type controls. As the seed-storage proteins extracted from the untransformed control were processed similar to the transgenic samples (i.e., treated with similar amounts of pepsin, trypsin and chymotrypsin as transgenic samples).

When PE-FmPep and EP-HvB2 expression lines were analyzed, 42.39%–57.57% reductions in the curve area for wheat transformants Fme113–13 and Fme108–10 was observed (Fig. 4; Table 3), this diminution in curve area could be attributed to the decrease in the median peptide length due to glutenase activity. For Fme108–10, which express PE-FmPep alone, an attenuation in peak area only after 15 min treatment with pre-activated EP-HvB2 enzyme was observed (Fig. S6). The two progeny plants of Fme162, Fme162–6 and Fme162–48 showed no difference from the control plant. This result led us to conclude that although these genotypes contained both transgenes, these were present either in truncated or non-functional forms not permitting accumulation of the encoded protein products (Fig. 4).

Anyway, our activity profiling through RP-HPLC demonstrated that it is possible to express glutenase combinations and that both PE-PfuPep + Ep-HvB2 and PE-FmPep + Ep-HvB2 permit hydrolytic degradation and detoxification of immunogenic gliadin peptides. This is obvious from the disappearance of the peptide peak at 15 min that was earlier confirmed to include most of the immunogenic peptides in partially digested gluten samples (Gass et al. 2006). In addition, protein extracts from the selected transformants (after necessary activation and treatment with digestive enzymes) were used for ELISA. For this purpose, a Veratox gliadin R5 kit (product # 8510) was used. This kit is based on a monoclonal antibody R5, which was raised against the ethanolic fraction of rye secalins. The core epitopes targeted by the antibody are QQPEP, QQQFP, LQPFP, and QLPFP, which occurs repeatedly in alpha-, gamma- and omega gliadins, and specifically overlap with immunogenic epitopes. Therefore, this assay is endorsed by Food and Drug Administration to determine amount of immunogenic epitopes in the allegedly ‘glutenfree’ products. Results of ELISA test showed up to 72% reduction in the amount of immunogenic peptides in Pfu11 (Table 3). Consistent with the data reported in Figure 3 and elsewhere (Gass et al. 2006), these results highlighted that due to the combinatorial effect of EPHvB2 with PE-FmPep/PE-PfuPep it is possible to severely reduce the amount of Pro/Gln-rich immunogenic peptides *in statu nascendi*.

Since the extraction buffer used to extract proteins from wheat grains was neutral to slightly basic in nature different salts were used to adjust its pH for activation of EP-HvB2 proenzyme and to create gastrointestinal conditions for the action of gastric and pancreatic enzymes. The additional salts might have an influence on the digestion process or subsequent analysis of the protein samples. Moreover, it is hard to anticipate, if SDS and DTT present in the extraction buffer exert any effect on the effective amount and action of the glutenases present in the protein sample derived from the transgenic wheat grains, but due to the limited amount available, further analyses were not possible. Nevertheless, since the extraction conditions were the same for all samples including controls it might have equally influenced both the controls and transgenic samples and have thus allowed a fair assessment of wheat transformants expressing glutenases.

Discussion

Elimination of gluten proteins has been repeatedly attempted by screening of wheat germplasm and cytogenetic stocks for reduced-gluten genotypes, induced mutagenesis, transgenic silencing or site-directed mutagenesis of genes encoding gluten proteins or regulators of prolamin genes (Sánchez-León et al. 2018; Gilissen et al. 2014; Rosell et al. 2013; Osorio et al. 2012). Our own research and parallel studies performed elsewhere clearly showed that it is possible to obtain 'reduced-gluten wheat genotypes/varieties' (Wen et al. 2012; also cf. Ribeiro et al. 2018). These lines are neither fully depleted of gluten nor do they completely lack the well-known highly immunogenic gluten peptides, except the recently developed wheat lines that lack highly immunogenic 33-mer gliadin peptide (Sánchez-León et al. 2018). Moreover, previously established lines still contained higher molecular weight (HMW) glutenins, which are indispensable for baking and were thus far supposed to be largely non-immunogenic. However, there is a small proportion of celiac patients who showed sensitivity to HMW glutenins and could not consume wheat genotypes containing these proteins (Barro et al. 2016; Ribeiro et al. 2018).

It has been demonstrated previously that proteolytically resistant proline-glutamine rich peptides can be completely detoxified within 10 min at a gluten:EP-HvB2:PE-Pep weight ratio of 75:3:1 (Bethune et al. 2006; Siegel et al. 2006). Based on these findings, medical pills or dietary enzyme supplements are currently being used or developed and subjected to clinical trials. Each of these therapeutic agents contains 'glutenases' and need to be administered along with or shortly before each meal, which is a bit of inconvenience and fairly expensive (Sollid and Khosla 2011). Additionally, these food supplements are mostly based on single enzymes of bacterial or fungal origin, whose activity is largely dependent on the meal contents making them less successful (Rood 2017; Rey et al. 2016). The preliminary clinical trials performed using a combination of glutenases dubbed ALV003 showed attenuation of gluten-induced small intestinal mucosal injury and decreased immune response to gluten in celiac patients, however it did not improve their clinical response (Tye-Din et al. 2010; Siegel et al. 2012; Lähdeaho et al. 2014). Therefore, from a conservationist viewpoint, currently, the oral enzyme therapy can be considered effective in subsiding the effects of accidental gluten exposure of susceptible individuals.

An alternative strategy for treating gluten-induced disorders such as celiac disease or to prevent damage to the small intestine from accidental gluten ingestion was developed in the current work. This strategy makes use of transgenic plants expressing combinations of glutenase enzymes, such as prolyl endopeptidase from *F. meningosepticum* (PE-FmPep) and barley cysteine endoprotease B2 (EP-HvB2), in the native context of the intact wheat grain and without distorting the balance of storage proteins or destroying the gluten network (Fig. S5), which contribute to the baking properties, while exploiting the inherent properties of the selected enzymes. Conceptually, these enzymes should get active only in the intestine and when in contact with their target substrate. Both criteria are met in the established transgenic lines. EPHvB2 that belong to the C1A cysteine protease family is encoded as a proenzyme, where the propeptide or the prosegment serves as both inhibitor and chaperone to respectively facilitate spatiotemporal regulation of the proteolytic activity and proper folding of the protease (Cambra et al. 2012; Schilling et al. 2009; Bethune et al. 2006; Cappetta et

al. 2002). Both of these properties have immense importance in the present research, as the propeptide keeps the enzyme in an inactive state in grains and likely facilitates its refolding after thermal denaturation. The propeptide inhibits enzyme activity at basic, neutral and slightly acidic pH. At acidic pH 4, however, the propeptide is removed by an autocatalytic mechanism. As the pH of mature wheat 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 the enzyme, no degradation of prolamins occurs (Leenhardt et al. 2005; Dominguez et al. 1999). In addition, PE-FmPep and PE-PfuPep due to their strict preference for substrates with 33 amino acids in size can only degrade peptides generated by EP-HvB2 (Gass and Khosla 2007), thus permitting both of these enzymes to accumulate within protein bodies containing the gluten proteins without degrading them (Fig. S5) and affecting the baking properties of the flour. Furthermore, the propeptide also serves as an intramolecular chaperone and helps the enzyme to refold into its native state after thermal denaturation, such that essentially no loss of enzyme activity is expected to occur during baking. The 'mature/active' cysteine endoproteases from wheat grains have previously been demonstrated to retain their activities at 60°C (Hartmann et al. 2006). Similarly, recombinant PE-FmPep with 12 amino acid substitutions exhibited a ~1200fold increase in its half-life at 60°C (Hamamatsu et al. 2005), which in combination with the sequestration of these two enzymes in protein bodies are expected to add to their thermal stabilities allowing them to survive the baking process with no effect on their catalytic activities.

Considering these aspects, native version of the proEP-HvB2 gene and of a recombinant PEFmPep gene were introduced into the wheat genome and the presence and maintenance of the transgenes confirmed in subsequent generations. Total wheat grain proteins were then extracted from the stable transformants and tested for the presence of immunogenic prolamins by reversed phase high performance liquid chromatography and ELISA with R5 antibodies under experimental conditions reflecting those of the gastrointestinal tract. More importantly, quantification of immunogenic gliadin-derived peptides showed 33.57% to 67.69% reductions respectively, in the amount of indigestible gluten peptides for Pfu2 and PfuC11 grains and up to 72% reduction in the content of major immunogenic peptides in PfuC11 (Fig. 4, Table 3). Because of the known substrate specificity of prolyl endopeptidase, preferentially cleaving peptides of about 13–33 amino acids in length, these results can only be explained by the joint action of both glutenases, EP-HvB2 and EP-FmPep/EP-PfuPep in the generated transgenic lines (Fig. 4). Thus, although we were unable to directly show accumulation of Coomassie-stainable protein bands representing these enzymes, the more sensitive activity assays are convincing and indicative of faithful protein expression, folding, substrate recognition and conversion. Through the combination of 'glutenase genes', including one for a prolyl endopeptidase from *F. meningosepticum* (PE-FmPep) or *P. furiosus* (PE-PfuPep) and one for a barley cysteine endoprotease B2 (EP-HvB2) introduced by biolistic transformation it is thus possible to engineer wheat varieties with preconditioned gluten detoxifiers that can serve in dietary therapy of celiac disease while retaining their agronomical properties (Table S2). It is important to point out in this context that most transgenic crops currently in use have been generated for the benefit of producers, while wheat genotypes that express glutenases will benefit not only the consumer

but particularly celiac patients. In the light of these considerations, the authors hope for a general acceptance of their work by both regulatory authorities and public means.

Prospectives

It is estimated that >3 million individuals suffering from celiac disease live in the US (https://www.cureceliacdisease.org/wp-content/uploads/341_CDCFactSheets8_FactsFigures.pdf) and that the number of gluten-intolerant humans is close to 76 million worldwide (Gujral et al. 2012). Today there are neither any drugs or other medical treatments for celiac disease, except for conventional dietary exclusion of gluten from food. A major challenge therefore is the development of alternative therapeutic strategies for this immune disorder. Co-expression of glutenase enzymes in wheat grains seems to be a feasible approach. Due to the observed cosequestration of substrate (gluten) and respective hydrolytic enzymes that can degrade the gluten-derived immunogenic gluten peptides *in statu nascendi*, an efficient mechanism is established to dampen or even fully drop the symptoms normally observed during celiac disease. By co-expressing recombinant barley endoprotease B2 (EP-HvB2) and prolyl endopeptidase (PE-Pep) from *F. meningosepticum* or *P. furiosus* an efficient mechanism is set on that degrades immunogenic gluten peptides once they are formed during ingestion in the intestine. The whole detoxification mechanism remains silent as long as both the substrate (gluten) and respective endoprotease/endopeptidase combination rest in protein bodies of the seed. Moreover, due to the complementary physicochemical properties and thermo-stability as well as refolding capacity of the barley cysteine endoprotease B2 (EP-HvB2) and *F. meningosepticum* or *P. furiosus* prolyl endopeptidase (PE-FmPep/PE-PfuPep) it is expected to obtain wheat genotypes with further reduced content of immunogenic gluten peptides than available in the current lines (between 32%–66%). Availability to such wheat varieties with preconditioned gluten detoxifiers would permit the large number of gluten-sensitive patients in the US and worldwide to return to a more balanced, wheat-based diet and experience the same life quality as healthy individuals or it will at least allow prevention of symptoms in celiac patients upon accidental exposure to gluten proteins. Expressing ‘glutenases’ as those reported here in wheat grains significantly improves digestibility of the normally indigestible gluten peptides, which in turn improve bioavailability of these proteins to the consumer. Since this approach does not require consumers to take costly dietary supplements, it is expected to reach the public more easily and reduce the healthcare expenses. The above mentioned properties of the dietary approach make it unique in the direction of improving the general public health, especially in resource-deprived countries.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors would like to thank Ellison Mackenzie and Rhoda Brew-Appiah for laboratory assistance, Richa Gemini for the bioinformatics support, Pat Reisenauer for greenhouse assistance, Robert Zemetra for help with the wheat genetic transformation, and the two anonymous reviewers whose comments helped us in improving the manuscript. Financial support by NIH grants 1R01 GM080749–01A2, and Clemson Faculty Succeeds grant 15–202-EQUIP-5701–430-1502211 is gratefully acknowledged.

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Fig. 1.
Constructs used for the genetic transformation of wheat cv. Brundage 96.

Fig. 2.

The prolyl endopeptidase and cysteine endoprotease cleavage sites in the 33-mer peptide of the $\alpha 2$ -gliadin.

Fig. 3.

In-silico simulated digestion of wheat gliadins to identify best combination of glutenases for expression in wheat grains.

Treatment 1 = pepsin + trypsin + chymotrypsin + carboxypeptidase A + elastase

Treatment 2 = pepsin + EP-HvB2 + trypsin + chymotrypsin + carboxypeptidase A + elastase

Treatment 3 = pepsin + EP-HvB2 + trypsin + chymotrypsin + carboxypeptidase A + elastase + prolyl endopeptidase (PE-Pep) (Modified from Osorio et al. 2012)

Fig. 4.

Total reduction in the content of the prolamin peptides in wheat transformants expressing A. PE-PfuPep and EP-HvB2, and B. PE-FmPep and EP-HvB2. Total protein was extracted from T₂ grains, treated with gastric enzymes and quantified by RP-HPLC. Proteins were measured in µg/mL and expressed as percent reduction in comparison with control line.

Table 1.

Molar ratios of constructs used for wheat genetic transformation.

Construct	pHMWg::EP- FmPep::nos + pHMWg::EPH vB2::nos + pDPG165	pHMWg::PE- PfuPep::nos + pHMWg::EPHvB 2::nos + pDPG165	pHMWg::PE- FmPep:: nos::pHMWg::EPHv B2::nos + pDPG165	pHMWg::PE- PfuPep::nos::pHMW g::EPHvB2::nos + pDPG165
Molar ratio	1:2:2	1:2:2	1:2	1:2

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

Table 2.

Transformants obtained after biolistic transformation of wheat scutellar calli.

Method	Total number of embryos [§]	No. of candidate transformants	Positive T ₀ plants [*]	Single transformants [‡]	Double transformants [‡]	Efficiency (%)
Combination of circular constructs and pDPG165	300	77	35	30	5 (1.67%)	11.67
Linear construct and pDPG165	300	91	12	-	12 (4.0%)	4.00

^{*} PCR with construct-specific primers and DNA sequencing

[§] 6 plates with 50 embryos each

[‡] Single transformants = presence of one transgene confirmed; double transformant = presence of both transgenes confirmed

Table 3.

List of seven transformants (T₂ grains) showing the gliadin peptide content determined via reversed-phase high-performance liquid chromatography (RP-HPLC) and R5 enzyme-linked immunosorbent assay (ELISA). The RP-HPLC values are presented in percent in relation to the control and the R5 ELISA values are expressed in parts per million (ppm)±S.D. After extraction proteins were treated with digestive enzymes to simulate conditions reflecting those of the gastrointestinal. Analysis was performed on C18 column or using the Veratox gliadin R5 kit from Neogen Corporation. Non-transgenic sibling line was used as control to determine the percent reductions.

Genotype	PCR analysis with gene-specific primers	Peptide content (% reduction in gluten peptides)		
		Based on peak area in chromatogram	Based on amount (µg/mL), determined using BSA	Based on R5 ELISA (ppm)±S.D.
Pfu2	PE-PfuPep, EP-HvB2 (L)	64.918 (35.08)	66.434 (33.57)	820.61±52.25 (36.4)
Pfu4	PE-PfuPep, EP-HvB2 (L)	51.826 (48.17)	58.440 (41.56)	605.83±72.67 (53.0)
Pfu11	PE-PfuPep, EP-HvB2 (L)	30.542 (69.46)	32.307 (67.69)	361.24±129.57 (72.0)
108_10	PE-FmPep (C)	42.113 (57.89)	42.432 (57.57)	504.09±64.12 (60.9)
113_13	PE-FmPep, EP-HvB2 (C)	59.498 (40.50)	57.612 (42.39)	788.02±149.52 (38.9)
145_43	PE-FmPep, EP-HvB2 (L)	54.153 (45.85)	48.659 (51.34)	620.81±86.75 (51.9)
162_6*	PE-PfuPep, EP-HvB2 (L)	93.627 (6.37)	90.725 (9.27)	1136.71±124.45 (11.9)
162_48*	PE-PfuPep, EP-HvB2 (L)	98.878 (1.12)	97.629 (2.37)	1266.26±29.48 (1.8)

* Same transformation event. (C) = circular construct and (L) = Linear construct