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(57) Abstract: The present disclosure concerns the specific silencing of at least one of, but preferable all, of α (alpha), β (beta), γ (gamma), and ω (omega) gliadins and the LMW (low molecular weight) glutenin subunits of hard wheat and flour by interference RNA (iRNA) through use of a polynucleotide that is transcribed into an hpRNA (hairpin RNA). Furthermore, the present disclosure also concerns a vector, cell, plant, or seed comprising the polynucleotide. Expression can be specifically directed to particular tissues of wheat seeds through gene expression-regulating sequences such as, for example, the promoter of the gene that codes for a D-hordein.

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TARGETING OF PROLAMIN BY RNAI IN BREAD WHEAT

FIELD OF THE INVENTION

The present disclosure concerns the specific silencing of at least one of, but preferable all, of α (alpha), β (beta), γ (gamma), and ω (omega) gliadins and the LMW (low molecular weight) glutenin subunits of hard wheat and flour by interference RNA (iRNA) through use of a polynucleotide that is transcribed into an hpRNA (hairpin RNA). Furthermore, the present disclosure also concerns a vector, cell, plant, or seed comprising the polynucleotide. Expression can be specifically directed to particular tissues of wheat seeds through gene expression-regulating sequences such as, for example, the promoter of the gene that codes for a D-hordein.

BACKGROUND OF THE INVENTION

Cereal grains contain about 10-15% (dry weight) of protein, from which gluten is the most important fraction as it is the major determinant of the technological properties of baking cereals. However, gluten is not a single protein but a complex mix of proteins, which are deposited in the starchy endosperm during grain development. Gluten proteins are divided into two major fractions: the gliadins and the glutenins, which are different in terms of structure and functionality. In turn, gliadins are formed by three different fractions/types; ω-, γ-, and α gliadins (the wheat α gliadins, are sometimes also referred to as α/β gliadins based on their separation by acid electrophoresis. However, both α and β gliadins have a very similar primary structure and for these reasons are currently considered a single gliadin type (α/β type). Therefore, the terms α-gliadins and α/β-gliadins are interchangeable). The glutenins comprise two fractions; the high molecular weight (HMW) and the low molecular weight (LMW) subunits. The gliadins are generally present as monomers and contribute extensibility to wheat flour dough. The glutenins contribute elasticity to dough and form large polymers linked by disulphide bonds.

These proteins make up a complex mixture that in a typical bread wheat cultivar may be comprised of up to 45 different gliadins, 7 to 16 LMW glutenin subunits, and 3 to 6 HMW glutenin subunits. Gliadins and glutenins are not present at the same amount in the grain of cereals, and their proportions can vary within a broad range depending on

both genotype (variety) and growing conditions (soil, climate, fertilisation, etc.). The ratio of gliadins to glutenins was examined in a range of cereals (Wieser and Koehler, 2009), and hexaploid common wheat showed the lowest ratio (1.5 - 3.1), followed by durum wheat (3.1 - 3.4), emmer wheat (3.5 – 7.6) and einkorn wheat (4.0 – 13.9).

In addition to their unique viscoelastic properties, gluten proteins are responsible for triggering certain pathologies in susceptible individuals: i) coeliac disease (CD), which affects both children and adults throughout the world at various frequencies (from 0.1% to >2.8%) (Mustalahti *et al.*, 2010; Abadie *et al.*, 2011), and ii) non-coeliac gluten sensitivity, a newly-recognised pathology of intolerance to gluten (Sapone *et al.*, 2011) with an estimated prevalence of 6% for the USA population. However, gliadins and glutenins do not contribute equally to CD, and gliadins are indubitably the main toxic component of gluten since most (DQ2 or DQ8)-specific CD4+ T lymphocytes obtained from small intestinal biopsies from coeliac patients seem to recognize this fraction (Arentz Hansen *et al.*, 2002). In the immune epitope database (IEDB) (http://www.iedb.org/) 190 T-lymphocytes stimulating epitopes related to CD can be found. Of these, 180 (95%) map to gliadins while only 10 (5%) map to glutenins.

However, not all gliadin epitopes are equally important in triggering CD. The α-2-gliadin family contain the 33-mer peptide, present in the N-terminal repetitive region, with six overlapping copies of three different DQ2-restricted T-cell epitopes with high stimulatory properties and highly resistant to human intestinal proteases (Shan *et al.*, 2002; Tye-Din *et al.*, 2010). The α-gliadins also contain the peptide p31-43, which has been reported to induce mucosal damage via a non-T-cell-dependent pathway (innate response) (Maiuri *et al.*, 2003; Di Sabatino and Corazza, 2009). Moreover, an additional DQ2-restricted epitope (DQ2.5-glia-α3) which partially overlaps with 33-mer peptide (Vader *et al.*, 2002) is present in α-2-gliadins.

Tye-Din *et al.* (Tye-Din *et al.*, 2010) comprehensively assessed the potentially toxic peptides contained within wheat, but also barley, and rye, and identified which ones stimulate T-cells from coeliac disease patients. They found that the 33-mer peptide from wheat α-gliadin was highly stimulatory, and another peptide (QPFPQPEQPFPW) (SEQ ID NO: 28) from ω-gliadin/C-hordein was immunodominant after eating wheat, barley and rye. These two peptides present in wheat, plus another from barley, can elicit 90% of the immunogenic response induced by wheat, barley and rye (Tye-Din *et*

al., 2010). These findings showed that the immunotoxicity of gluten could be reduced to three highly immunogenic peptides, which make the development of varieties with low-toxic epitopes more affordable.

RNA interference (RNAi) is a system of degradation of messenger RNA mediated by double-stranded RNA that allows the specific silencing of particular genes. Its discovery has made it possible to design vectors composed of a promoter and termination signals including the sequence of the gene one wishes to silence and having sense and antisense sequences separated by a spacer sequence of variable length.

siRNAs (small interfering RNA or short interfering RNA) are molecules of double-stranded RNA (dsRNA, the English abbreviation for double-stranded RNA) of 21-25 nucleotides (nt) that originate from a longer precursor dsRNA. Precursor dsRNAs may be of endogenous origin, in which case they are referred to as miRNAs (encoded in the genome of the organism) or of exogenous origin (such as viruses or transgenes). Both siRNA and miRNA are types of iRNA (interference RNA). iRNA suppresses the post-transcriptional expression of a particular mRNA (messenger RNA) recognized by the iRNA sequence.

When a cell receives a dsRNA precursor (single-stranded RNAs do not produce this effect), which may be generated from an exogenous transgene, a viral agent, or an endogenous genetic element, it is fragmented into siRNAs through the action of an enzyme referred to as Dicer, a cytoplasmic enzyme of the RNAse III family. Dicer cleaves the dsRNA into double-stranded fragments of approximately 21-25 nucleotides (siRNA), with the 5' end phosphorylated and two unpaired nucleotides protruding at the 3' end. Of the two strands of siRNA, only one, referred to as the guide strand, is incorporated into the enzymatic complex RISC (RNA-induced silencing complex), while the other strand is degraded. The thermodynamic characteristics of the 5' end of the siRNA determine which of the two strands is incorporated into the RISC complex. The strand that is less stable at the 5' end is normally incorporated as the guide strand, either because it has a higher content of AU bases or because of imperfect pairings. The guide strand must be complementary to the mRNA to be silenced in order for post-transcriptional silencing to occur. Subsequently, the RISC complex binds to the complementary mRNA of the guide strand of the siRNA present in the complex, and

cleavage of the mRNA occurs. After this, the fragments obtained are degraded. In this manner, the siRNAs cause post-transcriptional silencing of the target nucleotide sequences so that the protein that would result from expression of these sequences is not obtained.

One promising approach for reducing gluten toxicity and, therefore the incidence of gluten-related intolerances in cereals, is the down-regulation of immunodominant peptides by RNAi. This technology was applied to separately down-regulate the expression of γ-gliadins (Gil-Humanes *et al.*, 2008), ω-5 gliadins (Altenbach and Allen, 2011) and α-gliadins (Becker *et al.*, 2012). In Gil-Humanes *et al.*, 2010 and WO 2010/089437 the technology was further used to silence α, β, γ and ω gliadins of hard wheat and flour through use of a polynucleotide that is transcribed into an hpRNA (hairpin RNA). These same sequences disclosed in WO 2010/089437 were later demonstrated to produce plants with increased lysine content (WO2014/202688). In all these reports, the silencing of gliadin fractions was accompanied by an increase in other storage proteins or non-gluten proteins (Rosell *et al.*, 2014). Protein extracts from transgenic lines with all three gliadin fractions down-regulated were tested for stimulation of DQ2- and DQ8-restricted T-cell clones of coeliac patients, and a pronounced reduction in proliferative responses was seen in some transgenic lines (Gil-Humanes *et al.*, 2010).

None of these approaches however, also targeted the glutenins. There therefore exists a need to reduce gliadins (preferably all gliadins) and glutenins, particularly LMW glutenins, which are the major proteins containing epitopes triggering coeliac disease.

It is therefore an object of the invention to address the shortcomings of the prior art, and preferably to provide plants or plant products, in particular wheat or wheat products with a reduced, gliadin and/or glutenin content or a reduced prolamin content.

However, obtaining plants having seeds with a highly reduced gliadin and/or glutenin content presents a number of technical difficulties, e.g., first, due to the increased number of genes coding for gliadins and glutenins, and second, due to the fact that bread wheat plants are hexaploid. Indeed, based on the transcript information, the genes encoding α-gliadins range from 25 to 150 copies per haploid genome (van Herpen *et al.,* 2006) from 15 to 18 copies for ω-gliadins and from 17 to 39 copies for γ-

gliadins (Qi *et al.,* 2009). Therefore, the total number of different genes encoding gliadins ranges from 57 to 207. Glutenin proteins are divided into two major fractions named high-molecular-weight glutenin subunits (HMW-GS) and low-molecular-weight glutenin sub-units (LMW-GS). Bread wheat contains six HMW-GS genes, with tightly linked pairs of genes encoding x- and y-type subunits being present at each of the Glu-A1, Glu-B1, and Glu-D1 loci on the long arms of chromosomes 1A, 1B, and 1D, respectively. Regarding LMW-GS, in bread wheat they are encoded by a multi-gene family located at Glu-A3, Glu-B3 and Glu-D3 loci on the short arms of homologous chromosomes 1A, 1B, and 1D, respectively. It is difficult to asses the exact copy number of the LMW-GS, but more than 15 LMW-GS genes were detected from a single wheat variety (Zhang et al, 2011). For this reason there are more difficulties involved in achieving stable transformation in wheat plants than in transformation of any other plant that have fewer copies of the genome. Thus, achieving silencing of at least one of the gliadins and glutenins, and preferably all at the same time, is a difficult task.

Here we report the effectiveness of seven plasmid combinations, encompassing RNAi fragments from α-, γ-, ω-gliadins, and LMW glutenin subunits, for silencing the expression of different prolamin fractions. All seven RNAi fragments were under the control of the same endosperm-specific promoter – hence all the results are comparable. Silencing patterns of 21 transgenic lines were analysed by gel electrophoresis, RP-HPLC, and mass spectrometry (LC-MS/MS) whereas gluten immunogenicity was assayed by G12 moAb (anti-gliadin 33-mer monoclonal antibody). Plasmid combinations 1 and 2 down-regulated only γ- and α-gliadins, respectively. Four plasmid combinations were highly effective in the silencing of ω-gliadins and γ-gliadins, and three of these also silenced α-gliadins. HMW glutenin expression was up-regulated in all but one plasmid combination, while LMW glutenins were only down-regulated in three plasmid combinations. Non-gluten proteins were up-regulated with all plasmid combinations but total protein and starch contents were unaffected regardless of the plasmid combination used. Six plasmid combinations provided strong reduction in the gluten content as measured by competitive anti-gliadin 33-mer moAb and for two combinations, this reduction was higher than 90% in comparison with the wild type. CD epitope analysis in peptides identified in LC-MS/MS showed that lines from three plasmid combinations were totally devoid of CD epitopes from the highly immunogenic α- and ω-gliadins. Our findings enable breeding wheat species with low

levels of harmful gluten, and of achieving the important goal of developing non-toxic wheat cultivars.

SUMMARY OF THE INVENTION

In one aspect, the invention relates to a polynucleotide that is at least 90% identical to a sequence comprising at least one sequence pair (a1-b1) separated by a spacer sequence in which a1 and b1 are selected from the group consisting of SEQ ID NO: 1, SEQ ID NO:2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 and SEQ ID NO: 8, wherein

- if a1 is SEQ ID NO: 1, b1 is SEQ ID NO: 5; if a1 is SEQ ID NO: 5, b1 is SEQ ID NO: 1
- if a1 is SEQ ID NO: 2, b1 is SEQ ID NO: 6; if a1 is SEQ ID NO: 6, b1 is SEQ ID NO: 2:
- if a1 is SEQ ID NO: 3, b1 is SEQ ID NO: 7; if a1 is SEQ ID NO: 7, b1 is SEQ ID NO: 3; and
- if a1 is SEQ ID NO: 4, b1 is SEQ ID NO: 8. If a1 is SEQ ID NO: 8, b1 is SEQ ID NO: 4.

In one embodiment, the sequence comprises two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, in which the sequences a1, a2, b1 and b2 differ among themselves and are selected from SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO:7 and SEQ ID NO: 8, in the following form:

- If a1 is SEQ ID NO: 3 or SEQ ID NO: 7, b1 is SEQ ID NO: 7 or SEQ ID NO: 3, and a2 is SEQ ID NO: 4 or SEQ ID NO: 8, b2 is SEQ ID NO: 8 or SEQ ID NO: 4.
- If a1 is SEQ ID NO: 4 or SEQ ID NO: 8, b1 is SEQ ID NO: 8 or SEQ ID NO: 4 and a2 is SEQ ID NO: 3 or SEQ ID NO: 7 and b2 is SEQ ID NO: 7 or SEQ ID NO: 3.

In a further embodiment, the spacer sequence is SEQ ID NO: 9.

In another embodiment, the sequence also comprises a gene expression-regulating sequence functionally linked to its 5' end. In a preferred embodiment, the gene expression-regulating sequence is SEQ ID NO: 10.

In a second aspect, the invention relates to an RNA sequence coded for by the polynucleotide of the invention, wherein the RNA sequence is capable of forming a hpRNA (hairpin RNA) in which the sequence coded for by a1 completely hybridizes with the sequence coded for by b1.

In one embodiment, the RNA sequence coded for by the polynucleotide of the present invention is capable of forming a hpRNA in which the sequence coded for by the pair a1-a2 completely hybridizes with the sequence coded for by the pair b2-b1.

In a third aspect, the invention relates to a hpRNA formed from the RNA sequence of the present invention.

In a fourth aspect, the invention relates to a siRNA generated from the sequence of the hpRNA of the present invention.

In a fifth aspect, the invention relates to an expression vector that comprises at least one polynucleotide of the present invention.

In one embodiment, the expression vector comprises a polynucleotide that comprises the sequence pair (a1-b1) separated by a spacer sequence, wherein

a1 is SEQ ID NO: 1 and b1 is SEQ ID NO: 5 or a1 is SEQ ID NO: 5 and b1 is SEQ ID NO: 1.

In another embodiment, the expression vector comprises the sequence pair (a1-b1) separated by a spacer sequence, wherein

a1 is SEQ ID NO: 2 and b1 is SEQ ID NO: 6 or a1 is SEQ ID NO: 6 and b1 is SEQ ID NO: 2.

In a further embodiment, the expression vector comprises a polynucleotide that comprises two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, wherein

If a1 is SEQ ID NO: 3 or SEQ ID NO: 7, b1 is SEQ ID NO: 7 or SEQ ID NO: 3, and a2 is SEQ ID NO: 4 or SEQ ID NO: 8, b2 is SEQ ID NO: 8 or SEQ ID NO: 4; or

If a1 is SEQ ID NO: 4 or SEQ ID NO: 8, b1 is SEQ ID NO: 8 or SEQ ID NO: 4 and a2 is SEQ ID NO: 3 or SEQ ID NO: 7 and b2 is SEQ ID NO: 7 or SEQ ID NO: 3.

In a sixth aspect, the invention relates to an isolated cell transfected with at least one expression vector as claimed in any one of claims 10 to 13.

In a seventh aspect, the invention relates to an isolated cell transfected with two expression vectors.

In one embodiment, the first expression vector comprises a polynucleotide that comprises the sequence pair (a1-b1) separated by a spacer sequence, wherein

a1 is SEQ ID NO: 1 and b1 is SEQ ID NO: 5 or a1 is SEQ ID NO: 5 and b1 is SEQ ID NO: 1.;

and the second expression vector comprises the sequence pair (a1-b1) separated by a spacer sequence, wherein

a1 is SEQ ID NO: 2 and b1 is SEQ ID NO: 6 or a1 is SEQ ID NO: 6 and b1 is SEQ ID NO: 2.

In another embodiment, the first expression vector comprises a polynucleotide that comprises the sequence pair (a1-b1) separated by a spacer sequence, wherein

a1 is SEQ ID NO: 1 and b1 is SEQ ID NO: 5 or a1 is SEQ ID NO: 5 and b1 is SEQ ID NO: 1,

and the second expression vector comprises a polynucleotide that comprises two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, wherein

If a1 is SEQ ID NO: 3 or SEQ ID NO: 7, b1 is SEQ ID NO: 7 or SEQ ID NO: 3, and a2 is SEQ ID NO: 4 or SEQ ID NO: 8, b2 is SEQ ID NO: 8 or SEQ ID NO: 4; or

If a1 is SEQ ID NO: 4 or SEQ ID NO: 8, b1 is SEQ ID NO: 8 or SEQ ID NO: 4 and a2 is SEQ ID NO: 3 or SEQ ID NO: 7 and b2 is SEQ ID NO: 7 or SEQ ID NO: 3.

In a further embodiment the isolated cell is transfected with two expression vectors,

- wherein the first expression vector comprises two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, in which the sequences a1,

a2, b1 and b2 differ among themselves and are selected from SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13 and SEQ ID NO: 14, in the following form: wherein,

if a1 is SEQ ID NO: 11 or SEQ ID NO: 12, b1 is SEQ ID NO: 12 or SEQ ID NO: 11, and a2 is SEQ ID NO: 13 or SEQ ID NO: 14, b2 is SEQ ID NO: 14 or SEQ ID NO: 13; or

a1 is SEQ ID NO: 13 or SEQ ID NO: 14, b1 is SEQ ID NO: 14 or SEQ ID NO: 13 and a2 is SEQ ID NO: 11 or SEQ ID NO: 12 and b2 is SEQ ID NO: 12 or SEQ ID NO: 11; and

- wherein the second expression vector comprises the sequence pair (a1-b1) separated by a spacer sequence, wherein
- a1 is SEQ ID NO: 15 and b1 is SEQ ID NO: 16 or a1 is SEQ ID NO: 16 and b1 is SEQ ID NO: 15.

In an eighth aspect, the invention relates to a genetically modified plant or plant cell wherein said plant comprises a transfected cell of the present invention.

In one embodiment there is provided a genetically modified plant or plant cell wherein the polynucleotide is integrated in a stable form.

In a further aspect, the invention relates to a genetically modified plant or plant cell with decreased gliadin and/or glutenin content comprising, in its genome, a polynucleotide of the present invention, wherein said plant exhibits reduced gliadin and/or glutenin content compared to a control plant.

In one embodiment, the plant belongs to the genus *Triticum.* In a further embodiment, the plant is selected from the species *Triticum aestivum* or *Triticum turgidum*. In a yet further embodiment, the plant is a Bobwhite cultivar or a Don Pedro cultivar.

In a further aspect, the invention relates to a seed derived from the plant of the present invention wherein said seed comprises the polynucleotide of the present invention.

In another aspect, the invention relates to the pollen, propagule, progeny, or part of the plant derived from any of the plants of the present invention wherein said pollen, propagule, progeny, or part comprise the polynucleotide of the present invention.

In a further aspect, the invention relates to the use of the polynucleotide of the present invention, a vector of the present invention or a cell of the present invention for silencing at least one of alpha-, gamma- and omega-gliadins or the LMW glutenin of *Triticum* spp.

In a further aspect, the invention relates to a method for silencing at least one of alpha-, gamma- and omega-gliadins or the LMW glutenin of *Triticum* spp using the polynucleotide of the present invention, a vector as claimed of the present invention or a cell of the present invention.

In a further aspect, the invention relates to the use of the seed of the present invention for the preparation of flour, a food composition, a vitamin or nutritional supplement.

In another aspect, the invention relates to food composition prepared from a seed of the present invention.

In yet a further aspect, the invention relates to method for obtaining the genetically modified plant of the present invention, comprising the following:

a. selecting a part of the plant,

b. transfecting the cells of the part of the plant of paragraph (a) with a vector of the present invention,

c. selecting the transfected cell of paragraph (b) that comprises a polynucleotide of the present invention,

d. regenerating at least one plant derived from the cell selected in paragraph (c),

e. selecting one or more plants regenerated according to paragraph (d) in which the polynucleotide is transcribed into an hpRNA, and

f. selecting one or more plants obtained according to paragraph (e) that show silencing of at least one of the following: alpha-, gamma-, omega-gliadins and LMW glutenin in its seeds.

In a further aspect, the invention relates to method for producing a food composition with reduced gliadin and/or glutenin content comprising producing a transgenic wheat plant with reduced gliadin and/or glutenin content comprising integrating a polynucleotide of the present invention into the genome of a plant and silencing at least

one of alpha-, gamma- and omega-gliadins and/or LMW glutenin in the seeds of said plant, producing seeds from said plant in which at least one of alpha-, gamma- and omega-gliadins and LMW glutenins are silenced and preparing a food composition from said seeds.

In another aspect, the invention relates to method for modulating an immune response to gliadins and/or glutenins, the method comprising providing a diet of a food composition of the present invention to a subject in need thereof.

In a final aspect, the invention relates to method for affecting or modulating a T-cell response to gluten in a subject, the method comprising providing a diet of a food composition of the present invention to a subject in need thereof.

DESCRIPTION OF THE FIGURES

The invention is further described in the following non-limiting figures:

Figure 1 shows the structure of α-, γ- and ω- gliadins and LMW glutenins and the gene regions where the RNAi sequences were designed.

Figure 1 (A) Structure of α-, γ-, and ω-gliadins, and LMW glutenins as reviewed in (Qi *et al.*, 2006), indicating the domains for each family where sequences for RNAi fragments were designated. (B) Sequences were or were not combined (same colour) into a unique RNAi fragment and inserted downstream a D-hordein promoter providing five different hairpin plasmids. (C) Plasmids were used alone or in combination into seven combinations where number 0 corresponds to BW208 wild type (wt).

Figure 2 shows the transgenic lines and effects of each plasmid combinations on the gliadin and glutenin fractions.

Box plots of gliadin (A) and glutenin (B) distribution in the grain of transgenic lines with seven plasmid combinations. Plasmid combinations are as indicated in Fig. 1C. Plasmid Combination 0 corresponds to BW208 wild type. Protein fractions are expressed as µg protein/mg flour. HMW and LMW denote the high molecular weight and low molecular weight subunits of glutenins, respectively.

Figure 3 shows the fold change in kernel composition of transgenic lines with the different plasmid conentrations.

Fold change in kernel composition and agronomic traits with the seven plasmid combinations as described in Fig. 1C. The ratio transgenic/wild type (obtained by dividing the value of the transgenic line by the value of the wild-type line) is presented for each parameter. Values above 1 represent higher values in the transgenic than the control, whereas values below 1 represent the opposite. Asterisks over a bar indicate that the transgenic line presents a significantly different value (p,0.05) with respect to its corresponding wild type as determined by the LSD all-pairwise comparisons test. Values for individual lines and averages for different plasmid combinations are shown, respectively, in Figures 8 and 9. Significant differences were identified at the 5% (*) and 1% (**) probability levels by the two-sided Dunnett's multiple comparisons with control (plasmid combination 0). PC, Plasmid Combination.

Figure 4 shows levels of reactive gluten identified by G12 moAb in each transgenic line.

Box plot of gluten content with seven plasmid combinations (see Fig. 1C) calculated by G12 competitive ELISA and expressed in parts per million (ppm).

Figure 5 shows the plasmid combinations and patterns of splicing.

Plasmid combinations and patterns of silencing. Left panels; A-PAGE and RP-HPLC chromatograms of gliadin extracts from wild type BW208 (*, and dashed line) and one transgenic line from each of the plasmid combinations; α-, γ-, and ω-gliadins are indicated. Right panels; SDS-PAGE and RP-HPLC chromatograms of glutenin extracts from wild type BW208 (*, and dashed line) and one transgenic line from each of the plasmid combinations; high molecular weight (HMW) and low molecular weight (LMW) glutenins are indicated. Plasmid combinations are as indicated in Fig. 1C.

Figure 6 shows the sequence of the inverted repeat (IR) regions.

Figure 7 shows the percentage identity among all inverted repeat (IR) sequences.

Figure 8 shows the transgenic lines of the invention.

Figure 9 shows the protein fractions and quality agronomic parameters of each transgenic line.

Figure 10 shows the average values for protein fractions and quality and agronomic parameters for each plasmid combination.

Figure 11 shows the number of CD immunogenic epitopes with one mismatch found in peptides identified by MS in the seven plasmid combinations.

Figure 12 is a table of the fragments and proteins identified by LC-MS/MS analysis in all seven plasmid combinations.

Figure 13 is a table of the number of CD immunogenic epitopes with perfect match and found in peptides identified by MS.

DETAILED DESCRIPTION OF THE INVENTION

The present invention will now be further described. In the following passages different aspects of the invention are defined in more detail. Each aspect so defined may be combined with any other aspect or aspects unless clearly indicated to the contrary. In particular, any feature indicated as being preferred or advantageous may be combined with any other feature or features indicated as being preferred or advantageous.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of botany, microbiology, tissue culture, molecular biology, chemistry, biochemistry and recombinant DNA technology, bioinformatics which are within the skill of the art. Such techniques are explained fully in the literature.

As used herein, the words "nucleic acid", "nucleic acid sequence", "nucleotide", "nucleic acid molecule" or "polynucleotide" are intended to include DNA molecules (e.g., cDNA or genomic DNA), RNA molecules (e.g., mRNA, siRNA, sRNA, dsRNA, miRNA), natural occurring, mutated, synthetic DNA or RNA molecules, and analogs of the DNA or RNA generated using nucleotide analogs. It can be single-stranded or double-stranded. Such nucleic acids or polynucleotides include, but are not limited to, coding sequences of structural genes, anti-sense sequences, and non-coding

regulatory sequences that do not encode mRNAs or protein products. These terms also encompass a gene. The term "gene" or “gene sequence“ is used broadly to refer to a DNA nucleic acid associated with a biological function. Thus, genes may include introns and exons as in the genomic sequence, or may comprise only a coding sequence as in cDNAs, and/or may include cDNAs, CDS or genomic DNA in combination with regulatory sequences.

For the purposes of the invention, "transgenic", “transgene” or "recombinant" means with regard to, for example, a nucleic acid sequence, specifically an RNA nucleic acid sequence or molecule, an expression cassette, gene construct, or a vector comprising the nucleic acid sequence or an organism transformed with the nucleic acid sequences, expression cassettes or vectors according to the invention, all those constructions brought about by recombinant methods in which either

(a) the nucleic acid sequences encoding proteins useful in the methods of the invention, or

(b) genetic control sequence(s) which is operably linked with the nucleic acid sequence according to the invention, for example a promoter, or

(c) a) and b)

are not located in their natural genetic environment or have been modified by recombinant methods, it being possible for the modification to take the form of, for example, a substitution, addition, deletion, inversion or insertion of one or more nucleotide residues. The natural genetic environment is understood as meaning the natural genomic or chromosomal locus in the original plant or the presence in a genomic library. In the case of a genomic library, the natural genetic environment of the nucleic acid sequence is preferably retained, at least in part. The environment flanks the nucleic acid sequence at least on one side and has a sequence length of at least 50 bp, preferably at least 500 bp, especially preferably at least 1000 bp, most preferably at least 5000 bp. A naturally occurring expression cassette – for example the naturally occurring combination of the natural promoter of the nucleic acid sequences with the corresponding nucleic acid sequence encoding a polypeptide useful in the methods of the present invention, as defined above – becomes a transgenic expression cassette when this expression cassette is modified by non-natural, synthetic ("artificial") methods such as, for example, mutagenic treatment. Suitable methods are described, for example, in US 5,565,350 or WO 00/15815 both incorporated by reference.

A transgenic plant for the purposes of the invention is thus understood as meaning, as above, that the nucleic acids used in the method of the invention are not at their natural locus in the genome of said plant, it being possible for the nucleic acids to be expressed homologously or heterologously. For the purposes of the invention, "transgenic", preferably means that the nucleic acids is expressed homologously. However, as mentioned, transgenic also means that, while the nucleic acids according to the different embodiments of the invention are at their natural position in the genome of a plant, the sequence has been modified with regard to the natural sequence, and/or that the regulatory sequences of the natural sequences have been modified. Transgenic is preferably understood as meaning the expression of the nucleic acids according to the invention at an unnatural locus in the genome, i.e. homologous or, preferably, heterologous expression of the nucleic acids takes place. According to the invention, the transgene is integrated into the plant in a stable manner and the plant is preferably homozygous for the transgene. As used herein, a "stably" transformed plant is a plant in which the exogenous DNA or RNA is heritable. The exogenous DNA or RNA may be heritable as a fragment of DNA maintained in the plant cell and not inserted into the host genome. Preferably, the stably transformed plant comprises the exogenous DNA or RNA. As used herein "progeny" means any subsequent generation, including the seeds and plants therefrom, which is derived from a particular parental plant or set of parental plants; the resultant progeny may be hybridized or substantially homozygous, depending upon pedigree. Progeny of a transgenic plant of this present invention can be, for example, self-pollinated, crossed to another transgenic plant, crossed to a non-transgenic plant, and/or back crossed to an ancestor.

As used herein “siNAs” of the invention refers to a double stranded oligonucleotide capable of mediating target mRNA cleavage via RNA interference.

The aspects of the invention involve recombination DNA technology and in a preferred embodiment exclude embodiments that are solely based on generating plants by traditional breeding methods.

In a first aspect the present invention provides a polynucleotide or variant thereof comprising at least one sequence pair (a1-b1) separated by a spacer sequence in which a1 and b1 are selected from the group consisting of SEQ ID NO: 1, SEQ ID NO:

2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 and SEQ ID NO: 8, wherein

- if a1 is SEQ ID NO: 1, b1 is SEQ ID NO: 5; if a1 is SEQ ID NO: 5, b1 is SEQ ID NO: 1
- if a1 is SEQ ID NO: 2, b1 is SEQ ID NO: 6; if a1 is SEQ ID NO: 6, b1 is SEQ ID NO: 2:
- if a1 is SEQ ID NO: 3, b1 is SEQ ID NO: 7; if a1 is SEQ ID NO: 7, b1 is SEQ ID NO: 3; and
- if a1 is SEQ ID NO: 4, b1 is SEQ ID NO: 8. If a1 is SEQ ID NO: 8, b1 is SEQ ID NO: 4.

In a further embodiment, there is provided a polynucleotide sequence of claim 1, wherein the sequence comprises two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, in which the sequences a1, a2, b1 and b2 differ among themselves and are selected from SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 7 and SEQ ID NO: 8, in the following form:

- If a1 is SEQ ID NO: 3 or SEQ ID NO: 7, b1 is SEQ ID NO: 7 or SEQ ID NO: 3, and a2 is SEQ ID NO: 4 or SEQ ID NO: 8, b2 is SEQ ID NO: 8 or SEQ ID NO: 4; and
- If a1 is SEQ ID NO: 4 or SEQ ID NO: 8, b1 is SEQ ID NO: 8 or SEQ ID NO: 4 and a2 is SEQ ID NO: 3 or SEQ ID NO: 7 and b2 is SEQ ID NO: 7 or SEQ ID NO: 3.

In one embodiment, the term ‘variant’ refers to a nucleotide sequence where the nucleotides are substantially identical to one of the above sequences. The variant may be achieved by modifications such as insertion, substitution or deletion of one or more nucleotides. In a preferred embodiment, the variant has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity to any one of the above sequences, SEQ ID NOs 1 to 8. In one embodiment, sequence identity is 100%.

Accordingly, in one embodiment, there is provided a polynucleotide that is at least 90% or 95% identical to a sequence comprising at least one sequence pair (a1-b1) separated by a spacer sequence in which a1 and b1 are selected from the group

consisting of SEQ ID NO: 1, SEQ ID NO:2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 and SEQ ID NO: 8, wherein

- if a1 is SEQ ID NO: 1, b1 is SEQ ID NO: 5; if a1 is SEQ ID NO: 5, b1 is SEQ ID NO: 1
- if a1 is SEQ ID NO: 2, b1 is SEQ ID NO: 6; if a1 is SEQ ID NO: 6, b1 is SEQ ID NO: 2:
- if a1 is SEQ ID NO: 3, b1 is SEQ ID NO: 7; if a1 is SEQ ID NO: 7, b1 is SEQ ID NO: 3; and
- if a1 is SEQ ID NO: 4, b1 is SEQ ID NO: 8. If a1 is SEQ ID NO: 8, b1 is SEQ ID NO: 4.

In a further embodiment, there is provided a polynucleotide that is at least 90 or 95% identical to a sequence comprising two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, in which the sequences a1, a2, b1 and b2 differ among themselves and are selected from SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 7 and SEQ ID NO: 8, in the following form:

- If a1 is SEQ ID NO: 3 or SEQ ID NO: 7, b1 is SEQ ID NO: 7 or SEQ ID NO: 3, and a2 is SEQ ID NO: 4 or SEQ ID NO: 8, b2 is SEQ ID NO: 8 or SEQ ID NO: 4; and
- If a1 is SEQ ID NO: 4 or SEQ ID NO: 8, b1 is SEQ ID NO: 8 or SEQ ID NO: 4 and a2 is SEQ ID NO: 3 or SEQ ID NO: 7 and b2 is SEQ ID NO: 7 or SEQ ID NO: 3.

Thus, the present disclosure concerns a polynucleotide comprising at least one or two sequence pairs, with each subsequence combining in a particular order to give rise to a sequence whose transcription into RNA is capable of generating hpRNA (hairpin RNA), e.g., RNA in the shape of a hairpin. This double-stranded RNA will be processed by endoribonucleases described in the prior art, which is used to generate the siRNAs that cause post-transcriptional silencing of all of the mRNAs (messenger RNA) that code for all types of wheat gliadins and glutenins.

By means of this polynucleotide, whose expression is specifically directed in particular to tissues of wheat seeds through gene expression-regulating sequences such as, for example, the promoter of the gene that codes for a D-hordein, one achieves post-transcriptional silencing of all of the genes of the species soft wheat and hard wheat in

an effective and synergistic manner, as one is able to silence a greater number of gliadin and glutenin genes. This is essentially due to the specific design of the sense and antisense subsequences whose generated siRNA hybridizes with all of the mRNA of at least one of α, β, γ, and ω gliadins and LMW glutenin of wheat in combination with promoters having higher levels of expression that can be induced in specific tissues of the wheat seed. For RNAi design, gliadin and LMW-glutenin sequences available at NCBI (National Center for Biotechnology Information) were aligned separately. For each group/type of proteins, conserved regions among all sequences by 80% or more were identified, regardless of gaps in the alignment. These regions were used for RNAi.

In the present invention, the terms DNA and RNA are used to refer to deoxyribonucleic acid and ribonucleic acid respectively.

The sequences a1-b1 or a1-a2 and b2-b1 (referred to in the following as the sequence pairs of the invention) are linked to form a linear and continuous nucleotide sequence in which the one or two pairs are linked by means of a spacer sequence at least one nucleotide in length. Preferably, the spacer sequence is a non-coding sequence that is eliminated after the process of forming dsRNA. The spacer sequence may be part of a sequence of an intron of a gene. The function of the spacer sequence is to act as a hinge for the sequence pairs described so that pairing or hybridization of the RNA sequences coding for the polynucleotide may take place.

SEQ ID NO: 1 is the sense sequence that comprises a fragment that covers part of the repetitive domain of α-gliadins plus the poly-Q domain and a small region of the non-repetitive domain I. SEQ ID NO: 5 is the antisense sequence of SEQ ID NO: 1.

SEQ ID NO: 2 is the sense sequence that comprises a fragment of the 3' end of the repetitive domain of ω-gliadins. SEQ ID NO: 6 is the antisense sequence of SEQ ID NO: 2.

SEQ ID NO: 3 is the sense sequence that also comprises a fragment of the 3' end of the repetitive domain of ω-gliadins. SEQ ID NO: 7 is the antisense sequence of SEQ ID NO: 3.

SEQ ID NO: 4 is the sense sequence that comprises a part of the signal peptide, the N terminal domain and part of the repetitive domain of LMW glutenins. SEQ ID NO: 8 is the antisense sequence of SEQ ID NO: 4.

The polynucleotides of the invention give rise to RNA in which the at least one, or two sequence pairs hybridize with each other, forming a hairpin. Therefore, according to these first two aspects of the present invention, the combinations of sequences by means of which RNA hairpins can be obtained are shown in Table 1, Table 2 and Table 3:

Table 1: Combinations of sequences in which a1 and b1 are sense or antisense sequences.

Combinations	a1	b1
1	SEQ ID NO: 1	SEQ ID NO:5
2	SEQ ID NO: 2	SEQ ID NO: 6
3	SEQ ID NO: 3	SEQ ID NO: 7
4	SEQ ID NO: 4	SEQ ID NO: 8
5	SEQ ID NO: 5	SEQ ID NO: 1
6	SEQ ID NO: 6	SEQ ID NO: 2
7	SEQ ID NO: 7	SEQ ID NO: 3
8	SEQ ID NO: 8	SEQ ID NO: 5

Table 2: Combinations of sequences in which a1-a2 and b2-b1 are sense and antisense sequences

Combinations	a1	a2	b2	b1
1	SEQ ID NO: 3	SEQ ID NO: 4	SEQ ID NO: 8	SEQ ID NO: 7
2	SEQ ID NO: 4	SEQ ID NO: 3	SEQ ID NO: 7	SEQ ID NO: 8
3	SEQ ID NO: 7	SEQ ID NO:8	SEQ ID NO: 4	SEQ ID NO: 3
4	SEQ ID NO: 8	SEQ ID NO: 7	SEQ ID NO: 3	SEQ ID NO: 4

Table 3: Combinations of sequences in which a1-a2 and b2-b1 are sense and antisense sequences

Combinations	a1	a2	b2	b1

1	SEQ ID NO: 3	SEQ ID NO: 8	SEQ ID NO: 4	SEQ ID NO: 7
2	SEQ ID NO: 7	SEQ ID NO: 4	SEQ ID NO: 8	SEQ ID NO: 3
3	SEQ ID NO: 4	SEQ ID NO: 7	SEQ ID NO: 3	SEQ ID NO: 8
4	SEQ ID NO: 8	SEQ ID NO: 3	SEQ ID NO: 7	SEQ ID NO: 4

In addition to the above, there is further provided the following combinations:

In one embodiment, there is provided a polynucleotide that is at least 90 or 95% identical to a sequence comprising two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, in which the sequences a1, a2, b1 and b2 differ among themselves and are selected from SEQ ID NOs: 1, 5, 4 and 8, in the following form:

If a1 is SEQ ID NO: 1 or SEQ ID NO: 5, b1 is SEQ ID NO: 5 or SEQ ID NO: 1, and a2 is SEQ ID NO: 4 or SEQ ID NO: 8, b2 is SEQ ID NO: 8 or SEQ ID NO: 4; and

If a1 is SEQ ID NO: 4 or SEQ ID NO: 8, b1 is SEQ ID NO: 8 or SEQ ID NO: 4 and a2 is SEQ ID NO: 1 or SEQ ID NO: 5 and b2 is SEQ ID NO: 5 or SEQ ID NO: 1.

In a further embodiment, there is provided a polynucleotide that is at least 90 or 95% identical to a sequence comprising two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, in which the sequences a1, a2, b1 and b2 differ among themselves and are selected from SEQ ID NOs: 1, 5, 3 and 7, in the following form:

If a1 is SEQ ID NO: 1 or SEQ ID NO: 5, b1 is SEQ ID NO: 5 or SEQ ID NO: 1 and a2 is SEQ ID NO: 3 or SEQ ID NO: 7, b2 is SEQ ID NO: 7 or SEQ ID NO: 3; and

If a1 is SEQ ID NO: 3 or SEQ ID NO: 7, b1 is SEQ ID NO: 7 or SEQ ID NO: 3 and a2 is SEQ ID NO: 1 or SEQ ID NO: 5 and b2 is SEQ ID NO: 5 or SEQ ID NO: 1.

In a further embodiment, there is provided a polynucleotide that is at least 90 or 95% identical to a sequence comprising two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, in which the sequences a1, a2, b1 and b2 differ among themselves and are selected from SEQ ID NOs: 1, 5, 2 and 6, in the following form:

If a1 is SEQ ID NO: 1 or SEQ ID NO: 5, b1 is SEQ ID NO: 5 or SEQ ID NO: 1 and a2 is SEQ ID NO: 2 or SEQ ID NO: 6, b2 is SEQ ID NO: 6 or SEQ ID NO: 2; and

If a1 is SEQ ID NO: 2 or SEQ ID NO: 6, b1 is SEQ ID NO: 6 or SEQ ID NO: 2 and a2 is SEQ ID NO: 1 or SEQ ID NO: 5 and b2 is SEQ ID NO: 5 or SEQ ID NO: 1.

In a further embodiment, there is provided a polynucleotide that is at least 90 or 95% identical to a sequence comprising two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, in which the sequences a1, a2, b1 and b2 differ among themselves and are selected from SEQ ID NOs: 4, 8, 2 and 6 in the following form:

If a1 is SEQ ID NO: 4 or SEQ ID NO: 8, b1 is SEQ ID NO: 8 or SEQ ID NO: 4 and a2 is SEQ ID NO: 2 or SEQ ID NO: 6, b2 is SEQ ID NO: 6 or SEQ ID NO: 2; and

If a1 is SEQ ID NO: 2 or SEQ ID NO: 6, b1 is SEQ ID NO: 6 or SEQ ID NO: 2 and a2 is SEQ ID NO: 4 or SEQ ID NO: 8 and b2 is SEQ ID NO: 8 or SEQ ID NO: 4.

In a further embodiment, there is provided a polynucleotide that is at least 90 or 95% identical to a sequence comprising two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, in which the sequences a1, a2, b1 and b2 differ among themselves and are selected from SEQ ID NOs: 2, 6, 3 and 7 in the following form:

If a1 is SEQ ID NO: 2 or SEQ ID NO: 6, b1 is SEQ ID NO: 6 or SEQ ID NO: 2 and a2 is SEQ ID NO: 3 or SEQ ID NO: 7, b2 is SEQ ID NO: 7 or SEQ ID NO: 3; and

If a1 is SEQ ID NO: 3 or SEQ ID NO: 7, b1 is SEQ ID NO: 7 or SEQ ID NO: 3 and a2 is SEQ ID NO: 2 or SEQ ID NO: 6 and b2 is SEQ ID NO: 6 or SEQ ID NO: 2.

In a further embodiment, there is provided a polynucleotide that is at least 90 or 95% identical to a sequence comprising two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, in which the sequences a1, a2, b1 and b2 differ among themselves and are selected from SEQ ID NOs: 1, 5, 15 and 16 in the following form:

If a1 is SEQ ID NO: 1 or SEQ ID NO: 5, b1 is SEQ ID NO: 5 or SEQ ID NO: 1 and a2 is SEQ ID NO: 15 or SEQ ID NO: 16, b2 is SEQ ID NO: 16 or SEQ ID NO: 15; and

If a1 is SEQ ID NO: 15 or SEQ ID NO: 16, b1 is SEQ ID NO: 16 or SEQ ID NO: 15 and a2 is SEQ ID NO: 1 or SEQ ID NO: 5 and b2 is SEQ ID NO: 5 or SEQ ID NO: 1.

In a further embodiment, there is provided a polynucleotide that is at least 90 or 95% identical to a sequence comprising two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, in which the sequences a1, a2, b1 and b2 differ among themselves and are selected from SEQ ID NOs: 1, 5, 11 and 12 in the following form:

If a1 is SEQ ID NO: 1 or SEQ ID NO: 5, b1 is SEQ ID NO: 5 or SEQ ID NO: 1 and a2 is SEQ ID NO: 11 or SEQ ID NO: 12, b2 is SEQ ID NO: 12 or SEQ ID NO: 11; and

If a1 is SEQ ID NO: 11 or SEQ ID NO: 12, b1 is SEQ ID NO: 12 or SEQ ID NO: 11 and a2 is SEQ ID NO: 1 or SEQ ID NO: 5 and b2 is SEQ ID NO: 5 or SEQ ID NO: 1.

In a further embodiment, there is provided a polynucleotide that is at least 90 or 95% identical to a sequence comprising two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, in which the sequences a1, a2, b1 and b2 differ among themselves and are selected from SEQ ID NOs: 1, 5, 13 and 14 in the following form:

If a1 is SEQ ID NO: 1 or SEQ ID NO: 5, b1 is SEQ ID NO: 5 or SEQ ID NO: 1 and a2 is SEQ ID NO: 13 or SEQ ID NO: 14, b2 is SEQ ID NO: 14 or SEQ ID NO: 13; and

If a1 is SEQ ID NO: 13 or SEQ ID NO: 14, b1 is SEQ ID NO: 14 or SEQ ID NO: 13 and a2 is SEQ ID NO: 1 or SEQ ID NO: 5 and b2 is SEQ ID NO: 5 or SEQ ID NO: 1.

In a further embodiment, there is provided a polynucleotide that is at least 90 or 95% identical to a sequence comprising two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, in which the sequences a1, a2, b1 and b2 differ among themselves and are selected from SEQ ID NOs: 4, 8, 15 and 16 in the following form:

If a1 is SEQ ID NO: 4 or SEQ ID NO: 8, b1 is SEQ ID NO: 8 or SEQ ID NO: 4 and a2 is SEQ ID NO: 15 or SEQ ID NO: 16, b2 is SEQ ID NO: 16 or SEQ ID NO: 15; and

If a1 is SEQ ID NO: 15 or SEQ ID NO: 16, b1 is SEQ ID NO:16 or SEQ ID NO: 15 and a2 is SEQ ID NO: 4 or SEQ ID NO: 8 and b2 is SEQ ID NO: 8 or SEQ ID NO: 4.

In a further embodiment, there is provided a polynucleotide that is at least 90 or 95% identical to a sequence comprising two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, in which the sequences a1, a2, b1 and b2 differ among themselves and are selected from SEQ ID NOs: 4, 8, 11 and 12 in the following form

If a1 is SEQ ID NO: 4 or SEQ ID NO: 8, b1 is SEQ ID NO: 8 or SEQ ID NO: 4 and a2 is SEQ ID NO: 11 or SEQ ID NO: 12, b2 is SEQ ID NO: 12 or SEQ ID NO: 11; and

If a1 is SEQ ID NO: 11 or SEQ ID NO: 12, b1 is SEQ ID NO:12 or SEQ ID NO: 11 and a2 is SEQ ID NO: 4 or SEQ ID NO: 8 and b2 is SEQ ID NO: 8 or SEQ ID NO: 4.

In a further embodiment, there is provided a polynucleotide that is at least 90 or 95% identical to a sequence comprising two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, in which the sequences a1, a2, b1 and b2 differ among themselves and are selected from SEQ ID NOs: 4, 8, 13 and 14 in the following form:

If a1 is SEQ ID NO: 4 or SEQ ID NO: 8, b1 is SEQ ID NO: 8 or SEQ ID NO: 4 and a2 is SEQ ID NO: 13 or SEQ ID NO: 14, b2 is SEQ ID NO: 14 or SEQ ID NO: 13; and

If a1 is SEQ ID NO: 13 or SEQ ID NO: 14, b1 is SEQ ID NO:14 or SEQ ID NO: 13 and a2 is SEQ ID NO: 4 or SEQ ID NO: 8 and b2 is SEQ ID NO: 8 or SEQ ID NO: 4.

In a further embodiment, there is provided a polynucleotide that is at least 90 or 95% identical to a sequence comprising two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, in which the sequences a1, a2, b1 and b2 differ among themselves and are selected from SEQ ID NOs: 3, 7, 15 and 16 in the following form:

If a1 is SEQ ID NO: 3 or SEQ ID NO: 7, b1 is SEQ ID NO: 7 or SEQ ID NO: 3 and a2 is SEQ ID NO: 15 or SEQ ID NO: 16, b2 is SEQ ID NO: 16 or SEQ ID NO: 15; and

If a1 is SEQ ID NO: 15 or SEQ ID NO: 16, b1 is SEQ ID NO: 16 or SEQ ID NO: 15 and a2 is SEQ ID NO: 3 or SEQ ID NO: 7 and b2 is SEQ ID NO: 7 or SEQ ID NO: 3.

In a further embodiment, there is provided a polynucleotide that is at least 90 or 95% identical to a sequence comprising two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, in which the sequences a1, a2, b1 and b2 differ among themselves and are selected from SEQ ID NOs: 3, 7, 11 and 12 in the following form:

If a1 is SEQ ID NO: 3 or SEQ ID NO: 7, b1 is SEQ ID NO: 7 or SEQ ID NO: 3 and a2 is SEQ ID NO: 11 or SEQ ID NO: 12, b2 is SEQ ID NO: 12 or SEQ ID NO: 11; and

If a1 is SEQ ID NO: 11 or SEQ ID NO: 12, b1 is SEQ ID NO: 12 or SEQ ID NO: 11 and a2 is SEQ ID NO: 3 or SEQ ID NO: 7 and b2 is SEQ ID NO: 7 or SEQ ID NO: 3.

In a further embodiment, there is provided a polynucleotide that is at least 90 or 95% identical to a sequence comprising two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, in which the sequences a1, a2, b1 and b2 differ among themselves and are selected from SEQ ID NOs: 3, 7, 13 and 14 in the following form:

If a1 is SEQ ID NO: 3 or SEQ ID NO: 7, b1 is SEQ ID NO: 7 or SEQ ID NO: 3 and a2 is SEQ ID NO: 13 or SEQ ID NO: 14, b2 is SEQ ID NO: 14 or SEQ ID NO: 13; and

If a1 is SEQ ID NO: 13 or SEQ ID NO: 14, b1 is SEQ ID NO: 14 or SEQ ID NO: 13 and a2 is SEQ ID NO: 3 or SEQ ID NO: 7 and b2 is SEQ ID NO: 7 or SEQ ID NO: 3.

In a further embodiment, there is provided a polynucleotide that is at least 90 or 95% identical to a sequence comprising two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, in which the sequences a1, a2, b1 and b2 differ among themselves and are selected from SEQ ID NOs: 2, 6, 15 and 16 in the following form:

If a1 is SEQ ID NO: 2 or SEQ ID NO: 6, b1 is SEQ ID NO: 6 or SEQ ID NO: 2 and a2 is SEQ ID NO: 15 or SEQ ID NO: 16, b2 is SEQ ID NO: 16 or SEQ ID NO: 15; and

If a1 is SEQ ID NO: 15 or SEQ ID NO: 16, b1 is SEQ ID NO: 16 or SEQ ID NO: 15 and a2 is SEQ ID NO: 2 or SEQ ID NO: 6 and b2 is SEQ ID NO: 6 or SEQ ID NO: 2.

In a further embodiment, there is provided a polynucleotide that is at least 90 or 95% identical to a sequence comprising two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, in which the sequences a1, a2, b1 and b2 differ among themselves and are selected from SEQ ID NOs: 2, 6, 11 and 12 in the following form:

If a1 is SEQ ID NO: 2 or SEQ ID NO: 6, b1 is SEQ ID NO: 6 or SEQ ID NO: 2 and a2 is SEQ ID NO: 11 or SEQ ID NO: 12, b2 is SEQ ID NO: 12 or SEQ ID NO: 11; and

If a1 is SEQ ID NO: 11 or SEQ ID NO: 12, b1 is SEQ ID NO: 12 or SEQ ID NO: 11 and a2 is SEQ ID NO: 2 or SEQ ID NO: 6 and b2 is SEQ ID NO: 6 or SEQ ID NO: 2.

In a further embodiment, there is provided a polynucleotide that is at least 90 or 95% identical to a sequence comprising two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, in which the sequences a1, a2, b1 and b2 differ among themselves and are selected from SEQ ID NOs: 2, 6, 13 and 14 in the following form:

If a1 is SEQ ID NO: 2 or SEQ ID NO: 6, b1 is SEQ ID NO: 6 or SEQ ID NO: 2 and a2 is SEQ ID NO: 13 or SEQ ID NO: 14, b2 is SEQ ID NO: 14 or SEQ ID NO: 13; and

If a1 is SEQ ID NO: 13 or SEQ ID NO: 14, b1 is SEQ ID NO: 14 or SEQ ID NO: 13 and a2 is SEQ ID NO: 2 or SEQ ID NO: 6 and b2 is SEQ ID NO: 6 or SEQ ID NO: 2.

In a further embodiment, there is provided a polynucleotide that is at least 90 or 95% identical to a sequence comprising two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, in which the sequences a1, a2, b1 and b2 differ among themselves and are selected from SEQ ID NOs: 15, 16, 11 and 12 in the following form:

If a1 is SEQ ID NO: 15 or SEQ ID NO: 16, b1 is SEQ ID NO: 16 or SEQ ID NO: 15 and a2 is SEQ ID NO: 11 or SEQ ID NO: 12, b2 is SEQ ID NO: 12 or SEQ ID NO: 11; and

If a1 is SEQ ID NO: 11 or SEQ ID NO: 12, b1 is SEQ ID NO: 12 or SEQ ID NO: 11 and a2 is SEQ ID NO: 15 or SEQ ID NO: 16 and b2 is SEQ ID NO: 16 or SEQ ID NO: 15.

In a further embodiment, there is provided a polynucleotide that is at least 90 or 95% identical to a sequence comprising two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, in which the sequences a1, a2, b1 and b2 differ among themselves and are selected from SEQ ID NOs: 15, 16, 13 and 14 in the following form:

If a1 is SEQ ID NO: 15 or SEQ ID NO: 16, b1 is SEQ ID NO: 16 or SEQ ID NO: 15 and a2 is SEQ ID NO: 13 or SEQ ID NO: 14, b2 is SEQ ID NO: 14 or SEQ ID NO: 13; and

If a1 is SEQ ID NO: 13 or SEQ ID NO: 14, b1 is SEQ ID NO: 14 or SEQ ID NO: 13 and a2 is SEQ ID NO: 15 or SEQ ID NO: 16 and b2 is SEQ ID NO: 16 or SEQ ID NO: 15.

The spacer is a length of nucleotides that separates the sense and antisense sequences of the target. Once transcribed, the spacer holds together the sense and antisense sequences and facilitates the formation of the double stranded RNA by forming the loop. The spacer may be any suitable sequence or length and can be designed using methods in the art. Having said that, the spacer should be very different in sequence from the sense and antisense sequences to prevent its own hybridization with these sequences. On the other hand, spacers that are too short are very inefficient at forming the loop and prevent the folding of sense and antisense sequences to form the double-stranded RNA. In one embodiment, the length of the spacer is up to, and including, 500 nucleotides. In a further preferred embodiment, the length of the spacer is between 500 and 1020 nucleotides. In another preferred embodiment the length of the spacer is 1011 nucleotides.

In a preferred embodiment, the spacer sequence is SEQ ID NO: 9. The sequence SEQ ID NO: 5 is a fragment of an intron of the gene Ubi1 that codes for maize ubiquitin. An intron is a region of DNA that is eliminated from the primary RNA transcript by a

process referred to as splicing, i.e., the intron does not code for any sequence of a protein. Ubiquitin is a protein that has the function of marking other proteins for destruction. Other suitable spacer sequences are known to the skilled person. Preferably, the spacer sequence is a non-coding sequence that is eliminated after the process of forming dsRNA.

Also included are the sequences that are complementary to any of the polynucleotides described herein.

Another preferred embodiment is a polynucleotide that also comprises a gene expression-regulating sequence functionally linked to its 5' end. In the present invention, the term gene expression-regulating sequence or promoter sequence refers to a nucleic acid sequence that affects the functionality of the gene with respect to the beginning of transcription of a DNA sequence or the beginning of translation of an RNA sequence or other un-described sequences. By way of example, the gene expression-regulating sequences covered by the present invention are promoters and other less common sequences such as certain introns. The regulatory sequence binds to the 5' end of the polynucleotide of the present invention in a functional manner, i.e., it is capable of directing the expression of the polynucleotide with an intensity and localization that depend on its own regulatory sequence. Thus, the expression-regulating sequence can be used to direct tissue-specific expression or enhance expression. Alternatively, the expression-regulating sequence can be used to permit inducible expression of the target.

According to a more preferable embodiment, the gene expression-regulating sequence is SEQ ID NO: 10. SEQ ID NO: 10 corresponds to the sequence of a promoter of the D-hordein gene (the second nucleotide of this gene has the accession number AY998009 and belongs to the species *Hordeum chilense*). In an alternative embodiment, the gene expression-regulating sequence is SEQ ID NO: 29 and corresponds to the sequence of a gliadin promoter such as the gamma, alpha or omega-gliadins. Both promoters are expressed in the endosperm of the seeds. In a further alternative embodiment the gene expression-regulating sequence may be any other endosperm-specific promoter known to the skilled person.

In the following, the terms "polynucleotides of the invention" or "polynucleotides of the present invention" will be used to refer to any of the above polynucleotides.

Another aspect of the present invention is an RNA sequence coded for by any of the polynucleotides of the invention and capable of forming an hpRNA in which the sequence coded for a1 hybridizes completely with b1 and the sequence coded for by the pair a1-a2 hybridizes completely with the sequence coded for by the pair b2-b1. Thus, the invention also relates to a hpRNA formed by an RNA sequence coded for by any of the polynucleotides of the invention.

An hpRNA (hairpin RNA) is a hairpin shape formed by hybridization of the transcribed sequences. An hpRNA is a double-stranded RNA (dsRNA) that is cleaved by an endoribonuclease, for example the endoribonuclease Dicer, resulting in fragments of approximately 21-25 nts. These fragments are known as siRNA. As has been described above, the siRNAs cause post-transcriptional silencing of the target nucleotide sequences so that the protein that would result from the expression of mRNA sequences is not obtained.

Another aspect of the present invention is at least one siRNA generated from the sequence of the hpRNA according to the previous aspect. The siRNA can also be referred to as RNAi. The siRNA is a double-stranded RNA of between 21 and 25 nucleotides, but is not limited to this number of nucleotides, and it is generated from the hpRNA sequence of the invention. In the present invention, in defining the approximate number of nucleotides of the siRNA (approximately 21 and 25 nucleotides), it is understood that there is another strand that is complementary to this sequence, i.e., that one can use the terms nucleotides or base pairs (bp) interchangeably.

As has been described, the Dicer enzyme cleaves the dsRNA into double-stranded fragments of approximately 21-25 nucleotides (siRNA), with the 5' end phosphorylated and two unpaired nucleotides protruding at the 3' end. Of the two strands of siRNA, only one, referred to as the guide strand, is incorporated into the enzymatic complex RISC, while the other is degraded. The thermodynamic characteristics of the 5' end of the siRNA determine which of the two strands is incorporated into the RISC complex. The strand that is less stable at the 5' end is normally incorporated as the guide strand.

The guide strand must be complementary to the mRNA that is to be silenced in order for post-transcriptional silencing to occur. Subsequently, the RISC complex binds to the complementary mRNA of the guide strand of the siRNA present in the complex, and cleavage of the mRNA occurs.

Another aspect of the present invention is an expression vector that comprises any of the polynucleotides of the invention. In one embodiment, the expression vector comprises at least one polynucleotide of the present invention. In one embodiment, the expression vector comprises a polynucleotide comprising at least one sequence pair, as defined above. In a second embodiment, the expression vector comprises two sequence pairs, as defined above.

Vector 1

In one embodiment, the expression vector comprises a polynucleotide that comprises the sequence pair (a1-b1) separated by a spacer sequence, wherein

- a1 is SEQ ID NO: 1 and b1 is SEQ ID NO: 5 or a1 is SEQ ID NO: 5 and b1 is SEQ ID NO: 1.

This expression vector is called Vector 1. In a preferred embodiment, the expression vector is pDhp_α/βZR. In this embodiment the gene expression-regulating sequence is SEQ ID NO: 10 – corresponding to the sequence of a promoter of the D-hordein gene. However, in an alternative embodiment, the gene expression regulating sequence can be SEQ ID NO: 29, corresponding to the sequence of a gliadin promoter.

Vector 2

In another embodiment, the expression vector comprises a polynucleotide that comprises the sequence pair (a1-b1) separated by a spacer sequence, wherein

- a1 is SEQ ID NO: 2 and b1 is SEQ ID NO: 6 or a1 is SEQ ID NO: 6 and b1 is SEQ ID NO: 2 ;

This expression vector is called Vector 2. In a preferred embodiment, the expression vector is pDhp_ω4ZR. In this embodiment the gene expression-regulating sequence is SEQ ID NO: 10 – corresponding to the sequence of a promoter of the D-hordein gene. However, in an alternative embodiment, the gene expression regulating sequence can be SEQ ID NO: 29, corresponding to the sequence of a gliadin promoter.

Vector 3

In a further embodiment, the expression vector comprises a polynucleotide that comprises two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, wherein

- a1 is SEQ ID NO: 3 or SEQ ID NO: 7, b1 is SEQ ID NO: 7 or SEQ ID NO: 3, and a2 is SEQ ID NO: 4 or SEQ ID NO: 8, b2 is SEQ ID NO: 8 or SEQ ID NO: 4; or
- a1 is SEQ ID NO: 4 or SEQ ID NO: 8, b1 is SEQ ID NO: 8 or SEQ ID NO: 4 and a2 is SEQ ID NO: 3 or SEQ ID NO: 7 and b2 is SEQ ID NO: 7 or SEQ ID NO: 3.

This expression vector is called Vector 3. In a preferred embodiment, the expression vector is pDhp_ω8ZR. In this embodiment the gene expression-regulating sequence is SEQ ID NO: 10 – corresponding to the sequence of a promoter of the D-hordein gene. However, in an alternative embodiment, the gene expression regulating sequence can be SEQ ID NO: 29, corresponding to the sequence of a gliadin promoter.

Vector 4

We also describe the use of the following expression vectors. In one aspect, the expression vector comprises two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, in which the sequences a1, a2, b1 and b2 differ among themselves and are selected from SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13 and SEQ ID NO: 14, in the following form:

wherein,

- a1 is SEQ ID NO: 11 or SEQ ID NO: 12, b1 is SEQ ID NO: 12 or SEQ ID NO: 11, and a2 is SEQ ID NO: 13 or SEQ ID NO: 14, b2 is SEQ ID NO: 14 or SEQ ID NO: 13; or
- a1 is SEQ ID NO: 13 or SEQ ID NO: 14, b1 is SEQ ID NO: 14 or SEQ ID NO: 13 and a2 is SEQ ID NO: 11 or SEQ ID NO: 12 and b2 is SEQ ID NO: 12 or SEQ ID NO: 11.

This expression vector is called Vector 4. In a preferred embodiment, the expression vector is called pDhp_ω/α. In this embodiment the gene expression-regulating sequence is SEQ ID NO: 10 – corresponding to the sequence of a promoter of the D-hordein gene. However, in an alternative embodiment, the gene expression regulating sequence can be SEQ ID NO: 29, corresponding to the sequence of a gliadin promoter.

For ease of reference, the combinations of sequences that can be obtained in Vector 4 is as follows:

Table 4: Combinations of sequences in which a1-a2 and b2-b1 are sense and antisense sequences

Combinations	a1	a2	b2	b1
1	SEQ ID NO: 13	SEQ ID NO: 11	SEQ ID NO: 12	SEQ ID NO: 14
2	SEQ ID NO: 11	SEQ ID NO: 13	SEQ ID NO: 14	SEQ ID NO: 12
3	SEQ ID NO: 12	SEQ ID NO: 14	SEQ ID NO: 13	SEQ ID NO: 11
4	SEQ ID NO: 14	SEQ ID NO: 12	SEQ ID NO: 11	SEQ ID NO: 13

Table 5: Combinations of sequences in which a1-a2 and b2-b1 are sense and antisense sequences

Combinations	a1	a2	b2	b1
1	SEQ ID NO: 13	SEQ ID NO: 12	SEQ ID NO: 11	SEQ ID NO: 14
2	SEQ ID NO: 12	SEQ ID NO: 13	SEQ ID NO: 14	SEQ ID NO: 11
3	SEQ ID NO: 11	SEQ ID NO: 14	SEQ ID NO: 13	SEQ ID NO: 12
4	SEQ ID NO: 14	SEQ ID NO: 11	SEQ ID NO: 12	SEQ ID NO: 13

Vector 5

In a further aspect, we describe the use of the following expression vector, wherein the expression vector comprises the sequence pair (a1-b1) separated by a spacer sequence, wherein

- a1 is SEQ ID NO: 15 and b1 is SEQ ID NO: 16 or a1 is SEQ ID NO: 16 and b1 is SEQ ID NO: 15.

This expression vector is called Vector 5. In a preferred embodiment, the expression vector is pghpg8.1. In this embodiment the gene expression-regulating sequence is SEQ ID NO: 29, corresponding to the sequence of a gliadin promoter (specifically the gamma-gliadin promoter). However, in an alternative embodiment, the gene expression regulating sequence can be SEQ ID NO: 10 – corresponding to the sequence of a promoter of the D-hordein gene.

The term "vector" or “expression vector” refers to a DNA fragment that has the capacity to replicate itself in a particular host, and, as the term indicates, may serve as a vehicle for multiplying another DNA fragment that has been fused to it (an insert). "Insert" refers to a DNA fragment that is fused to the vector; in the case of the present invention, the vector comprises the polynucleotide of the invention, which, when fused thereto, can replicate itself in the corresponding host. Vectors may be plasmids, cosmids, bacteriophages, or viral vectors, without excluding other types of vectors that meet the present definition of vector.

Another aspect of the present invention is an isolated cell transfected with at least one vector of the invention. In a further embodiment, there is provided an isolated cell transfected with at least two, preferably two, vectors of the invention. In one embodiment, the isolated cell is transfected with at least one expression vector selected from the following expression vectors: Vector 1, Vector 2, Vector 3, Vector 4 and Vector 5. In a second embodiment, the isolated cell is transfected with Vector 1 and Vector 2, as defined above. In another embodiment, the isolated cell is transfected with Vector 1 and Vector 3, as defined above. In a yet further embodiment, the isolated cell is transfected with Vector 4 and Vector 5, as defined above.

In addition to the above, there is also provided the following plasmid combinations:

Table 6: Plasmid combinations

Plasmid combination	Plasmid 1	Plasmid 2
0 (wt)	NA	NA
1	Vector 5	NA
2	Vector 1	NA
3	Vector 3	NA
4	Vector 4	NA

5	Vector 5	Vector 4
6	Vector 1	Vector 2
7	Vector 1	Vector 3
8	Vector 2	NA
9	Vector 5	Vector 1
10	Vector 5	Vector 3
11	Vector 5	Vector 2
12	Vector 1	Vector 4
13	Vector 3	Vector 2
14	Vector 3	Vector 4
15	Vector 2	Vector 4

The term "cell" as used in the present invention refers to a prokaryotic or eukaryotic cell. The cell may be a bacterium capable of replicating foreign DNA by transforming, for example, any of the strains of the species *Escherichia coli* or a bacterium capable of transferring the DNA of interest into the interior of a plant, such as for example *Agrobacterium tumefaciens*. Preferably, the cell refers to a eukaryotic plant cell, and within this group, more preferably to cells belonging to the kingdom *Plantae*. Therefore, in cases in which the cell is a plant cell, the term "cell" comprises at least a parenchyma cell, meristem cell, or a cell of any type, differentiated or undifferentiated. Thus, this definition also includes a protoplast (a plant cell lacking a cell wall).

The term "transfection" refers to the introduction of external genetic material into cells via plasmids, viral vectors (in this case one can also use the term "transduction"), or other means of transfer. The term "transfection by nonviral methods" is used with reference to mammalian eukaryotic cells, while the term "transformation" is preferred to describe nonviral transfers of genetic material into bacteria and non-animal eukaryotic cells such as yeasts, algae, and plants. In the case of the present invention, the term "transfection" is equivalent to the term "transformation."

Another aspect of the present invention is a genetically modified or altered plant that comprises the cell of the invention. The term "plant" includes every part of the plant, which may be preserved or cultivated either individually or in combination, as well as the germplasm. The germplasm is composed of biological material that contains interspecies genetic variability or the genetic materials that can perpetuate a species or

population of an organism (see seeds, propagule, or progeny below). The plant must comprise the cell of the present invention in a form that is expressed in a specific tissue (at a specific moment of plant development or depending on the environmental conditions in which it develops) or in a constitutive or epitopic form (expressed in other cells or tissues differing from those that are common and expected).

In a further aspect of the invention, there is provided a genetically modified or altered plant with decreased gliadin and/or glutenin content (or prolamin content) comprising, in its genome, a polynucleotide of the present invention, wherein said plant exhibits reduced gliadin and/or glutenin content compared to a control plant.

The plant of the invention may contain the polynucleotide of the invention in homozygosis, heterozygosis, or homozygosis. Preferably, the plants of the invention are homozygous for the stably integrated polynucleotide.

According to a preferred embodiment, the plant belongs to the genus *Triticum*. The plant may be selected from the list that includes, but is not limited to, *Triticum aestivum, T. aethiopicum, T. araraticum, T. boeoticum, T. carthlicum, T. compactum, T. dicoccoides, T. dicoccum, T. durum, T. ispahanicum, T. karamyschevii, T. macha, T. militinae, T. monococcum, T. polonicum, T. repens, T. spelta, T. sphaerococcum, T. timopheevii, T. turanicum, T. turgidum, T. urartu, T. vavilovii* and *T. zhukovskyi.*

According to another preferred embodiment, the plant is of the species *Triticum aestivum* or *Triticum turgidum*. According to another preferred embodiment, the plant belongs to the cultivar Bobwhite or the cultivar Don Pedro. More preferably, the cultivars BW208 and BW2003 (Bobwhite), which belong to the wheat species *Triticum aestivum* L. ssp *aestivum,* and the variety Don Pedro, which belongs to the wheat species *Triticum turgidum* L. ssp *durum*, are selected.

Bobwhite is the name of the cultivar obtained from the International Maize and Wheat Improvement Center (CIMMYT). BW208 and BW2003 are different Bobwhite lines. Don Pedro is a hard wheat variety, also from CIMMYT. Bobwhite and Don Pedro are public varieties.

The plant of the invention may be obtained by genetic transformation of plant cells by means of biolistics, *Agrobacterium tumefaciens,* or any other technique that allows integration of the polynucleotide of the invention into the DNA of the plant, whether this DNA be genomic, chloroplastic, or mitochondrial, followed, although not necessarily, by an *in vitro* regeneration program suited to the characteristics and requirements of the transformed plant species. Moreover, the plant may also be obtained by transference of any of the sequences of the invention by crossing, e.g., using pollen of the plant of the invention to pollinate any other plant that does not contain the polynucleotide of the invention, or pollinating the gynoecia of plants containing the polynucleotide of the invention with other pollen that does not contain these sequences. The methods for obtaining the plant of the invention are not exclusively limited to those described in this paragraph; for example, genetic transformation of germ cells from the ear of wheat could be carried out as mentioned, but without having to regenerate a plant afterward (see below). Moreover, a plant that comprises the cell of the present invention in a stable or transient form is also included.

Another aspect of the present invention is a seed from any of the plants of the invention. This will be referred to below as the "seed of the invention" or "seed of the present invention". The seed includes in its genome the polynucleotide of the invention and has a reduced gliadin and/or glutenin content compared to control or wild-type plants. The seed is preferably homozygous for the transgene.

Another aspect of the present invention is a grain of pollen, propagule, progeny, or plant part derived from any of the plants of the invention.

In the present invention, pollen is taken into account as a transmitter of the genetic and phenotypic characteristics that may result from pollination of any plant variety compatible with the pollen in question. In this manner, one obtains a plant that comprises the polynucleotide of the present invention, and, after the respective crosses and/or selections, one can obtain a plant in which the sequence is integrated in stable form (although it may also be expressed in transient form) and in a sufficient number of copies to obtain the same desirable characteristics in subsequent generations.

Propagules are parts of the plant that allow asexual propagation or reproduction in plants, whereby new individualized plants or organs are obtained. The tissues of the

separated portion must recover to the status of meristems in order to produce the entire group of organs of the plant.

The term "progeny" refers to the result of reproduction, i.e., the individual or individuals produced by the intervention of one or more parent individuals. For example, the progeny of plants obtained by sexual reproduction are seeds, but the progeny of a plant may be any cell resulting from the fusion of any cellular contents, plastid, cellular compartment, DNA, or any combinations thereof. In the processes of cellular division (such as *in vitro* cultivation, for example), the progeny are the cells resulting from the division.

Another aspect of the present invention is the use of the polynucleotide, vector, or cell of the invention for the silencing or substantially silencing of at least one of alpha-, gamma-, and omega-gliadins and LMW glutenin of *Triticum* spp. In a preferred embodiment there is provided the use of a polynucleotide, vector, or cell of the invention for the silencing of all of alpha-, gamma-, and omega-gliadins and LMW glutenin of *Triticum* spp. In an alternative embodiment, there is provided the use of a polynucleotide, vector, or cell of the invention for the silencing of only alpha-gliadin of *Triticum* spp.

In a further embodiment, there is provided a method for silencing or substantially silencing at least one of alpha-, gamma- and omega-gliadins or the LMW glutenin of *Triticum* spp using the polynucleotides, vectors or transfected cells of the invention.

Another aspect of the present invention is the use of the polynucleotide, vector, or cell of the invention to reduce gliadin and/or glutenin content in the *Triticum* spp. A further aspect of the present invention is the use of the polynucleotide, vector, or cell of the invention to reduce total prolamin content of *Triticum* spp. The reduction in gliadin and/or glutenin or total prolamin may be at least 75%, preferably at least 50% and more preferably at least 25% compared to wild-type or control levels (i.e. in a control plant. In one embodiment, the control plant is wild-type Bobwhite 208 (BW208).

A further aspect of the present invention is the use of the polynucleotide, vector, or cell of the invention to reduce gluten content in the *Triticum* spp. Measurement of gluten content can be carried out using techniques standard in the art and known to the

skilled person. However, in one embodiment, the gluten content is measured using the G12 and/or R5 monoclonal antibodies. The reduction in gluten levels may be at least 40%, preferably 80%, more preferably 90% compared to wild-type or control levels. In one preferred embodiment the reduction in gluten may be 92% compared to wild-type or control levels.

A control plant as used herein is a plant, which has not been modified according to the methods of the invention. Accordingly, the control plant has not been genetically modified to express a nucleic acid as described herein. In one embodiment, the control plant is preferably a wild type (WT) plant. For example, if the plant is wheat, then in one embodiment, then control plant is wild type wheat. In another embodiment, the control plant is a plant that does not carry a transgene according to the methods described herein, but expresses a different transgene. The control plant is typically of the same plant species, preferably the same variety or ecotype as the plant to be assessed.

A control plant or plant cell may thus comprise, for example: (a) a wild-type plant or cell, i.e., of the same genotype as the starting material for the genetic alteration which resulted in the subject plant or cell; (b) a plant or plant cell of the same genotype as the starting material but which has been transformed with a null construct (i.e., with a construct which has no known effect on the trait of interest, such as a construct comprising a marker gene); (c) a plant or plant cell which is a non-transformed segregant among progeny of a subject plant or plant cell; (d) a plant or plant cell genetically identical to the subject plant or plant cell but which is not exposed to conditions or stimuli that would induce expression of the gene of interest or (e) the subject plant or plant cell itself, under conditions in which the gene of interest is not expressed.

Another aspect of the present invention is the use of the seed of the invention to prepare a food composition (referred to in the following as the "composition of the invention" or "composition of the present invention"). The food composition is prepared from, but not limited to, the flour and/or semolina of the seeds of the invention, combined or not with other flours and/or semolinas, or other compounds.

The term "flour" as it is understood in the present invention refers to the product obtained by milling of any seed or plants of the genus *Triticum*, with the bran or husk of the seed removed to a greater or lesser degree.

The term "semolina" refers to coarse flour (slightly milled wheat seeds), i.e., fragments of the endosperm with a variable amount of seed husks.

The prepared food is selected from, but not limited to, the list comprising bread, bakery products, pastries, confectionery products, food pasta, food dough, grains, drinks, or dairy products.

Another aspect of the invention is use of the composition of the invention to prepare a food product, vitamin supplement, or nutritional supplement. As understood in the present invention, a food product fulfils a specific function, such as improving the diet of those who consume it. For this purpose, a vitamin and/or nutritional supplement may be added to the food product.

The food product that comprises the food composition of the present invention may be consumed even by persons who are allergic to gluten, i.e., suffer from celiac disease.

Another aspect of the present invention is a method for obtaining the plant of the invention, comprising the following:

a. selecting a part of the plant,

b. transfecting the cells of the part of the plant of paragraph (a) with a vector described herein,

c. selecting the transfected cell of paragraph (b) that comprises any polynucleotide of the present invention

d. regenerating at least one plant derived from the cell selected in paragraph (c),

e. selecting one or more plants regenerated according to paragraph (d) in which the polynucleotide is transcribed into an hpRNA, and

f. selecting one or more plants obtained according to paragraph (e) that show silencing of at least one, preferably all, of alpha-, gamma-, and omega-gliadins and LMW glutenin in its seeds.

In the case of wheat plants, preferably one may select the scutellum to be transfected by the vector of the invention. The insertion of the polynucleotide of the present invention into a vector may be carried out by cloning methods that are known in the art, by means of cleaving the polynucleotide and the vector with restriction enzymes (digestion) and subsequent ligation, such that the sequence of the vector comprises the polynucleotide of the invention. The vector was described in a previous paragraph.

The selection of the vector that comprises the selected sequence of the invention may be carried out by techniques such as the following:

- Selection of cells containing the vectors of the invention by means of adding antibiotics to the culture medium. The resistance of these cells to substances such as antibiotics is produced by the synthesis of molecules coded for by a sequence contained in the sequence of the vector.
- Digestion with restriction enzymes, by means of which one obtains a fragment of one of the sequences of the invention inserted into the vector.

The cell is obtained by any type of microbiological culture (for example, *E. coli* or *Agrobacterium tumefaciens*) or plant culture.

Genetic transformation of the cells is carried out using techniques known in the art, such as, for example, electroporation, genetic transformation by biolistics, *Agrobacterium tumefaciens* transformation, or any other technique that allows the integration of any of the sequences of the invention into the DNA of the cell. Preferably, transformation should be carried out by biolistics. By means of these techniques, one can obtain in a stable manner a vector that comprises any of the sequences of the invention, such that after successive cell divisions, the incorporated sequence continues to express itself. Cells including any of the sequences of the invention in a transient manner are also included.

The cell transformed with a vector that comprises any of the polynucleotides of the invention may incorporate the sequence in any type of cellular DNA: nuclear, mitochondrial, and/or chloroplastic, and in this case, one usually inserts the DNA, which comprises, among other sequences, the polynucleotide of the invention. Selection of cells that have incorporated any of the sequences of the invention is

carried out by adding antibiotics to the culture medium that provides nutrients to them. The resistance of these cells to substances such as antibiotics or herbicides is produced by the synthesis of molecules coded for by a sequence contained in the DNA sequence of the vector. One may also select the cell that comprises the polynucleotide of the invention by any other technique that allows its presence or absence and/or its expression to be distinguished.

The plant cells selected may be subjected to a program of organogenesis or somatic embryogenesis, thus giving rise to a complete plant that comprises the genetic material of the original cell from which it originated. This is possible because of the fact that plant cells are totipotent, i.e., by means of a suitable combination of hormones, they can be dedifferentiated, thus generating embryonic cells that, because they contain a complete copy of the genetic material of the plant to which they belong, have the potential to regenerate a complete new plant. Light and temperature conditions suited to each plant species are also required. Once the plant originating from the selected plant cell has regenerated itself, one can carry out an analysis of the presence and/or expression of the nucleotide sequence that codes for the polynucleotide of the invention or any other sequence of the present invention (promoter sequence, etc.).

The method also includes the selection of a plant that shows substantial silencing of at least one, preferably all of, the alpha/beta-, gamma-, and omega-gliadins and LMW glutenin in its seeds. Preferably, plants showing virtually complete or complete silencing of all the gliadins and/or LMW glutenin, of the seeds should be selected. The reduction in total gliadin and/or glutenin content compared to a control plant (a plant not including the polynucleotide of the invention) is greater than or equal to 90%. The control plants preferably do not contain the polynucleotide of the invention in the plant cell. Prior to being transformed, the control may also be a wild-type plant that has undergone the same *in vitro* cultivation steps as the plants of the invention or has not undergone these cultivation steps.

As a consequence of the gliadins/LMW silencing other protein groups in the grain are up-regulated. This can be observed in the HMW fraction, which for all plasmid combinations, except one, the content of HMW is higher than that of the wild type. Albumins and globulins, not implicated in coeliac disease, are also up-regulated, as described previously (Gil-Humanes, *et al.*, 2011) maintaining the total nitrogen in the

grain at the same level in both transgenic and control/wild type plants. Implications of the up-regulation of HMW are on the technological properties of doughs prepared from the low-gliadin lines, in which a general weakening effect can be observed. In contrast, the stability of doughs was increased significantly in some of the transgenic lines, indicating better tolerance to over-mixing (Gil-Humanes, *et al.*, 2014).

The transfected cells may be germ cells from the ear of the plant, and in this case, at least one plant derived from the seeds generated by said ear of the plant would be regenerated, and one would select at least one plant showing silencing of at least one, preferably all, of alpha-, gamma-, and omega-gliadins and LMW glutenin in its seeds.

In a further aspect there is provided a method of reducing the gluten content in a plant (such as the plants described herein). There is also provided a method of reducing the number of disease-related, preferably coeliac disease (CD) epitopes in a plant (such as the plants described herein). The epitopes may be T-stimulating epitopes. The epitopes may be from α- or ω-gliadins. For either aspect, the method comprises:

a. selecting a part of a first plant,

b. transfecting the cells of the part of the plant of paragraph (a) with a vector as described herein,

c. selecting the transfected cell of paragraph (b) that comprises the polynucleotide as described herein,

d. regenerating at least one (new) plant derived from the cell selected in paragraph (c),

e. selecting one or more plants regenerated according to paragraph (d) in which the polynucleotide is transcribed into an hpRNA, and

f. selecting one or more plants obtained according to paragraph (e) that show silencing of at least one of the following: alpha-, gamma-, omega-gliadins and LMW glutenin in its seeds.

In a final aspect, there is provided a method for modulating an immune response to gliadins and/or glutenins, the method comprising providing a diet of the food composition of the invention to a subject in need thereof. There is also provided a method for affecting or modulating a T-cell response to gluten in a subject, the method comprising providing a diet of the food composition of the invention to a subject in need thereof. The T-cell may be a $CD4^+$ T-lymphocyte.

Throughout the description and the claims, the word "comprise" and its variants is not intended to exclude other technical characteristics, additives, components, or steps. For the person skilled in the art, other objects, advantages, and characteristics of the invention will be obvious partly from the description and partly from the practice of the invention. The following figures and examples are provided by way of illustration, and they are not intended to limit the scope of the present invention. In addition, all references cited are incorporated herein.

EXAMPLE

Plasmid design and construction

For the synthesis of the RNAi vectors, conserved regions of the α-, γ-, and ω-gladins were PCR amplified from genomic DNA isolated from the bread wheat (*Triticum aestivum*) cv. Bobwhite 208 (BW208) and cloned in sense and antisense orientation, separated by the *Ubi1* intron to form the inverted repeat (IR) sequences. The relative position of each IR on their corresponding gliadin sequences is shown in Fig. 1A. In total, we synthesised 5 different plasmids (Fig. 1B). The synthesis of plasmids pghpg8.1 and pDhp_ω/α was described in (Gil-Humanes *et al.*, 2008; 2011), respectively. The vectors pDhp_α/βZR, pDhp_ω4ZR, and pDhp_ω8ZR were synthesised for this work by using the GATEWAY (Invitrogen) recombination technology. Three different pairs of primers were used for the amplification of the IR fragments of these vectors: primers alpha/betaF (AATTGCAGCCACAAAATCCATCTCAG) (SEQ ID NO: 17) and alpha/betaR (CATCCMTGCATGGAATCAGTTGTTG) (SEQ ID NO: 18) for the alpha/betaZR fragment; primers omega8F (CCTATCTTTGTCCTCCTTGCC) (SEQ ID NO: 19) and omega8R (CATCGTTACATTGAACGCTCA) (SEQ ID NO: 20) for the omega8 and LMW fragments; and primers omega4F (CAACAATCCCCTGAACAACA) (SEQ ID NO: 21) and omega4R (GCTGGGGTGGGTATGGTATT) (SEQ ID NO: 22) for the omega4 fragment. The expression of the IRs was driven by an endosperm-specific D-hordein promoter (Piston *et al.*, 2008), with the nopaline synthase (nos) as terminator sequence.

Plant material and genetic transformation

All lines described in this work are transgenic lines derived from bread wheat cv. BW208. Lines C655, D623, 28A, 28B, D783, E33, E42, E82, and E83 were reported

previously (Piston *et al.*, 2013). The rest of transgenic lines were produced in the present work using immature scutella as explants for genetic transformation as described in León *et al.*, 2009. Plasmids carrying the RNAi fragments were used in combination with plasmid pAHC25 containing the selectable *bar* gene (Christensen and Quail, 1996). For bombardment, plasmids were precipitated onto 0.6 µm gold particles at 0.5 pmol mg^{-1} gold for pAHC25 and 0.75 pmol mg^{-1} gold for the plasmids containing the RNAi fragments. Putative transgenic plants were then transferred to soil and grown to maturity in the greenhouse. Homozygous progeny of plants containing the RNAi plasmids were first identified by PCR by using the forward primer prHorD*3 (GGGGTACCCATTAATTGAACTCATTCGGGAAGC) (SEQ ID NO: 23) and one of the reverse primers specific for each RNAi construct: SUbiR (GCGTACCTTGAAGCGGAGGTGGTCGACTCTAGATTGCAACACCAATGATCTGAT CG) (SEQ ID NO: 24) for the pghpg8.1 plasmid, alpha/betaR for the pDhp_α/βZR plasmid, omega8R for the pDhp_ω8ZR plasmid, omega4R for the pDhp_ω4ZR plasmid, and omega_III_R_overlapping (CAGTTGTTGTTGAAATGGTTGTTGCGATGG) (SEQ ID NO: 25) for the pDhp_ω/α plasmid. Then, PCR-positive transgenic plants were analysed by A-PAGE of endosperm proteins in lines produced by single half-seed descent. Homozygous lines were self-pollinated for three generations and assayed as described below.

Experimental design and statistical analysis

The homozygous transgenic lines were assayed using randomized complete block designs with three replicates of five plants each. Data were analysed with the statistical software R version 3.0.1 (Ihaka and Gentleman, 1996). Figures were drawn using the user interface GrapheR (Hervé, 2013). The differences in the data were assessed using analysis of the variance (ANOVA) (function *aov*, package *agricolae*), followed by the two-tailed Dunnett's *post hoc* test for median multiple comparisons. *P* values lower than 0.05 were considered significant.

Polyacrylamide gel electrophoresis analysis

Mature wheat grains were crushed into a fine powder and used to extract the endosperm storage proteins. Gliadins and glutenins were sequentially extracted, and separated in A-PAGE and SDS-PAGE gels, respectively, as described in (Gil-Humanes *et al.*, 2012).

Reversed-phase high-performance liquid chromatography (RP-HPLC)

Gliadins and glutenins were extracted and quantified by RP-HPLC following the protocol reported by (Piston *et al.*, 2011). Three independent repetitions were carried out for each transgenic line and control.

Mass spectrometry analysis

Total protein was extracted from 1 g of flour with 10 ml of SDS buffer (0.5% SDS, 0.1 M sodium phosphate pH 6.9) shaking (120 rpm) at 60°C during 80 min. Sonication (Ultrasonic cleaner USLU-5.7, Fungilab SA, Barcelona, Spain) was carried out at medium intensity during 1 min at 24°C, and the supernatant was collected centrifuging at 16,000 *g* during 15 min. The extraction was performed twice using the pellet to finally obtain 30 ml of extract. Proteins were precipitated adding four volumes of cold acetone and kept at -20°C during 30 min. After centrifuging at 16,000 *g* during 15 min, proteins were dissolved in 5 ml of 0.01 M acetic acid.

Pepsin digestion was carried out adjusting the pH to 1.8 with hydrochloric acid, adding pepsin (Sigma, St. Louis, Missouri, USA) at a 1:100 ratio (w/w) and stirring during 4 h at 37°C. The pepsin digestion was finished by bringing the pH to 7.8 with sodium hydroxide solution. A second digestion with trypsin (Sigma, 1:100 ratio [w/w]) was performed at the same incubation conditions. Trypsin was deactivated by heating at 85°C for 45 min. The pH of the peptic-tryptic (PT) digest was adjusted to 4.5 with hydrochloric acid, dialyzed (1 kDa cut-off) against 0.01 M ammonium bicarbonate and lyophilised.

The PT-digest was cleaned using a SEP-PAK C18 cartridge (Waters, MA, USA) and 1.5 µg of total peptide was injected into Eksigent NanoLC-1D Plus (AB SCIEX, CA, USA) coupled to TripleTOF® 5600 System (AB SCIEX) (LC-MS/MS). The HPLC pre-column was Acclaim® PepMap 100, 100 µm x 2 cm (Thermo Fisher Scientific, MA, USA) and HPLC column NanoACQUITY UPLC® 1.7 µm BEH130 C18, 75 µm x 150 mm (Waters). All Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) datasets were searched using MASCOT version 2.4 (http://www.matrixscience.com/) against NCBI protein database of the species *Triticum* without any enzyme restriction, peptide error tolerance of 25 ppm and MS/MS fragment error tolerance of 0.05 Da. Only peptides with scores higher than 20 were extracted for further analyses.

CD epitope analysis

The peptides identified in LC-MS/MS analysis were BlastP searched against CD epitopes described by (Sollid *et al.*, 2012) and the α-gliadin peptide 31–43 (p31-43; PGQQQPFPPQQPY (SEQ ID NO: 26) (Maiuri *et al.*, 1996) and LGQQQPFPPQQPY (SEQ ID NO: 27) (Maiuri *et al.*, 2003)), able to induce innate immune response, setting the parameters: -task blastp-short, -ungapped, -seg no, -max_target_seqs 5 and allowing one mismatch. CD epitopes were searched in peptides longer than 8 amino acids as described above.

Seed quality determinations

Kernel weight (g) was determined using 1000 seeds from each sample. Test weight (g l^{-1}) was calculated by weighing 100 ml of cleaned grains from each sample. Two measurements were carried out for each sample.

The protein content of whole flour was calculated from the Kjeldahl nitrogen content (%N x 5.7) according to the standard ICC method no. 105/2 (ICC, 1994), and the starch content was determined according to the standard ICC method no. 123/1 (ICC, 1994). Both parameters were expressed on a 14% moisture basis.

The non-gluten proteins, expressed in percentage of dried weight (% DW), were calculated as follow: [Total protein in % - (Prolamin content in µg/mg*10)/(100 - moisture in %)]. The sodium dodecyl sulphate sedimentation (SDSS) volume was determined as described by (Williams *et al.*, 1988). Three technical replicates were carried out for each biological sample.

Gluten content determination by competitive ELISA

Gluten proteins were extracted according to the manufacturer's instructions using Universal Gluten Extraction Solution UGES (Biomedal SL, Seville, Spain). Maxisorp microtiter plates (Nunc, Roskilde, Denmark) were coated with Prolamin Working Group (PWG) gliadin solution and incubated overnight at 4 °C. The plates were washed with PBS-Tween 20 buffer and blocked with blocking solution (phosphate-buffered saline (PBS)-5% non-fat dry milk) for 1 hr at RT. Different dilutions of each sample as well as standard solution of PWG gliadin were made in PBS-bovine serum albumin 3%, to each of which was added horseradish peroxidase–conjugated G12 moAb solution. The samples were pre-incubated for 2 h at RT with gentle stirring, and then added to the wells. After 30 min of incubation at RT, the plates were washed, and 3,3′,5,5′-

tetramethylbenzidine (TMB) substrate solution (Sigma, St. Louis, Missouri, USA) was added. After 30 min of incubation at RT in the dark, the reaction was stopped with 1 M sulphuric acid, and the absorbance at 450 nm was measured (microplate reader UVM340, Asys Hitech GmbH, Eugendorf, Austria). Results were expressed in parts per million (ppm) in dry matter.

Results

RNAi sequence combinations and plasmid design

Fig. 1A shows the structure of the α-, γ-, and ω-gliadins, and LMW glutenins indicating the gene regions where seven RNAi sequences were designed. These RNAi sequences varied in length from 109 to 377 (nucleotide sequences provided in Figure 6) and were cloned from different domains of the gliadin sequences. The *alpha/beta ZR* RNAi fragment (SEQ ID NOs 1 and 5) covered part of the repetitive domain of α-gliadins plus the poly-Q domain, and a small region of the non-repetitive domain I, whereas the *alpha* RNAi fragment (SEQ ID NOs 11 and 12) was PCR amplified from the non-repetitive domain I. The γ-gliadin RNAi (*g8.1*) sequence (SEQ ID NOs 15 and 16) encompassed part of the non-repetitive domain I of the γ-gliadins, plus the Q-rich domain and part of the non-repetitive domain II. Among the ω-gliadins the *omega* RNAi sequence (SEQ ID NOs 13 and 14) was designed containing part of the signal peptide, the N terminal domain, and part of the repetitive domain; whereas both the *omega4* (SEQ ID NOs 2 and 6) and *omega8* (SEQ ID NOs 3 and 7) were cloned from the 3' end of the repetitive domain of the ω-gliadins. The *LMW* fragment (SEQ ID NOs 4 and 8) contained part of the signal peptide, the N terminal domain, and part of the repetitive domain of the LMW glutenins. The identity observed between the IR fragments and the non-targeted prolamin fractions was in all cases between 40-60% (Figure 7). The RNAi sequences were in some cases combined by fusion PCR to provide five different RNAi fragments, which were inserted downstream a D-hordein promoter to produce five hairpin plasmids (Fig. 1B). In one embodiment, the plasmids contain at least one RNAi sequence. In an alternative embodiment, the plasmids contain two or three RNAi sequences. The possible RNAi combinations are presented in Table 1.

Table 7: RNAi fragment combinations

Combination	RNAi fragment 1	RNAi fragment 2
1	alpha/beta ZR	LMW
2	alpha/beta ZR	omega 8

Combination	RNAi fragment 1	RNAi fragment 2
3	alpha/beta ZR	omega 4
4	LMW	omega 8
5	LMW	omega 4
6	omega 8	omega 4
7	alpha/beta ZR	g8.1
8	alpha/beta ZR	alpha
9	alpha/beta ZR	omega
10	LMW	g8.1
11	LMW	alpha
12	LMW	omega
13	omega 8	g8.1
14	omega 8	alpha
15	omega 8	omega
16	omega 4	g8.1
17	omega 4	alpha
18	omega 4	omega
19	g8.1	alpha
20	g8.1	omega

Plasmids were further used alone or combined with others providing seven plasmid combinations (Fig. 1C). Plasmids pghpg8.1 and pDhp_ω/α were described previously (Gil-Humanes *et al.*, 2008; 2010) whereas all other plasmids and plasmid combinations are compared for the first time in this work. In total, 21 transgenic wheat lines (Figure 8) containing any of the seven combination of plasmids were assayed for the down-regulation of gliadins and effects on other protein fractions, agronomic parameters, and the content of coeliac disease related gliadins as determined using the G12 monoclonal antibody and CD immunogenic peptides identified by LC-MS/MS.

Plasmid combinations and patterns of silencing

Silencing patterns in all 21 transgenic lines were analysed by A-PAGE and SDS-PAGE, and the gliadin and glutenin fractions quantified by RP-HPLC and compared with the untransformed wild type BW208 (denoted as plasmid combination 0). Examples of gliadin and glutenin patterns obtained with all seven plasmid combinations are in Figure 5. Detailed results for each line are in Figure 9. Fig. 2 shows the

transgenic lines and the effects of each plasmid combinations on the gliadin and glutenin fractions. Plasmid Combination 1 clearly targeted the γ-gliadin fraction while ω-, and α-gliadins were-up regulated. This Plasmid Combination did not significantly affect the total amount of the glutenin fractions. However, not all LMW glutenins were equally affected: while the amount of LMW glutenins appearing between 45 and 60 minutes retention time decreased, LMW peaks between 35 and 45 minutes retention time were slightly increased compared with the wild type (Figure 5). Plasmid Combination 2 achieved a greater reduction in the α-gliadin fraction, with no major effects on other gliadin fractions, and an important increment in the HMW glutenin fraction compared with the wild type. Plasmid Combination 3 provided a significant reduction in all three ω-, γ-, and α-gliadin fractions, and also a strong reduction in the LMW glutenin content, but an important increment in the HMW glutenin fraction (Fig. 2B). In contrast to Plasmid Combination 1, Combination 3 affected mainly to the LMW in the region between 35 and 45 minutes retention time (Figure 5). Plasmid Combination 4 also provided a strong reduction in all three gliadin fractions, and a significant increment in the HMW glutenin fraction. However, in contrast to Plasmid Combination 3, the LMW glutenins were not reduced. Plasmid Combinations 5, 6, and 7 were equally effective in the down-regulation of ω-gliadins (Fig. 2A), although Combination 5 was more effective in the down-regulation of γ-gliadins, and Combinations 6 and 7 in the down-regulation of α-gliadins (Fig. 2A). These plasmid combinations (5, 6, and 7) also had an important impact in the glutenin fraction; while all three plasmid combinations decreased the content of LMW glutenins (with wide differences within Combination 5), only Combination 7 showed a strong increment in the HMW glutenin fraction (Fig. 2B).

Protein fraction redistributions kernel composition and agronomic traits

Fig. 3 shows the fold change in kernel composition of transgenic lines with the different plasmid combinations. Gliadins, Gli/Glu ratio, and total prolamin content were significantly reduced in all the transgenic combinations compared with the control (Combination 0). Plasmid Combinations 5, 6, and 7, all three combinations of two different plasmids, were the most effective in the down-regulation of total gliadin content in transgenic wheat (Figure 10). Only three plasmid combinations, 2, 4, and 6 had significant effect on total glutenin content; plasmid Combinations 2, and 4 increased the total glutenin content in transgenic wheat whereas Combination 6 decreased it (Fig. 3). The Gli/Glu ratio decreased significantly in all seven plasmid

combinations compared to the wild type, with the largest differences found for Combinations 4, 5, and 7. As consequence of these changes, the prolamin fraction (comprising gliadins plus glutenins) was strongly decreased when Plasmid Combinations 5, 6, and 7 were used. In contrast, non-gluten proteins, which represented about 4.3% DW in the wild type (Plasmid Combination 0), increased up to 9.9, 10.6, and 9.4% DW in Plasmid Combinations 5, 6, and 7, respectively (Figure 10). Non-gluten proteins compensated the down-regulation of prolamins, and total protein was not affected by any of the seven plasmid combinations, all of them showing protein contents around 13% DW. The major component of wheat grain, starch, was not significantly affected by the changes in the protein fraction observed with the seven combinations of plasmids. Hence, the lowest value for starch content was 62.8% DW for Plasmid Combinations 4 and 6, which was not significantly different to the 66.0% DW of the wild type (Figure 10).

Agronomic traits such as days to anthesis, kernel weight, and test weight were determined in transgenic plants from all seven plasmid combinations and compared with that of the wild type. As showed in Figure 10, the number of days from sowing to anthesis was around the 149 days in the wild type, with no significant differences for transgenic plants from all seven plasmid combinations. Kernel weights showed higher values than the wild type in Plasmid Combination 1 whereas combinations 5, 6, and 7 had lower weights (Fig. 3). With respect to kernel weight, these differences were significant for Combinations 1, 5, 6, and 7, and in the case of test weight for Combinations 5 and 6.

LC-MS/MS analysis of the protein fraction in plasmid combinations

Total protein extracted from all plasmid combinations was also analysed by LC-MS/MS after PT-digestion. The total fragments detected by MS ranged from 46,287 (Plasmid Combination 1) to 52,129 (Plasmid Combination 7) (Figure 12). Approximately 3 to 10% of these fragments were identified using the NCBI protein database restricted to *Triticum* species, comprising 146 to 198 proteins. More than 70% and 38% of the identified fragments and proteins, respectively, were seed storage proteins (SSPs). Although e Plasmid Combination 1 targets γ-gliadins, the number of identified γ-gliadin fragments was not as low as would be expected (Figure 12). In addition, the number of LMW glutenins was higher and that of ω-gliadins was lower. In Plasmid Combination 2, the number of α-gliadin peptides was lower but that of γ- and ω-gliadins and glutenins

were similar to the wild type. The profile of gliadin fraction in Plasmid Combination 3 was similar to the control but the proportion of fragments derived from LMW glutenin was lower and that of non-gluten proteins was higher. In contrast to Plasmid Combination 3, in Plasmid Combination 4 the number of gliadin fragments was lower and that of HMW glutenins was higher, while LMW glutenins were similar to the wild type. In this combination, the proportion of non-gluten fragments was also higher. In Plasmid Combinations 5 to 7, the gliadin fraction was greatly decreased, affecting mainly the α-gliadin in Combinations 6 and 7 and all gliadins in the Combination 5. The proportion of LMW glutenin peptides was also decreased with higher effect in Plasmid Combination 7, and with a strong increase in HMW glutenins in Plasmid Combination 5. There was also an increase in the proportion of non-gluten protein fragments in these three combinations. In general, the higher proportion of non-gluten fragments was due to the increase in Serpins, Globulins and Triticins.

CD immunotoxic peptides in fragments identified by LC-MS/MS

In peptides identified by LC-MS/MS analysis against *Triticum* proteins, CD immunogenic epitopes (Sollid *et al.*, 2012) and the p31-43 fragment of α-gliadins were searched allowing up to one mismatch (Figure 13 and Figure 11). In the wild type, a total of 645 epitopes with perfect match were found in 3,240 identified peptides, being most of them epitopes reported in γ-gliadins (482 epitopes) followed by α-gliadin (92 epitopes) and ω-gliadin (24 epitopes) (Figure 13). HMW glutenin epitopes were not found in these peptides. In transgenic lines, total perfect match epitopes ranged from 55 in Plasmid Combination 5 to 849 in Plasmid Combination 3 (Figure 13), γ-gliadin epitopes being the most abundant in all combinations. α-gliadin epitopes were not found in the identified peptides in Plasmid Combinations 2, 5 and 6, and were much lower than wild type in Combinations 4 and 7. Furthermore, the number of ω-gliadin epitopes found was lower in Plasmid Combinations 4, 5 and 7. Plasmid Combination 5 also exhibited the lowest number of γ-gliadin epitopes and an increase in HMW glutenin epitopes. The p31-43 fragment was lower in all transgenic lines except in Plasmid Combination 1, and it was not found in Plasmid Combinations 2, 6 and 7. Of the α-gliadin epitopes, the most abundant was DQ2.5-glia-α2, followed by DQ2.5-glia-α1b and DQ2.5-glia-α1a (Figure 13). Among the γ-gliadin epitopes, DQ2.5-glia-γ4c/DQ8-glia-γ1a and DQ2.5-glia-γ5 were the majority. LMW glutenin epitopes were

not found in Combinations 3 and 4, were lower in Combinations 2 and 7, and were similar to wild type levels in 5 and 6, and higher than wild type in Plasmid Combination 1. Epitopes matched to that reported in Hordeins were lower in all transgenics.

The epitopes with one mismatch also showed a similar pattern to those with perfect match. A total of 842 epitopes were found in the wild type, where 603 were γ-gliadin epitopes, 95 of ω-gliadin and 82 of α-gliadin epitopes (Figure 11). Similarly, a drastic decrease in all gliadin epitopes and an increase in HMW glutenin epitopes were observed in Plasmid Combination 5. Epitopes with one mismatch of all gliadins were also decreased in Combinations 4 and 7 compared to the wild type, with a decrease of LMW glutenin epitopes in the latter. Plasmid Combinations 2, 3 and 6 presented lower number of α-gliadin epitopes, but differing in γ-gliadin (slightly lower in combination 2) and ω-gliadin(slightly increased in Combinations 3 and 6) epitopes. In Plasmid Combination 1, γ- and ω-gliadin epitopes were slightly decreased but LMW glutenin was increased compared to wild type. A drastic increase in Hordein epitopes was observed in Plasmid Combination 2. These results suggest that the Plasmid Combinations 4 and 5 have lower CD toxicity than wild type and other transgenics.

Transgenic lines and gluten immunogenicity by anti-gliadin 33-mer moAb

The 33-mer peptide derived from α-2 gliadin (residues 57-89) is one of the most highly antigenic peptides identified to date (Shan *et al.*, 2002; Tye-Din *et al.*, 2010). This peptide was identified as the primary initiator of the inflammatory response to gluten in CD patients This 33-mer fragment is naturally formed by digestion with gastric and intestinal proteases, binds to DQ2 after deamidation by tissue transglutaminases (tTG), contains a cluster of 6 T-cell epitopes, and is the most immunodominat peptide in patients with CD after eating wheat based products (Shan *et al.*, 2002; Tye-Din *et al.*, 2010).

The G12 monoclonal antibody (moAb) is able to recognize with great sensitivity peptides (besides the 33-mer) inmunotoxic for patients with CD. The sensitivity and epitope preferences of this antibody was found to be useful for detecting gluten-relevant peptides to infer the potential toxicity of cereals for patients with CD (Morón *et al.*, 2008*b*; Comino *et al.*, 2011). Immunotoxic properties of the seven lines were studied with G12 in order to study the reduced toxicity profile.

As shown in Fig. 4, the reactive gluten identified by G12 moAb strongly decreased in transgenic lines for Plasmid Combinations 3, 4, 5, 6, and 7, but increased in wheat lines for Combination 2. We observed reductions of 84, 93, 88, and 92% gluten content by competitive G12 moAb in transgenic lines for plasmid Combinations 4, 5, 6, and 7, respectively, in comparison with the wild type. G12 values for individual lines are shown in Figure 9.

Discussion

Gluten proteins are responsible for the unique viscoelastic properties of wheat flour but also for triggering pathologies in susceptible individuals, of which coeliac disease (CD) and non-coeliac gluten sensitivity may affect up to 8% of the population. Currently, the only effective treatment for affected persons is a strict gluten-free diet.

IR sequences were designed using different regions of the gliadin and LMW coding sequences and combined to form 7 different RNAi constructs (Fig. 1). These combinations promoted differential changes in the prolamin (gliadins and glutenins) composition of the transgenic plants generated (Fig. 2). Interestingly, pleotropic effects (i.e. stronger down-regulation of the targeted gliadin groups) were observed when two RNAi constructs with IR fragments targeting different groups were combined in the same transgenic plant. For example, down-regulation of α- and γ-gliadins was stronger in Combination 5 (pDhp_ω/α and the pghpg8.1 vectors) than when they were used alone (Combinations 4 and 1, respectively). Similarly, the down-regulation of ω-gliadins was significantly stronger in the Combination 7, in which the pDhp_ω8ZR was co-transformed with the pDhp_αβ/ZR, than when only the pDhp_ω8ZR was used (Combination 3). The same was observed in the α-gliadins, with a stronger down-regulation in the Combinations 6 and 7 (both with the pDhp_αβ/ZR plasmid plus a second plasmid targeting different prolamins) than in the Combination 2 (the pDhp_αβ/ZR plasmid alone).

In summary, the on-target silencing was normally higher when a second IR sequence (targeting a different gliadin group) was co-transformed, which might be a consequence of 1) off-target activity of the second IR fragment, 2) reduction in the cells of potential off-target mRNA sequences that compete with the on-target gliadin mRNAs for recognition and degradation by the post-transcriptional silencing machinery, or 3) a

combination of both effects. When short interfering RNAs (siRNAs) from a certain double stranded RNA (dsRNA) sequence are formed, they lead the RNA-induced silencing complex (RISC) to recognise homologous mRNA sequences for their cleavage and degradation (Hammond *et al.*, 2000). It has been previously reported that non-specific down-regulation can occur during RNAi since 100% identity between the silencing siRNA and the target gene is not absolutely required to promote gene silencing (Xu *et al.*, 2006; Senthil-Kumar *et al.*, 2007). In fact, only 14 nucleotides (or even less) of sequence complementarity between siRNA and mRNA can lead to gene silencing in plants (Jackson and Linsley, 2004; Jackson *et al.*, 2006). Therefore, by reducing the number of possible off-target sequences in the cell, the probability of the siRNA to find the on-target sequences is enhanced, and so is the efficiency of the gene silencing.

The identity observed between the IR fragments and the non-targeted prolamin fractions (40-60%), would make it possible for there to be, among the pool of siRNAs formed from each IR fragment, some with total (or almost total) identity with non-targeted prolamins. This fact could explain the off-target silencing observed with some plasmid combinations. For example, α- and γ-gliadins were down-regulated with |Combination 3 that only contains the LMW and omega8 IR fragments (Fig. 2). Similarly, Combination 6 showed off-target reduction of γ-gliadins and LMW glutenins (Fig. 2). These results fit with the off-target down-regulation of γ-gliadins and LMW glutenins reported in previous studies when using the IR ω/α (omega and alpha RNAi fragments) (Gil-Humanes *et al.*, 2010; 2014) (Gil-Humanes *et al.*, 2011). By contrast, Plasmid Combinations 1 and 2 were highly specific to their on-target gliadin group (γ- and α-gliadins, respectively), and did not silence any of the other prolamin groups. Moreover, the high number of gamma gliadin fragments found by LS-MS / MS for Combination 1 could be explained as a consequence of the high specificity of the pgphp8.1 fragment in silencing just some of the proteins in this family. The analysis of the 11 active genes reported for the cultivar Chinese Spring (Anderson *et al.*, 2013) shows that the identity between those sequences and the pgphp8.1 fragment varies from less than 60% to nearly 100%. Two of the proteins with lower identity (ɣ-gliadin 3, 60.4%, and ɣ-gliadin 4, 61.6%) are those containing an odd number of cysteine residues, supporting the hypothesis that the amount of gamma gliadin as measured by RP-HPLC in our lines may be underestimated because they could be part of the polymer fraction.

The silencing of gliadins and LMW glutenins did not affect total protein and starch content or tkernel weight In contrast, quality parameters like SDSS and the gliadins to glutenins ratio were affected by the silencing of gliadins. The SDS sedimentation test provides information on the protein quantity and the quality of ground wheat and flour samples (Carter *et al.*, 1999). Positive correlations were observed between sedimentation volume and gluten strength, and hence, SDSS test should be an effective small-scale test for quality assessment. As Plasmid Combinations 3, 5, 6 and 7 had lower SDSS values than wild type, they might exhibit also lower gluten strength. The fact that Plasmid Combination 4 showed a SDSS value similar to the wild type, with lower gliadin content but significantly higher contents of glutenins may indicate that glutenins are major determinants of SDSS as previously reported (Piston *et al.*, 2011).

Because we did not perform quantitative LC-MS/MS analysis, the number of protein fragments and immunotoxic epitopes identified do not necessarily reflect their abundance in the total gluten protein. In addition, , not all epitopes are equally immunogenic for CD (Tye-Din *et al.*, 2010). The number of α-gliadin peptides and epitopes were well correlated with α-gliadin quantified by RP-HPLC. The DQ2.5-glia-α3 epitope, downstream of the 33-mer fragment, was the least abundant probably due to its digestibility with Trypsin. Because γ-gliadins contain 5 to 10 epitopes per sequence (Salentijn *et al.*, 2012) a high number of these epitopes was identified in almost all lines analysed, most prevalent being the epitopes DQ2.5-glia-γ4c/DQ8-glia-γ1a and DQ2.5-glia-γ5. In general, a lower number of CD epitopes was observed in transgenic lines generated using two plasmids, with the exception of Plasmid Combination 6.

As expected, the changes observed in the prolamin fractions led to changes in the immuno toxicity of lines generated from the different combinations of plasmids, as determined by the G12 moAb assay (Fig. 4). The 33-mer peptide is highly resistant to digestion and one of the main immunodominant toxic peptide in celiac patients (Tye-Din *et al.*, 2010). The sensitivity and epitope preferences of the G12 antibody has been found to be useful for detecting gluten-relevant peptides to predict the potential toxicity of cereal foods for patients with CD (Morón *et al.*, 2008*a*). Moreover, the reactivity of G12 moAb with cereal storage proteins of different varieties of cereals has been correlated with the known dietary immunotoxicity of the different grains (Comino *et al.*, 2011). All the combinations of plasmids, except Combination 1, resulted in a reduction

of the reactivity of the G12 moAb, and consequently a decrease in the immunotoxicity of the gluten proteins. The level of reduction of the different combinations was strongly correlated (92.38%, $p<0.001$) with the level of reduction of the α-gliadin fraction, and was also well correlated with the number of α-gliadin epitopes found in MS analysis. This observation may be explained by the fact that the G12 moAb mainly recognises the hexapeptide QPQLPY found in the 33-mer peptide of α2-gliadin, although it also binds to other related peptide variants of immunotoxic gluten proteins (Morón *et al.*, 2008*a*).

Our present results indicate that RNAi can be a very effective approach for obtaining wheat lines without, or with very low levels of gliadins and LMW glutenins, the major gluten proteins containing epitopes triggering coeliac disease. Six of the plasmid combinations tested showed strong reduction in the gluten content as measured by competitive anti-gliadin 33-mer moAb and in two combinations, this reduction was higher than 90% by comparison with the wild type. Transgenic lines from three plasmid combinations were found to be totally devoid of CD epitopes from the highly immunogenic α- and ω-gliadins. These lines have potential to be used directly in breeding programs for obtaining wheat cultivars suitable for coeliac or other glutenintolerant patients, and the effective Plasmid Combinations identified may be used to down-regulate toxic epitopes in other wheat backgrounds.

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CLAIMS:

1. A polynucleotide that is at least 90% identical to a sequence comprising at least one sequence pair (a1-b1) separated by a spacer sequence in which a1 and b1 are selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 and SEQ ID NO: 8, wherein
   - if a1 is SEQ ID NO: 1, b1 is SEQ ID NO: 5; if a1 is SEQ ID NO: 5, b1 is SEQ ID NO: 1
   - if a1 is SEQ ID NO: 2, b1 is SEQ ID NO: 6; if a1 is SEQ ID NO: 6, b1 is SEQ ID NO: 2:
   - if a1 is SEQ ID NO: 3, b1 is SEQ ID NO: 7; if a1 is SEQ ID NO: 7, b1 is SEQ ID NO: 3; and
   - if a1 is SEQ ID NO: 4, b1 is SEQ ID NO: 8. If a1 is SEQ ID NO: 8, b1 is SEQ ID NO: 4.

2. The polynucleotide of claim 1, wherein the sequence comprises two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, in which the sequences a1, a2, b1 and b2 differ among themselves and are selected from SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO:7 and SEQ ID NO: 8, in the following form:
   - If a1 is SEQ ID NO: 3 or SEQ ID NO: 7, b1 is SEQ ID NO: 7 or SEQ ID NO: 3, and a2 is SEQ ID NO: 4 or SEQ ID NO: 8, b2 is SEQ ID NO: 8 or SEQ ID NO: 4.
   - If a1 is SEQ ID NO: 4 or SEQ ID NO: 8, b1 is SEQ ID NO: 8 or SEQ ID NO: 4 and a2 is SEQ ID NO: 3 or SEQ ID NO: 7 and b2 is SEQ ID NO: 7 or SEQ ID NO: 3.

3. The polynucleotide as claimed in any of claims 1 or 2, wherein the spacer sequence is SEQ ID NO: 9.

4. The polynucleotide as claimed in any of claims 1 to 3, wherein the sequence also comprises a gene expression-regulating sequence functionally linked to its 5' end.

5. The polynucleotide as claimed in any of claims 1 to 4, wherein the gene expression-regulating sequence is SEQ ID NO: 10.

6. An RNA sequence coded for by the polynucleotide of claim 1, wherein the RNA sequence is capable of forming a hpRNA (hairpin RNA) in which the sequence coded for by a1 completely hybridizes with the sequence coded for by b1.

7. An RNA sequence coded for by the polynucleotide as claimed in claim 2, capable of forming a hpRNA in which the sequence coded for by the pair a1-a2 completely hybridizes with the sequence coded for by the pair b2-b1.

8. A hpRNA formed from the RNA sequence of claim 6 or 7.

9. A siRNA generated from the sequence of the hpRNA of claim 8.

10. An expression vector that comprises at least one polynucleotide as claimed in any of claims 1 to 5.

11. The expression vector of claim 10, wherein the expression vector comprises a polynucleotide that comprises the sequence pair (a1-b1) separated by a spacer sequence, wherein
a1 is SEQ ID NO: 1 and b1 is SEQ ID NO: 5 or a1 is SEQ ID NO: 5 and b1 is SEQ ID NO: 1.

12. The expression vector of claim 10, wherein the expression vector comprises the sequence pair (a1-b1) separated by a spacer sequence, wherein
a1 is SEQ ID NO: 2 and b1 is SEQ ID NO: 6 or a1 is SEQ ID NO: 6 and b1 is SEQ ID NO: 2.

13. The expression vector of claim 10, wherein the expression vector comprises a polynucleotide that comprises two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, wherein
a1 is SEQ ID NO: 3 or SEQ ID NO: 7, b1 is SEQ ID NO: 7 or SEQ ID NO: 3, and a2 is SEQ ID NO: 4 or SEQ ID NO: 8, b2 is SEQ ID NO: 8 or SEQ ID NO: 4; or

a1 is SEQ ID NO: 4 or SEQ ID NO: 8, b1 is SEQ ID NO: 8 or SEQ ID NO: 4 and a2 is SEQ ID NO: 3 or SEQ ID NO: 7 and b2 is SEQ ID NO: 7 or SEQ ID NO: 3.

14. An isolated cell transfected with at least one expression vector as claimed in any one of claims 10 to 13.

15. The isolated cell of claim 14, wherein the cell is transfected with two expression vectors, wherein the expression vectors are the vectors described in claims 11 and 12.

16. The isolated cell of claim 14, wherein the cell is transfected with two expression vectors, wherein the expression vectors are the vectors described in claims 11 and 13.

17. An isolated cell transfected with two expression vectors,

- wherein the first expression vector comprises two sequence pairs (a1-a2) and (b2-b1) separated by a spacer sequence, in which the sequences a1, a2, b1 and b2 differ among themselves and are selected from SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13 and SEQ ID NO: 14, in the following form: wherein,

  a1 is SEQ ID NO: 11 or SEQ ID NO: 12, b1 is SEQ ID NO: 12 or SEQ ID NO: 11, and a2 is SEQ ID NO: 13 or SEQ ID NO: 14, b2 is SEQ ID NO: 14 or SEQ ID NO: 13; or

  a1 is SEQ ID NO: 13 or SEQ ID NO: 14, b1 is SEQ ID NO: 14 or SEQ ID NO: 13 and a2 is SEQ ID NO: 11 or SEQ ID NO: 12 and b2 is SEQ ID NO: 12 or SEQ ID NO: 11; and
- wherein the second expression vector comprises the sequence pair (a1-b1) separated by a spacer sequence, wherein
- a1 is SEQ ID NO: 15 and b1 is SEQ ID NO: 16 or a1 is SEQ ID NO: 16 and b1 is SEQ ID NO: 15.

18. A genetically modified plant wherein said plant comprises the transfected cell as claimed in any of claims 14 to 17.

19. A genetically modified plant according to claim 18 wherein the polynucleotide is integrated in a stable form.

20. A genetically modified plant with decreased gliadin and/or glutenin content comprising, in its genome, a polynucleotide as defined in any one of claims 1 to 5, wherein said plant exhibits reduced gliadin and/or glutenin content compared to a control plant.

21. The plant as claimed in any of claims 18 to 20, wherein said plant belongs to the genus *Triticum.*

22. The plant as claimed in claim 21 wherein said plant is selected from the species *Triticum aestivum* or *Triticum turgidum*.

23. The plant as claimed in claim 22, wherein said plant is a Bobwhite cultivar or a Don Pedro cultivar.

24. A seed derived from the plant as claimed in any of claims 18 to 23 wherein said seed comprises the polynucleotide of any of claims 1 to 5.

25. The pollen, propagule, progeny, or part of the plant derived from any of the plants as claimed in any of claims 18 to 23 wherein said pollen, propagule, progeny, or part comprise the polynucleotide of any of claims 1 to 5.

26. The use of the polynucleotide as claimed in any of claims 1 to 5, a vector as claimed in any of claims 10 to 13 or a cell as claimed in any of claims 14 to 17 for silencing at least one of alpha-, gamma- and omega-gliadins or the LMW glutenin of *Triticum* spp.

27. A method for silencing at least one of alpha-, gamma- and omega-gliadins or the LMW glutenin of *Triticum* spp using the polynucleotide of any of claims 1 to 5, a vector as claimed in any of claims 10 to 13 or a cell as claimed in any of claims 14 to 17.

28. The use of the seed as claimed in claim 24 for the preparation of flour, a food composition, a vitamin or nutritional supplement.

29. A food composition prepared from a seed as claimed in claim 24.

30. A method for obtaining the genetically modified plant as claimed in any of claims 18 to 23, comprising the following:

a. selecting a part of the plant,

b. transfecting the cells of the part of the plant of paragraph (a) with a vector as claimed in any of claims 10 to 13,

c. selecting the transfected cell of paragraph (b) that comprises the polynucleotide as claimed in any of claims 1 to 5,

d. regenerating at least one plant derived from the cell selected in paragraph (c),

e. selecting one or more plants regenerated according to paragraph (d) in which the polynucleotide is transcribed into an hpRNA, and

f. selecting one or more plants obtained according to paragraph (e) that show silencing of at least one of the following: alpha-, gamma-, omega-gliadins and LMW glutenin in its seeds.

31. A method for producing a food composition with reduced gliadin and/or glutenin content comprising producing a transgenic wheat plant with reduced gliadin and/or glutenin content comprising integrating the polynucleotide of any of claims 1 to 5 into the genome of a plant and silencing at least one of alpha-, gamma- and omega-gliadins and LMW glutenin in the seeds of said plant, producing seeds from said plant in which at least one of alpha-, gamma- and omega-gliadins and LMW glutenins are silenced and preparing a food composition from said seeds.

32. A method for modulating an immune response to gliadins and/or glutenins, the method comprising providing a diet of a food composition according to claim 29 to a subject in need thereof.

33. A method for affecting or modulating a T-cell response to gluten in a subject, the method comprising providing a diet of a food composition according to claim 29 to a subject in need thereof.

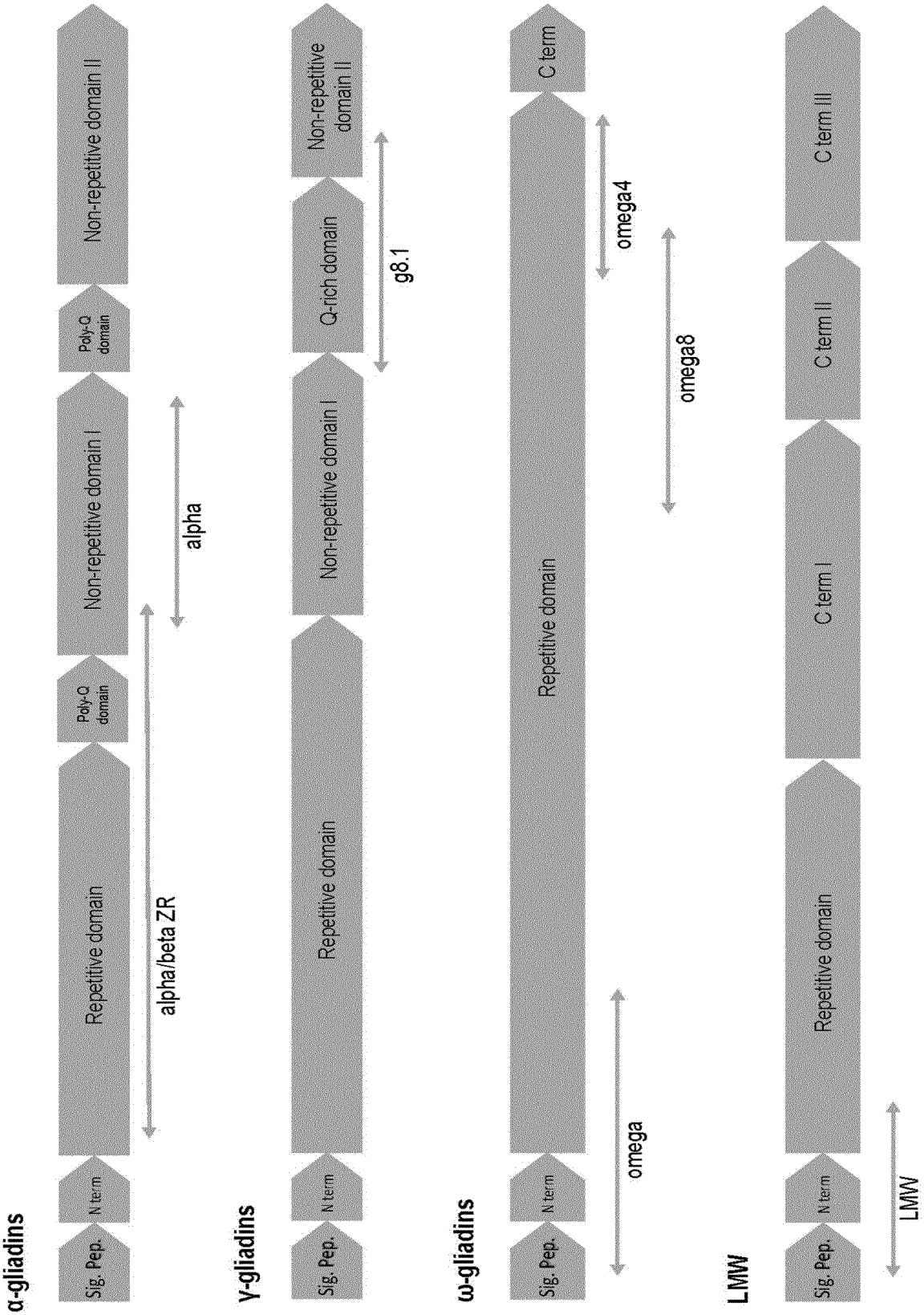

α-gliadins
Sig. Pep.
N term
Repetitive domain
Poly-Q domain
Non-repetitive domain I
Poly-Q domain
Non-repetitive domain II
alpha/beta ZR
alpha
γ-gliadins
Sig. Pep.
N term
Repetitive domain
Non-repetitive domain I
Q-rich domain
Non-repetitive domain II
g8.1
ω-gliadins
Sig. Pep.
N term
Repetitive domain
C term
omega
omega4
omega8
LMW
Sig. Pep.
N term
Repetitive domain
C term I
C term II
C term III
LMW

FIG. 1A

Plasmid name	Sequence name	Sequence length (bp)	RNAi fragment length (bp)
pghpg8.1	g8.1	169	169
pDhp_α/βZR	alpha/beta ZR	377	377
pDhp_ω8ZR	LMW	132	305
	omega8	173	
pDhp_ω4ZR	omega4	109	109
pDhp_ω/α	alpha	170	361
	omega	191	

FIG. 1B

Plasmid Combination	Plasmid 1	Plasmid 2
0 (wt)	NA	NA
1	pghpg8.1	NA
2	pDhp_α/βZR	NA
3	pDhp_ω8ZR	NA
4	pDhp_ω/α	NA
5	pghpg8.1	pDhp_ω/α
6	pDhp_α/βZR	pDhp_ω4ZR
7	pDhp_α/βZR	pDhp_ω8ZR

FIG. 1C

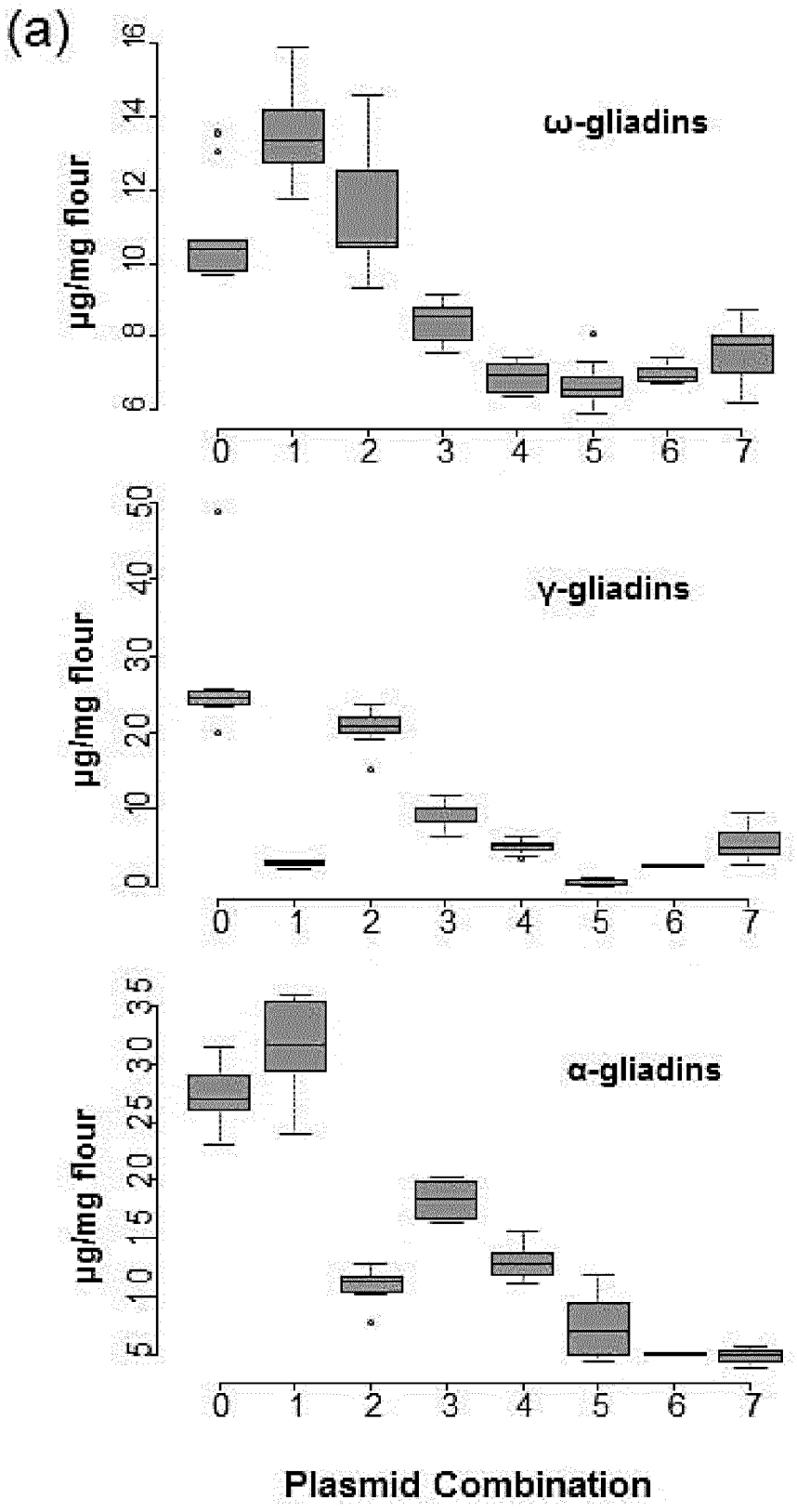

(a)
ω-gliadins
γ-gliadins
α-gliadins
µg/mg flour
Plasmid Combination

FIG. 2

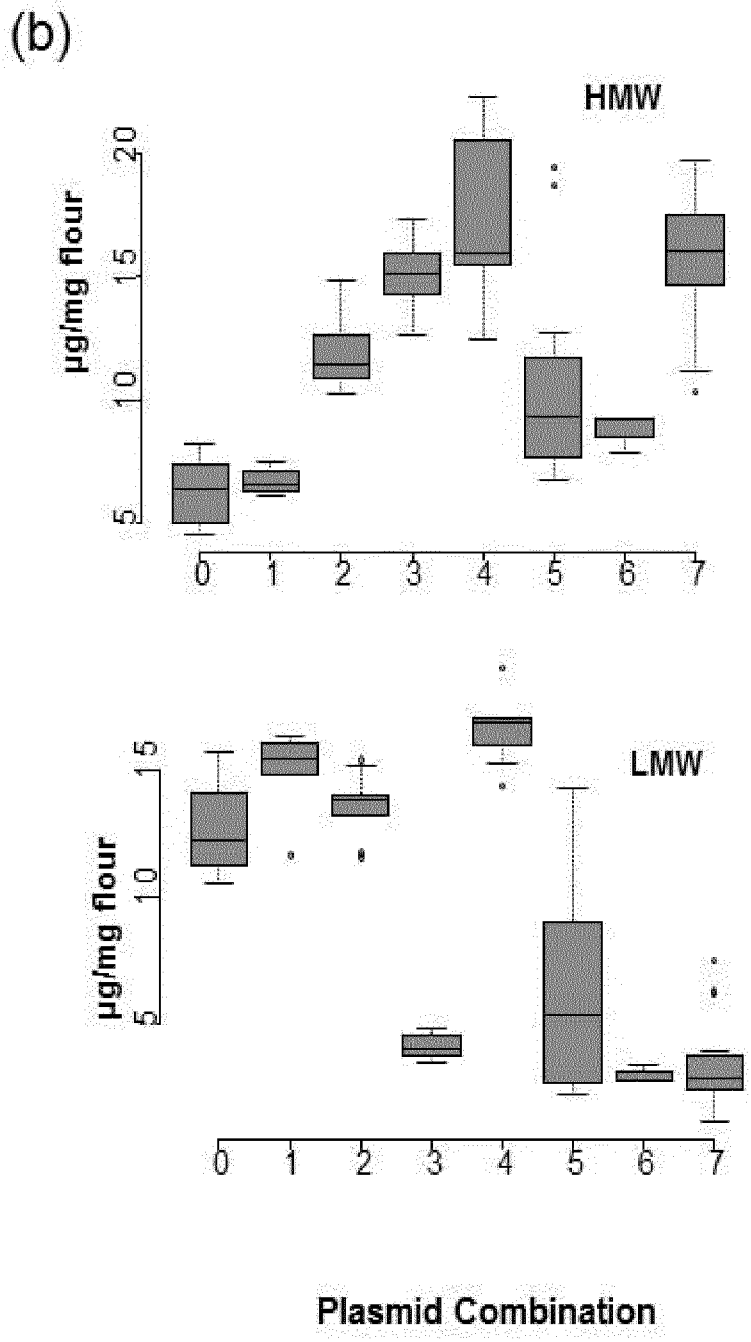

(b)
HMW
µg/mg flour
5
10
15
20
0
1
2
3
4
5
6
7
LMW
µg/mg flour
5
10
15
0
1
2
3
4
5
6
7
Plasmid Combination

FIG. 2 (cont.)

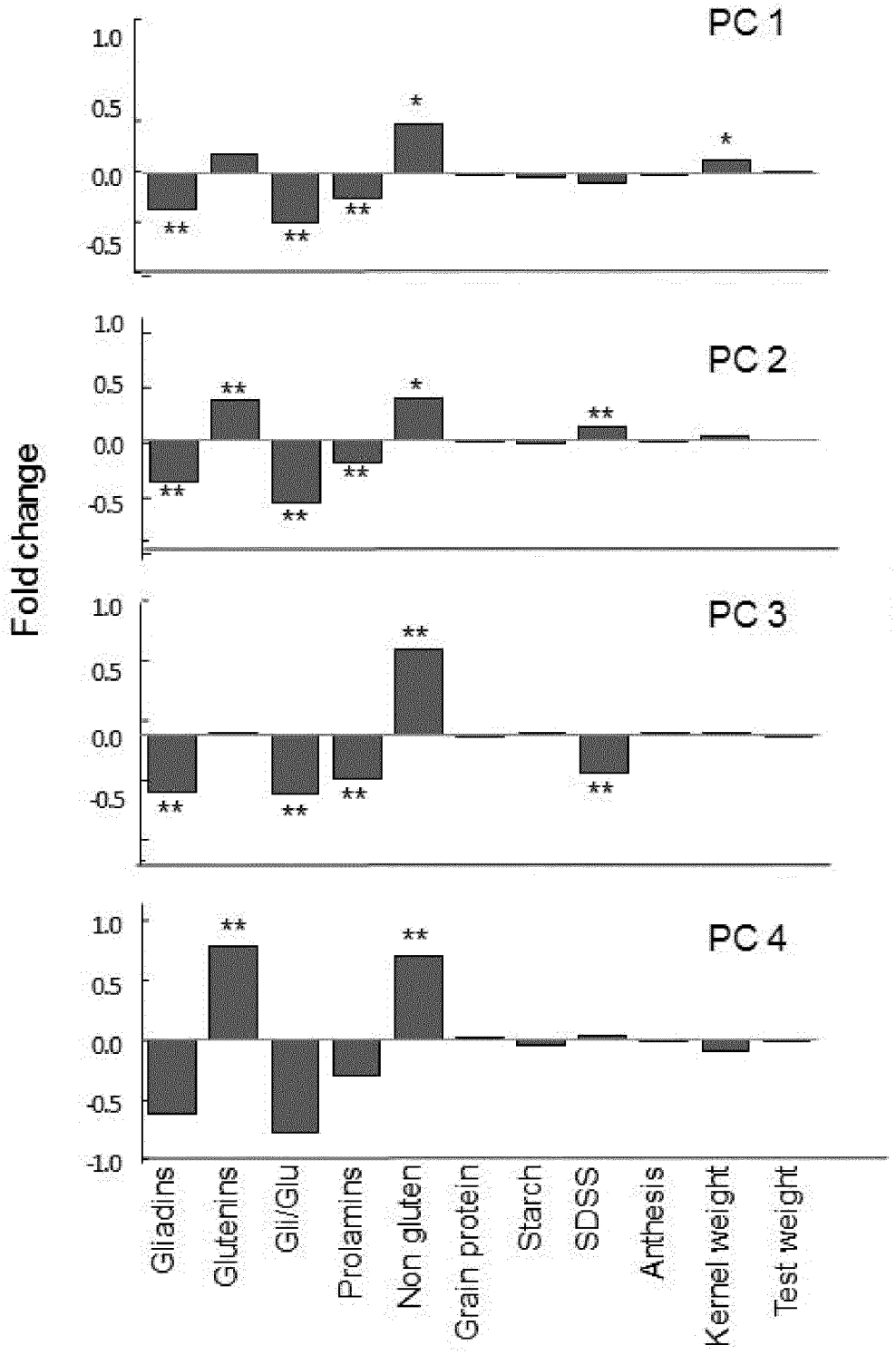

PC 1
PC 2
PC 3
PC 4
Fold change
1.0
0.5
0.0
-0.5
-1.0
Gliadins
Glutenins
Gli/Glu
Prolamins
Non gluten
Grain protein
Starch
SDSS
Anthesis
Kernel weight
Test weight

FIG. 3

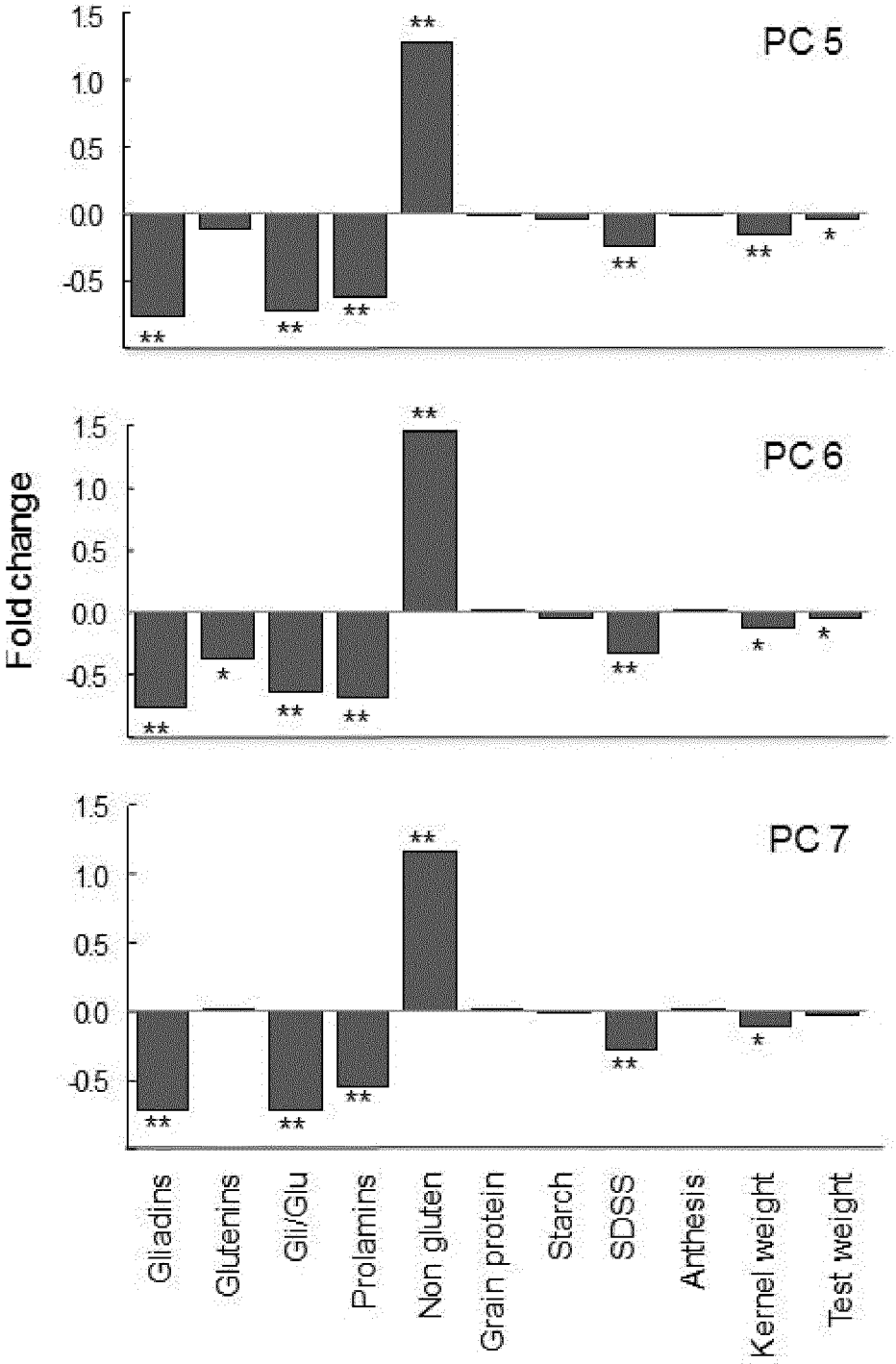

PC 5
PC 6
PC 7
Fold change
1.5
1.0
0.5
0.0
-0.5
Gliadins
Glutenins
Gli/Glu
Prolamins
Non gluten
Grain protein
Starch
SDSS
Anthesis
Kernel weight
Test weight

FIG. 3 (cont.)

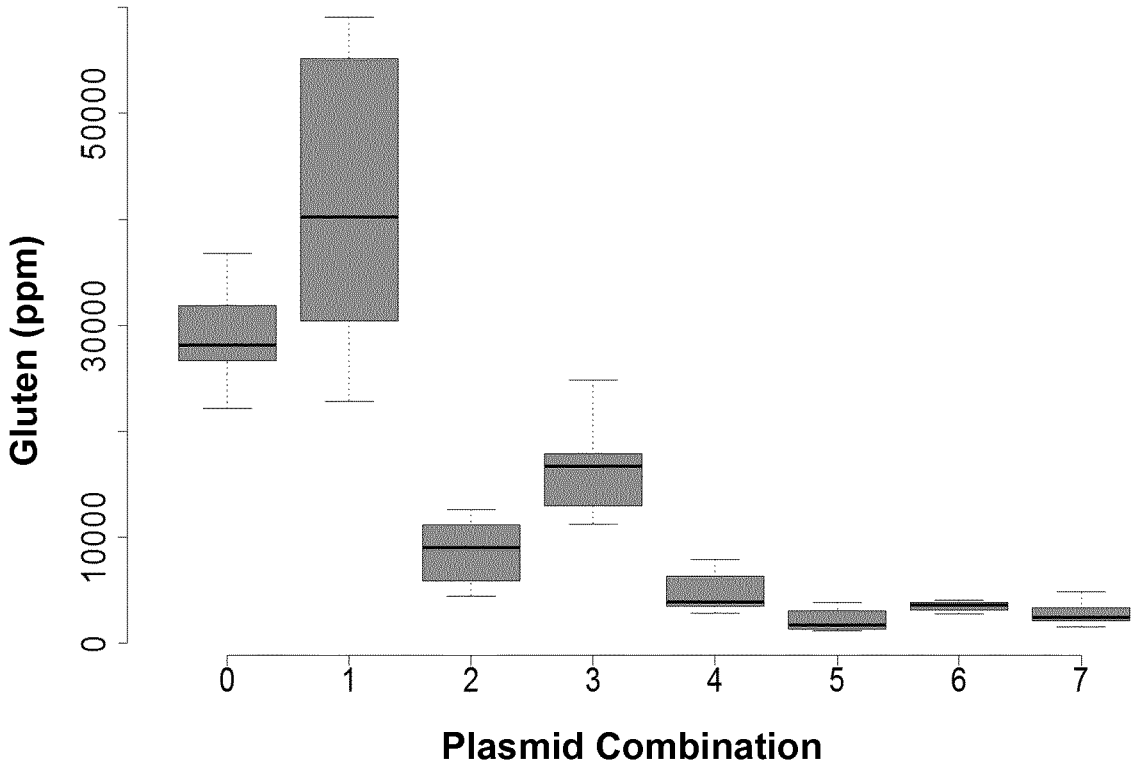

Gluten (ppm)
0
10000
30000
50000
0
1
2
3
4
5
6
7
Plasmid Combination

FIG. 4

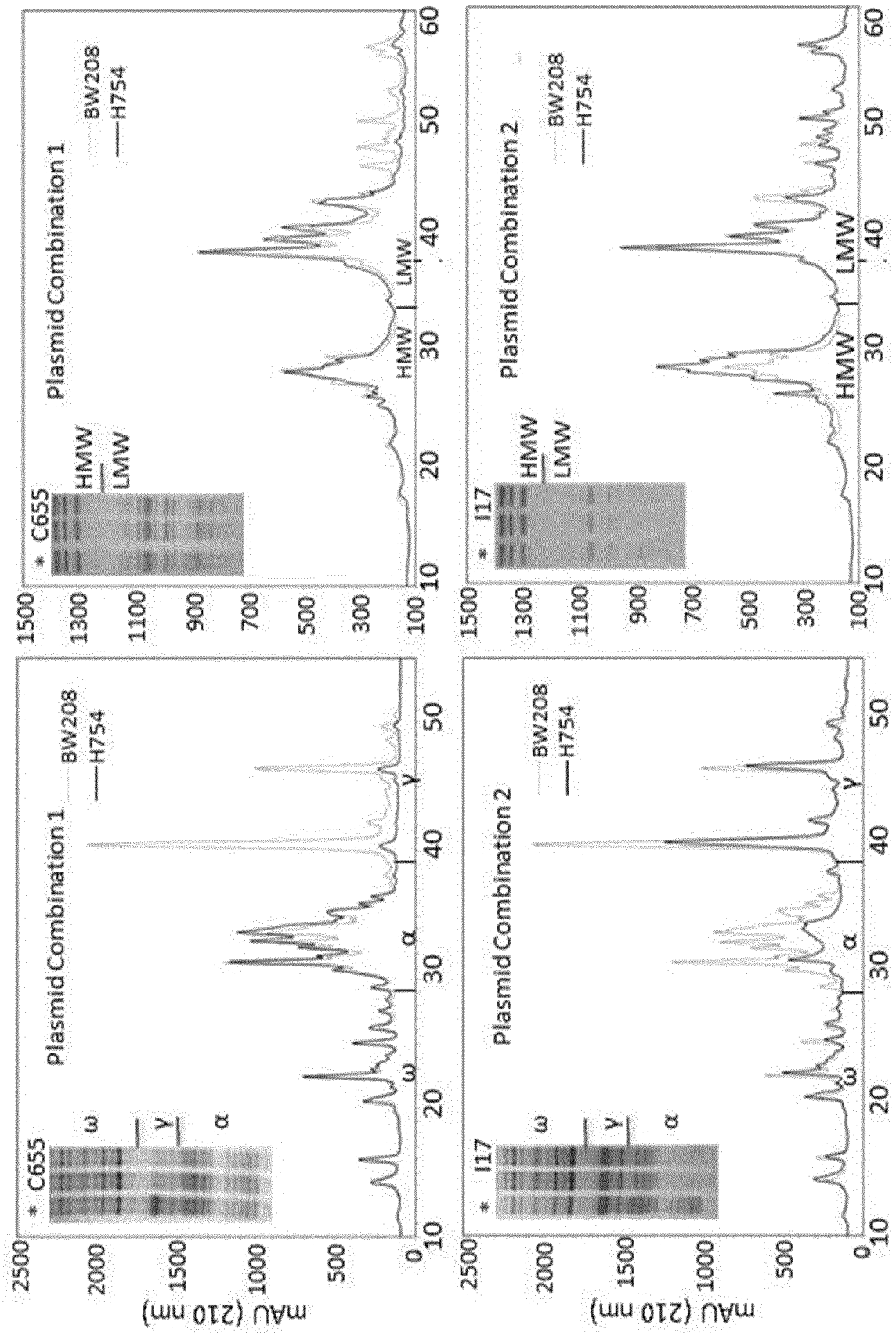

Plasmid Combination 1
Plasmid Combination 2
BW208
H754
* C655
* I17
ω
γ
α
HMW
LMW
mAU (210 nm)

FIG. 5

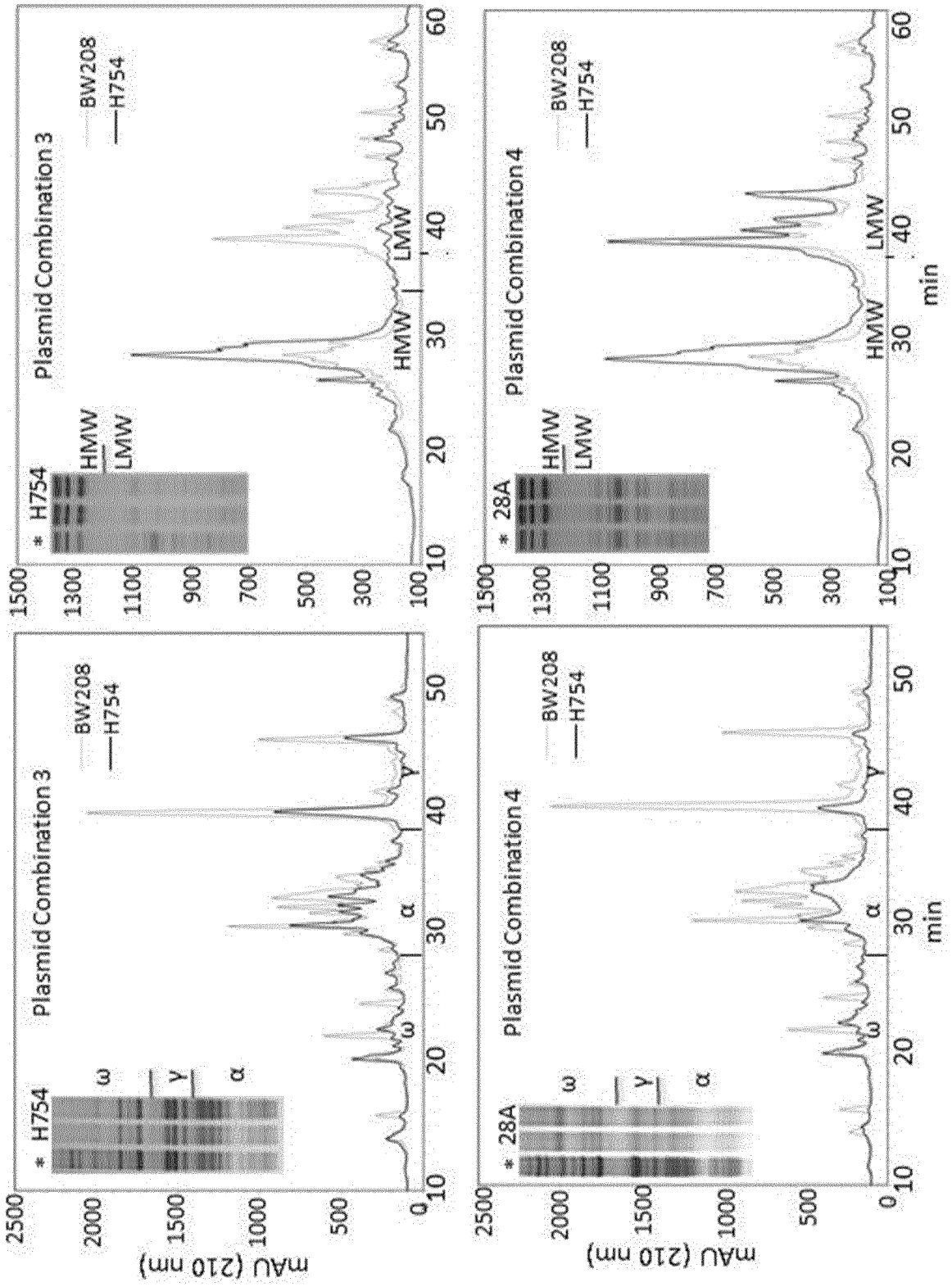

Plasmid Combination 3
Plasmid Combination 4
BW208
H754
* H754
* 28A
HMW
LMW
ω
γ
α
mAU (210 nm)
min

FIG. 5 (cont.)

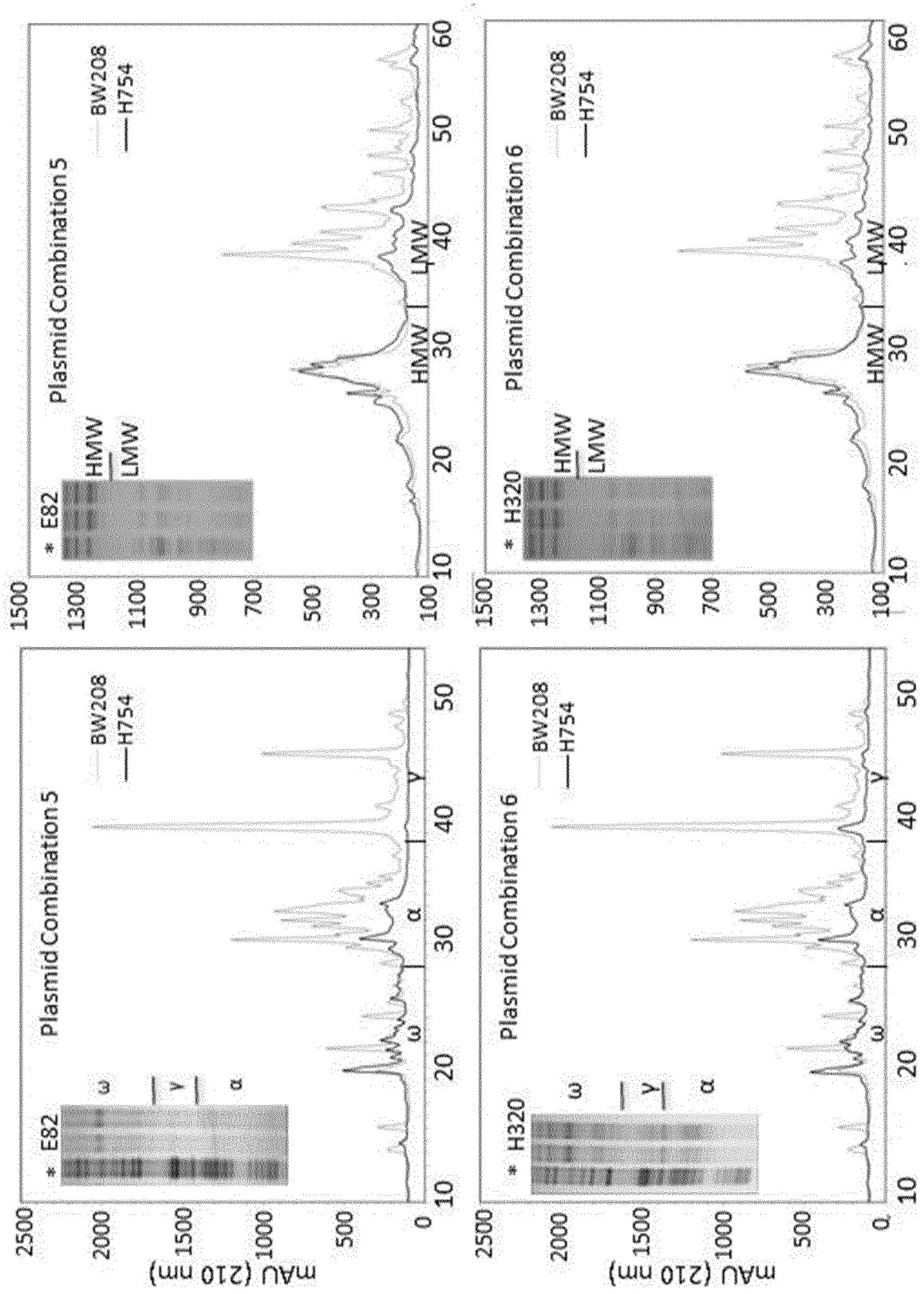

Plasmid Combination 5
BW208
H754
* E82
ω
γ
α
HMW
LMW
mAU (210 nm)
Plasmid Combination 6
* H320

FIG. 5 (cont.)

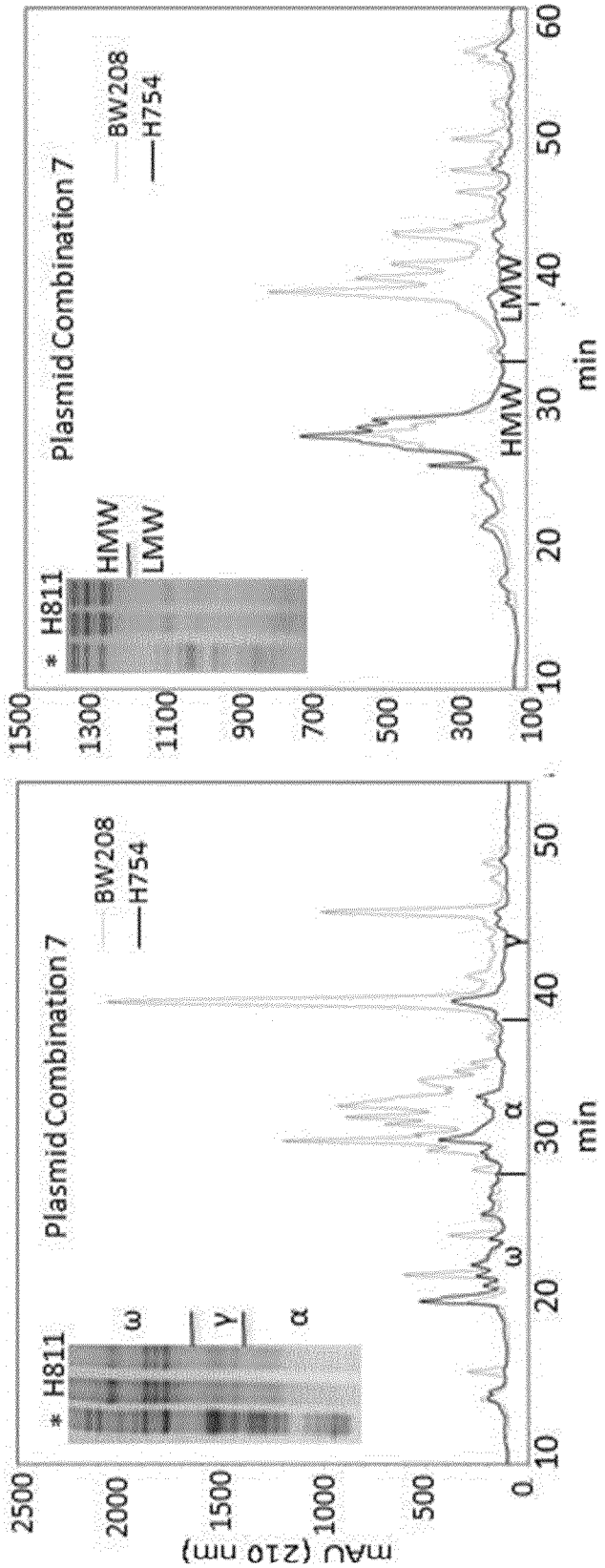

Plasmid Combination 7
BW208
H754
* H811
ω
γ
α
HMW
LMW
mAU (210 nm)
min

FIG. 5 (cont.)

g8.1

CATCATCATGCAGCAGCAGCAGCAACAACAACAACAACAAGGCATGCATATCTTTCTGCCA
CTATCTCAGCAGCAACAGGTGGGTCAAGGTTCTCTAGTCCAAGGCCAGGGCATCATCCAA
CCACAACAACCAGCTCAATTGGAGGCGATCAGATCATTGGTGTTGCAA

alpha/beta ZR

ATTGCAGCCACAAAATCCATCTCAGCAACAGCCACAAGAGCAAGTTCCATTGGTACAACAA
CAACAATTTCCAGGGCAGCAACAACAATTTCCACCACAACAGCCATATCCGCAGCCGCAAC
CATTTCCATCACAACAACCATATCTGCAACTGCAACCATTTCCGCAGCCGCAACCATTTCC
GCCACAACTACCATATCCGCAGCCGCAATCATTTCCACCACAACAACCATATCCACAACAG
CAACCACAGTATCTACAACCACAACAACCAATTTCGCAGCAACAAGCACAACAACAACAAC
AACAACAACAACAACAACAACAACAAATCCTTCAACAAATTTTGCAACAACAACTGATTCCA
TGCATGGATG

LMW

CCTATCTTTGTCCTCCTTGCCGTTGCAGCGACAAGTGCCATTGCACAAATGGAGACTAGCC
ACATCCCTGGCTTGGAGAAACCATCGCAACAACAACCATTACCACTACAACAAATATTATG
GTACCACCAA

omega8

CAGCAACCCCAACAACCATTCCCCCAGCAACCACACCAACCTCAACAACCATATCCACAAC
AACAACCATATGGGAGTAGTCTTACAAGCATCGGTGGCCAATGAAATGAACACCTGTAATA
CAAGACGGATGGATCATCGTTGTTTAGTCAATGAGCGTTCAATGTAACGATG

omega4

TATGGTATTGGAAATTGTTGTTGGGGGAATTGTTGTGGTGGTTGCTGTGGGAATTGTTGTT
GGGGGAATTGTTGTTGTTCAGGGGATTGTTGAAGGGCGAATTCGGAGC

alpha

CAACAACAACTGATTCCATGCAGGGATGTTGTATTGCAACAACACAGCATAGCGTAT
GGAAGCTCACAAGTTTTGCAACAAAGTACTTACCAGCTGGTGCAACAATTGTGTTGTCAGC
AGCTGTGGCAGATCCCCGAGCAGTCGCGGTGCCAGGCCATCCACAATGTTAT

omega

CCTTCCTCATCTTTGTCCTCCTTGCCATGGCGATGAAGATCGCCACTGCCGCTAGG
GAGTTAAACCCTAGCAACAAAGAGTTACAATCACCTCAACAATCATTTTCCCATCAACAACA
ACCATTTCCACAGCAGCCATATCCACAACAACCATATCCATCACAGCAACCATATCCATCG
CAACAACCATTT

FIG. 6

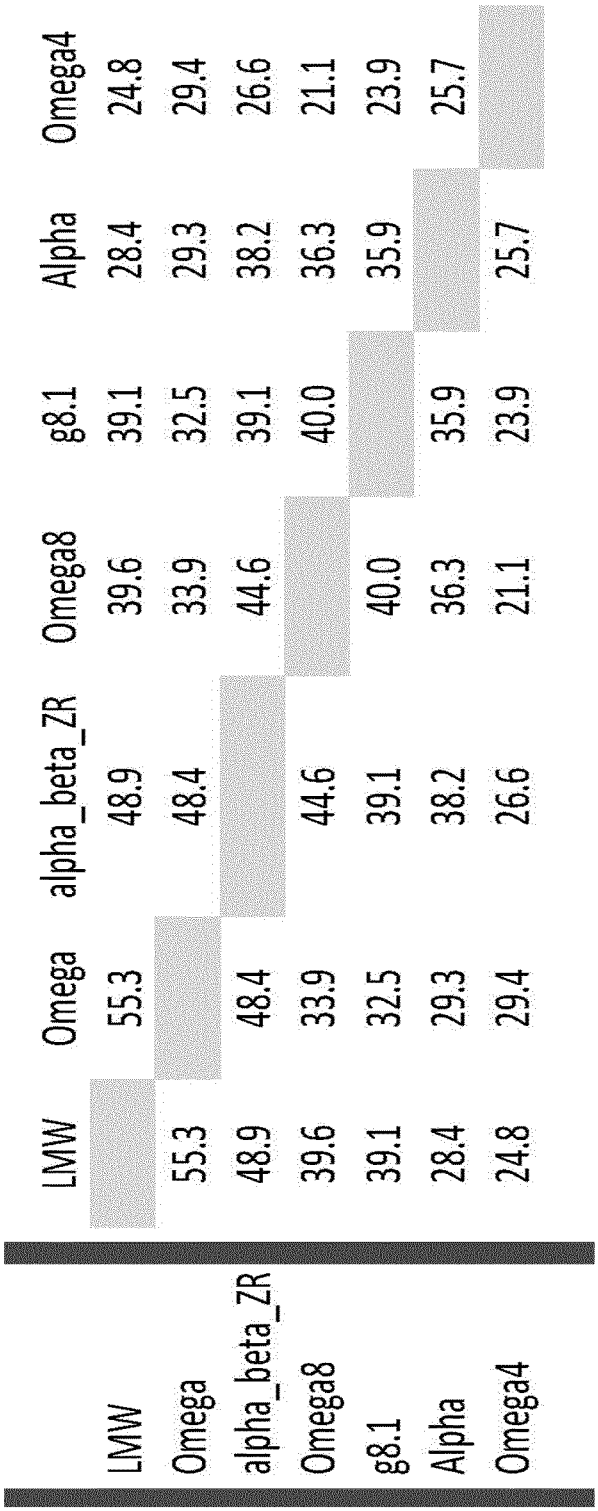

LMW
Omega
alpha_beta_ZR
Omega8
g8.1
Alpha
Omega4
LMW 55.3 48.9 39.6 39.1 28.4 24.8
Omega 55.3 48.4 33.9 32.5 29.3 29.4
alpha_beta_ZR 48.9 48.4 44.6 39.1 38.2 26.6
Omega8 39.6 33.9 44.6 40.0 36.3 21.1
g8.1 39.1 32.5 40.0 35.9 23.9
Alpha 28.4 29.3 38.2 36.3 35.9 25.7
Omega4 24.8 29.4 26.6 21.1 23.9 25.7

FIG. 7

Line	Plasmid combination
BW208	**0**
C655	1
D623	1
G804	2
I17	2
I20	2
H980	3
H754	3
28A	4
28B	4
D783	4
E33	5
E42	5
E82	5
E83	5
H320	6
H687	7
H702	7
H964	7
H801	7
H811	7
H761	7

FIG. 8

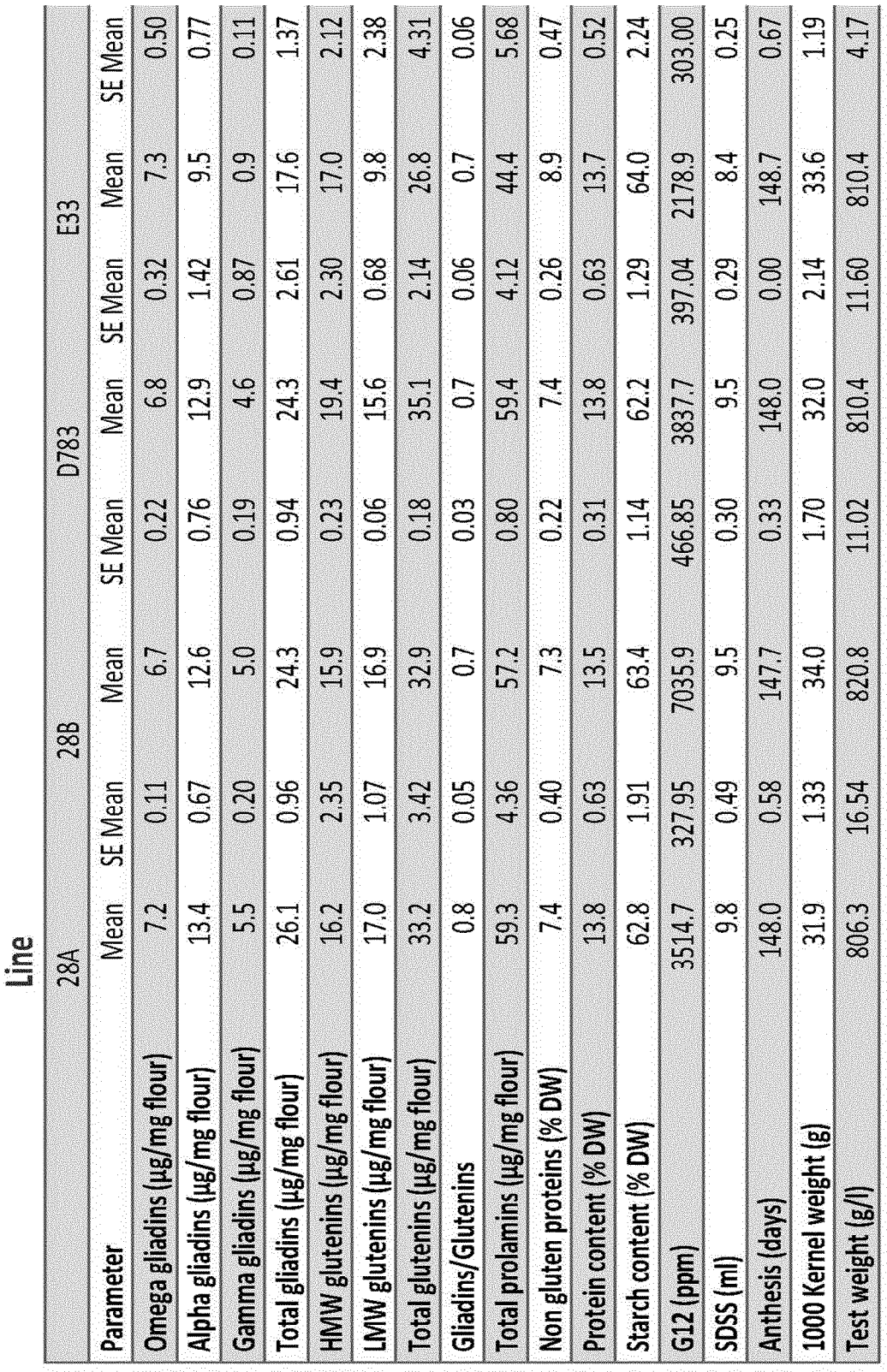
FIG. 9

	Line							
	BW208		C655		D623		G804	
Parameter	Mean	SE Mean	Mean	SE Mean	Mean	SE Mean	Mean	SE Mean
Omega gliadins (µg/mg flour)	10.8	0.49	13.9	0.99	13.2	0.72	11.4	0.94
Alpha gliadins (µg/mg flour)	27.5	0.87	33.1	1.88	29.6	3.31	11.5	0.09
Gamma gliadins (µg/mg flour)	26.7	2.83	3.0	0.18	2.8	0.39	21.5	0.37
Total gliadins (µg/mg flour)	65.0	3.08	50.0	2.85	45.6	4.29	44.4	1.12
HMW glutenins (µg/mg flour)	6.3	0.44	6.7	0.43	6.8	0.17	10.9	0.36
LMW glutenins (µg/mg flour)	12.6	0.62	14.2	1.34	15.6	0.46	14.6	0.69
Total glutenins (µg/mg flour)	18.9	1.02	20.9	0.95	22.4	0.61	25.4	1.01
Gliadins/Glutenins	3.5	0.19	2.4	0.25	2.0	0.22	1.7	0.05
Total prolamins (µg/mg flour)	84.0	3.55	70.9	1.90	68.0	3.97	69.9	2.01
Non gluten proteins (% DW)	4.3	0.48	5.7	0.26	6.0	0.26	5.5	0.45
Protein content (% DW)	13.4	0.26	13.4	0.19	13.3	0.23	13.1	0.26
Starch content (% DW)	66.0	0.95	64.8	1.25	64.3	0.51	63.8	1.50
G12 (ppm)	29494.0	1667.30	53750.0	3569.60	28913.0	3172.70	11915.0	427.60
SDSS (ml)	9.3	0.19	8.7	0.20	8.5	0.25	10.6	0.38
Anthesis (days)	149.0	0.69	148.3	0.33	146.0	1.53	150.3	0.33
1000 Kernel weight (g)	36.3	0.88	40.4	1.47	39.4	0.64	40.6	1.40
Test weight (g/l)	829.2	4.66	843.8	6.25	847.9	7.51	841.7	8.33

DW, Dried Weight; ppm, parts per million; SDSS, sodium dodecyl sulphate sedimentation

Line	I17		I20		H980		H754	
Parameter	Mean	SE Mean	Mean	SE Mean	Mean	SE Mean	Mean	SE Mean
Omega gliadins (µg/mg flour)	10.8	0.93	12.1	1.31	8.5	0.47	8.3	0.34
Alpha gliadins (µg/mg flour)	9.5	0.86	12.0	0.65	18.7	1.18	17.7	0.87
Gamma gliadins (µg/mg flour)	18.2	1.48	22.1	1.03	10.2	0.06	10.7	0.59
Total gliadins (µg/mg flour)	38.5	3.07	46.1	2.89	37.4	1.71	36.7	1.67
HMW glutenins (µg/mg flour)	13.3	0.79	11.3	0.71	14.5	1.44	15.5	0.52
LMW glutenins (µg/mg flour)	13.1	0.77	13.1	0.70	4.3	0.36	4.3	0.27
Total glutenins (µg/mg flour)	26.4	0.18	24.3	1.21	18.8	1.72	19.9	0.42
Gliadins/Glutenins	1.5	0.11	1.9	0.21	2.0	0.15	1.9	0.12
Total prolamins (µg/mg flour)	64.9	3.21	70.5	1.74	56.2	2.98	56.6	1.34
Non gluten proteins (% DW)	6.3	0.47	5.8	0.28	7.4	0.42	6.7	0.25
Protein content (% DW)	13.3	0.26	13.4	0.46	13.4	0.74	12.8	0.37
Starch content (% DW)	66.5	1.37	61.7	0.48	65.3	3.38	68.6	2.27
G12 (ppm)	5222.2	490.76	8395.3	1318.40	20872.0	2078.20	15783.0	1440.90
SDSS (ml)	10.1	0.23	10.6	0.14	6.3	0.23	6.7	0.09
Anthesis (days)	153.7	1.67	136.0	18.61	152.0	0.00	150.7	0.67
1000 Kernel weight (g)	35.8	0.12	36.2	1.17	34.8	1.91	38.0	1.70
Test weight (g/l)	831.3	3.61	831.3	3.61	822.9	12.67	835.4	8.33

FIG. 9 (cont.)

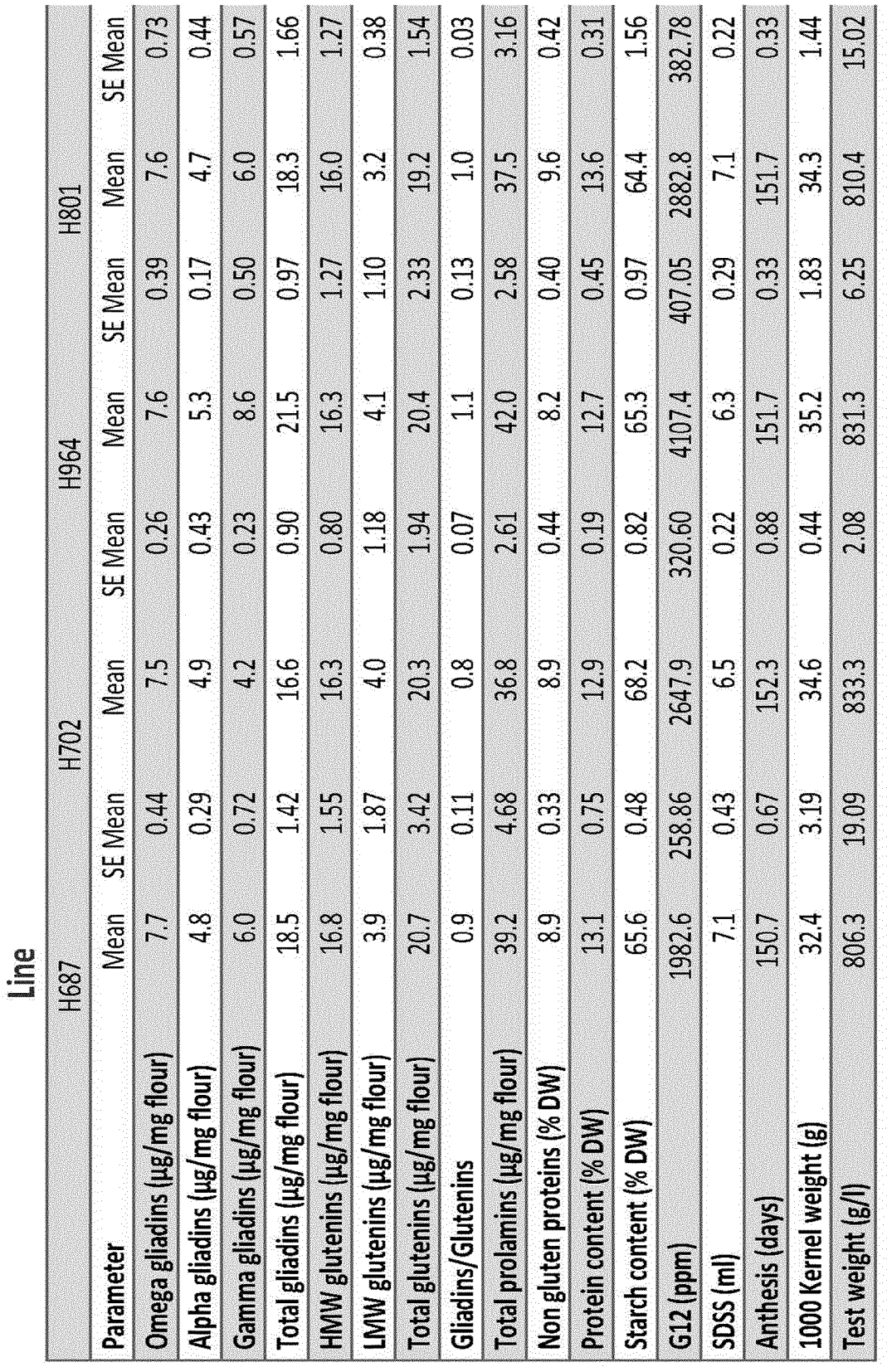


Line	28A		28B		D783		E33	
Parameter	Mean	SE Mean	Mean	SE Mean	Mean	SE Mean	Mean	SE Mean
Omega gliadins (µg/mg flour)	7.2	0.11	6.7	0.22	6.8	0.32	7.3	0.50
Alpha gliadins (µg/mg flour)	13.4	0.67	12.6	0.76	12.9	1.42	9.5	0.77
Gamma gliadins (µg/mg flour)	5.5	0.20	5.0	0.19	4.6	0.87	0.9	0.11
Total gliadins (µg/mg flour)	26.1	0.96	24.3	0.94	24.3	2.61	17.6	1.37
HMW glutenins (µg/mg flour)	16.2	2.35	15.9	0.23	19.4	2.30	17.0	2.12
LMW glutenins (µg/mg flour)	17.0	1.07	16.9	0.06	15.6	0.68	9.8	2.38
Total glutenins (µg/mg flour)	33.2	3.42	32.9	0.18	35.1	2.14	26.8	4.31
Gliadins/Glutenins	0.8	0.05	0.7	0.03	0.7	0.06	0.7	0.06
Total prolamins (µg/mg flour)	59.3	4.36	57.2	0.80	59.4	4.12	44.4	5.68
Non gluten proteins (% DW)	7.4	0.40	7.3	0.22	7.4	0.26	8.9	0.47
Protein content (% DW)	13.8	0.63	13.5	0.31	13.8	0.63	13.7	0.52
Starch content (% DW)	62.8	1.91	63.4	1.14	62.2	1.29	64.0	2.24
G12 (ppm)	3514.7	327.95	7035.9	466.85	3837.7	397.04	2178.9	303.00
SDSS (ml)	9.8	0.49	9.5	0.30	9.5	0.29	8.4	0.25
Anthesis (days)	148.0	0.58	147.7	0.33	148.0	0.00	148.7	0.67
1000 Kernel weight (g)	31.9	1.33	34.0	1.70	32.0	2.14	33.6	1.19
Test weight (g/l)	806.3	16.54	820.8	11.02	810.4	11.60	810.4	4.17

FIG. 9 (cont.)

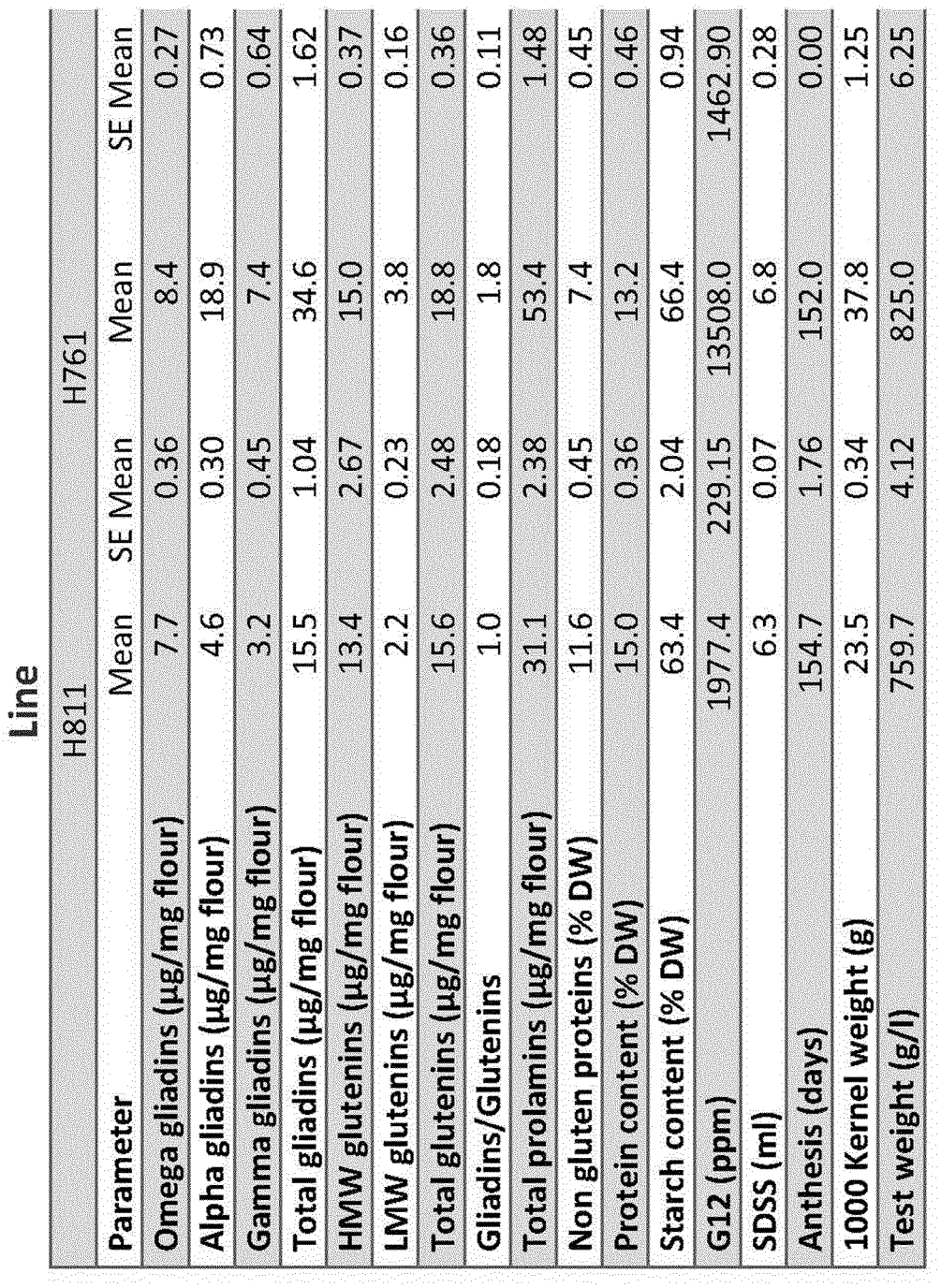
Line	E42		E82		E83		H320	
Parameter	Mean	SE Mean	Mean	SE Mean	Mean	SE Mean	Mean	SE Mean
Omega gliadins (µg/mg flour)	6.3	0.19	6.8	0.14	6.4	0.26	7.0	0.22
Alpha gliadins (µg/mg flour)	9.5	1.17	5.5	0.23	4.7	0.15	5.0	0.07
Gamma gliadins (µg/mg flour)	0.4	0.13	0.1	0.04	0.1	0.04	2.6	0.11
Total gliadins (µg/mg flour)	16.2	1.39	12.4	0.38	11.2	0.38	14.6	0.34
HMW glutenins (µg/mg flour)	9.0	1.17	8.8	0.52	7.9	0.44	8.8	0.44
LMW glutenins (µg/mg flour)	8.4	1.27	2.5	0.10	3.4	0.62	3.0	0.22
Total glutenins (µg/mg flour)	17.4	2.44	11.3	0.63	11.3	1.05	11.8	0.59
Gliadins/Glutenins	1.0	0.25	1.1	0.05	1.0	0.08	1.3	0.07
Total prolamins (µg/mg flour)	33.7	1.08	23.7	0.94	22.5	1.28	26.4	0.65
Non gluten proteins (% DW)	9.1	0.30	11.0	0.20	10.8	0.13	10.6	0.23
Protein content (% DW)	12.7	0.26	13.5	0.20	13.2	0.15	13.5	0.27
Starch content (% DW)	63.7	1.54	61.3	0.76	65.3	2.83	62.8	1.82
G12 (ppm)	3616.1	137.13	1359.7	96.92	1428.3	88.89	3479.3	397.47
SDSS (ml)	7.7	0.23	5.9	0.22	5.8	0.24	6.2	0.27
Anthesis (days)	147.3	0.67	148.3	0.33	148.3	0.33	152.0	0.00
1000 Kernel weight (g)	28.4	1.68	28.0	0.43	30.9	0.98	31.4	1.30
Test weight (g/l)	802.1	9.08	777.1	2.08	800.0	14.43	791.7	9.08

FIG. 9 (cont.)



Line	H687		H702		H964		H801	
Parameter	Mean	SE Mean	Mean	SE Mean	Mean	SE Mean	Mean	SE Mean
Omega gliadins (µg/mg flour)	7.7	0.44	7.5	0.26	7.6	0.39	7.6	0.73
Alpha gliadins (µg/mg flour)	4.8	0.29	4.9	0.43	5.3	0.17	4.7	0.44
Gamma gliadins (µg/mg flour)	6.0	0.72	4.2	0.23	8.6	0.50	6.0	0.57
Total gliadins (µg/mg flour)	18.5	1.42	16.6	0.90	21.5	0.97	18.3	1.66
HMW glutenins (µg/mg flour)	16.8	1.55	16.3	0.80	16.3	1.27	16.0	1.27
LMW glutenins (µg/mg flour)	3.9	1.87	4.0	1.18	4.1	1.10	3.2	0.38
Total glutenins (µg/mg flour)	20.7	3.42	20.3	1.94	20.4	2.33	19.2	1.54
Gliadins/Glutenins	0.9	0.11	0.8	0.07	1.1	0.13	1.0	0.03
Total prolamins (µg/mg flour)	39.2	4.68	36.8	2.61	42.0	2.58	37.5	3.16
Non gluten proteins (% DW)	8.9	0.33	8.9	0.44	8.2	0.40	9.6	0.42
Protein content (% DW)	13.1	0.75	12.9	0.19	12.7	0.45	13.6	0.31
Starch content (% DW)	65.6	0.48	68.2	0.82	65.3	0.97	64.4	1.56
G12 (ppm)	1982.6	258.86	2647.9	320.60	4107.4	407.05	2882.8	382.78
SDSS (ml)	7.1	0.43	6.5	0.22	6.3	0.29	7.1	0.22
Anthesis (days)	150.7	0.67	152.3	0.88	151.7	0.33	151.7	0.33
1000 Kernel weight (g)	32.4	3.19	34.6	0.44	35.2	1.83	34.3	1.44
Test weight (g/l)	806.3	19.09	833.3	2.08	831.3	6.25	810.4	15.02

FIG. 9 (cont.)



Line	H811		H761	
Parameter	Mean	SE Mean	Mean	SE Mean
Omega gliadins (µg/mg flour)	7.7	0.36	8.4	0.27
Alpha gliadins (µg/mg flour)	4.6	0.30	18.9	0.73
Gamma gliadins (µg/mg flour)	3.2	0.45	7.4	0.64
Total gliadins (µg/mg flour)	15.5	1.04	34.6	1.62
HMW glutenins (µg/mg flour)	13.4	2.67	15.0	0.37
LMW glutenins (µg/mg flour)	2.2	0.23	3.8	0.16
Total glutenins (µg/mg flour)	15.6	2.48	18.8	0.36
Gliadins/Glutenins	1.0	0.18	1.8	0.11
Total prolamins (µg/mg flour)	31.1	2.38	53.4	1.48
Non gluten proteins (% DW)	11.6	0.45	7.4	0.45
Protein content (% DW)	15.0	0.36	13.2	0.46
Starch content (% DW)	63.4	2.04	66.4	0.94
G12 (ppm)	1977.4	229.15	13508.0	1462.90
SDSS (ml)	6.3	0.07	6.8	0.28
Anthesis (days)	154.7	1.76	152.0	0.00
1000 Kernel weight (g)	23.5	0.34	37.8	1.25
Test weight (g/l)	759.7	4.12	825.0	6.25

FIG. 9 (cont.)

Parameters	Plasmid Combination							
	0 (wt)	1	2	3	4	5	6	7
Total gliadins (µg/mg flour)	65	47.8 **	43.0 **	36.2 **	24.9 **	14.4 **	14.6 **	18.1 **
Total glutenins (µg/mg flour)	18.9	21.6	25.4 **	19.2	33.7 **	16.7	11.8 *	19.2
Gli/Glu ratio	3.5	2.2 **	1.7 **	1.9 **	0.7 **	0.9 **	1.3 **	1.0 **
Total prolamins (µg/mg flour)	84.0	69.4 **	68.4 **	55.4 **	58.6 **	31.1 **	26.4 **	37.3 **
Non gluten proteins (% DW)	4.3	5.9 *	5.9 *	7.2 **	7.4 **	9.9 **	10.6 **	9.4 **
Grain protein (% DW)	13.4	13.3	13.2	13.1	13.7	13.3	13.5	13.4
Gluten (ppm)	29494	41332 **	8511 **	16721 **	4796 **	2146 **	3479 **	2720 **
Starch (% DW)	66.0	64.5	64.0	66.8	62.8	63.5	62.8	65.4
SDSS (ml)	9.3	8.6	10.4 **	6.6 **	9.6	7.0 **	6.2 **	6.6 **
Anthesis (days)	149	147	147	152	148	148	152	152
Kernel weight (g)	36.3	39.9 *	37.5	36.9	32.6	30.2 **	31.4 *	32.0 *
Test weight (g/l)	829.2	845.8	834.7	827.8	812.5	797.4 *	791.7 *	808.2

Plasmid combinations are as indicated in Figure 1C; DW, Dried Weight; ppm, parts per million; SDSS, sodium dodecyl sulphate sedimentation. Significant differences were identified at the 5% (*) and 1% (**) probability levels by the two-sided Dunnett's multiple comparisons with control indicated as (0) in plasmid combination.

FIG. 10

		Plasmid Combination	0	1	2	3	4	5	6	7
		Line	BW208	D623	I17	H754	D783	E82	H320	H811
Prolamin	Epitope	Deaminated sequence	Original sequence							
γ-gliadin	DQ2.5-glia-g4c/DQ8-glia-g1a	QQPEQPFPQ/EQPQQPFPQ	QQPQQPFPQ	262	203	225	383	72	18	272	148
γ-gliadin	DQ2.5-glia-g5	QQPFPEQPQ	QQPFPQQPQ	181	185	122	241	15	12	210	67
γ-gliadin	DQ2.5-glia-g1/DQ8.5-glia-g1	PQQSFPEQQ/PQQSFPEQE	PQQSFPQQQ	17	7	10	36	16	1	25	17
γ-gliadin	DQ2.5-glia-g3/DQ8-glia-g1b	QQPEQPYPQ/EQPQQPYPE	QQPQQPYPQ	7	5	3	15	8	0	14	8
γ-gliadin	DQ2.5-glia-g4a	SQPEQEFPQ	SQPQQQFPQ	5	0	5	8	1	0	3	5
γ-gliadin	DQ2.5-glia-g2	IQPEQPAQL	IQPQQPAQL	4	4	7	7	4	2	4	7
γ-gliadin	DQ2.5-glia-g4b	PQPEQEFPQ	PQPQQQFPQ	3	5	0	20	1	0	11	3
γ-gliadin	DQ2.5-glia-g4d	PQPEQPFCQ	PQPQQPFCQ	3	0	0	3	0	0	1	0
	Total γ-gliadin			**482**	**409**	**372**	**713**	**117**	**33**	**540**	**255**
α-gliadin	DQ2.5-glia-a2	PQPELPYPQ	PQPQLPYPQ	43	63	0	49	4	0	0	2
α-gliadin	DQ2.5-glia-a1b	PYPQPELPY	PYPQPQLPY	30	40	0	30	4	0	0	1
α-gliadin	DQ2.5-glia-a1a	PFPQPELPY	PFPQPQLPY	15	29	0	17	1	0	0	0

FIG. 11

α-gliadin	DQ2.5-glia-a3	FRPEQPYPQ	FRPQQPYPQ	0	3	0	2	0	0	0	0	0	0	0
α-gliadin	DQ8-glia-a1	EGSFQPSQE	QGSFQPSQQ	4	13	0	7	0	0	0	0	0	0	0
Total α-gliadin				**92**	**148**	**0**	**105**	**9**	**0**	**0**	**0**	**0**	**0**	**3**
ω-gliadin	DQ2.5-glia-w1	PFPQPEQPF	PFPQPQQPF	17	8	11	14	2	0	0	13	3		
ω-gliadin	DQ2.5-glia-w2	PQPEQPFPW	PQPQQPFPW	7	4	4	1	0	0	0	3	1		
Total ω-gliadin				**24**	**12**	**15**	**15**	**2**	**0**	**0**	**16**	**4**		
HMW-GS	**DQ8-glut-1**	**QGYYPTSPQ**	**QGYYPTSPQ**	**0**	**0**	**1**	**2**	**2**	**9**	**4**	**3**			
α-gliadin	p31-43	PGQQQPFPPQQPY	PGQQQPFPPQQPY	13	14	0	3	3	0	0	0	0		
α-gliadin	p31-43	LGQQQPFPPQQPY	LGQQQPFPPQQPY	8	6	0	6	3	1	0	0	0		
Total α-gliadin p31-43				**21**	**20**	**0**	**9**	**6**	**1**	**0**	**0**			
LMW-GS	DQ2.5-glut-L1	PFSEQEQPV	PFSQQQQPV	16	24	4	0	0	11	15	2			
LMW-GS	DQ2.5-glut-L2	FSQQQESPF	FSQQQQSPF	0	1	0	0	0	1	0	0			
Total LMW-GS				**16**	**25**	**4**	**0**	**0**	**12**	**15**	**2**			
Hordein	DQ2.5-hor-2	PQPEQPFPQ	PQPQQPFPQ	10	1	4	5	1	0	3	2			
Hordein	DQ2.5-hor-3	PIPEQPQPY	PIPQQPQPY	0	0	0	0	0	0	0	0			
Total Hordein				**10**	**1**	**4**	**5**	**1**	**0**	**3**	**2**			

FIG. 11 (cont.)

Plasmid Combination	0		1		2		3	
Line	BW208		D623		I17		H754	
Parameter	Fragments	Proteins	Fragments	Proteins	Fragments	Proteins	Fragments	Proteins
Total	**48405**	**ND**	**46287**	**ND**	**51660**	**ND**	**48480**	**ND**
Triticum	**3240**	**184**	**4832**	**198**	**1639**	**149**	**3436**	**197**
α-gliadins	1554	32	3107	44	21	2	1323	29
γ-gliadins	404	14	341	15	409	14	641	18
ω-gliadins	347	9	244	5	264	6	279	9
Total gliadins	**2305**	**55**	**3692**	**64**	**694**	**22**	**2243**	**56**
LMW	104	12	317	22	83	12	50	7
HMW	11	1	13	2	11	2	13	2
Total glutenins	**115**	**13**	**330**	**24**	**94**	**14**	**63**	**9**
Total prolamins	**2420**	**68**	**4022**	**88**	**788**	**36**	**2306**	**65**
γ-secalin	72	3	39	2	28	1	123	5
ω-secalin	93	3	36	1	82	3	51	3
γ-hordein-like	2	1	16	2	2	1	2	1
γ-gliadin-like	73	3	61	4	12	1	83	4
Gliadin/avenin-like	38	3	44	5	50	5	33	4
AAI	48	9	53	8	67	10	72	10
Gobulins	106	5	105	4	132	5	178	6
Triticins	8	1	10	1	17	1	18	1
Serpin	70	6	114	6	137	7	182	8
Non-Gluten Proteins	**270**	**24**	**326**	**24**	**403**	**28**	**483**	**29**
LTPs	3	1	3	2	4	1	4	1
β-amylase	44	2	47	2	41	2	62	2
Total SSPs	**2737**	**95**	**4398**	**116**	**1236**	**67**	**2855**	**97**

FIG. 12

Plasmid Combination	4		5		6		7	
Line	D783		E82		H320		H811	
Parameter	Fragments	Fragments	Fragments	Proteins	Fragments	Proteins	Fragments	Proteins
Total	**50980**	**ND**	**51221**	**ND**	**50365**	**ND**	**52129**	**ND**
Triticum	**1639**	**187**	**1464**	**162**	**2141**	**181**	**1616**	**146**
α-gliadins	262	11	37	3	0	0	7	1
γ-gliadins	189	13	33	5	555	20	361	13
ω-gliadins	22	2	10	1	226	6	99	4
Total gliadins	**473**	**26**	**80**	**9**	**781**	**26**	**467**	**18**
LMW	150	15	91	14	82	11	0	0
HMW	42	6	172	7	64	6	35	3
Total glutenins	**192**	**21**	**263**	**21**	**146**	**17**	**35**	**3**
Total prolamins	**665**	**47**	**343**	**30**	**927**	**43**	**502**	**21**
γ-secalin	12	1	0	0	109	3	20	1
ω-secalin	7	1	0	0	54	2	0	0
γ-hordein-like	7	2	5	2	2	1	0	0
γ-gliadin-like	22	2	7	1	55	2	85	4
Gliadin/avenin-like	41	5	29	4	24	4	46	4
AAI	69	11	78	13	71	11	77	10
Gobulins	157	5	210	6	211	6	228	8
Triticins	25	1	60	2	27	1	28	1
Serpin	231	9	314	9	247	8	255	8
Non-Gluten Proteins	**523**	**31**	**691**	**34**	**580**	**30**	**634**	**31**
LTPs	3	1	4	2	3	1	3	1
β-amylase	54	2	65	2	57	2	64	2
Total SSPs	**1245**	**81**	**1103**	**68**	**1567**	**76**	**1203**	**55**

FIG. 12 (cont.)

			Plasmid Combination	0	1	2	3	4	5	6	7
			Line	BW208	D623	I17	H754	D783	E82	H320	H811
Prolamin	Epitope	Deaminated sequence	Original sequence								
γ-gliadin	DQ2.5-glia-g4c/DQ8-glia-g1a	QQPEQPFPQ/EQPQQPFPQ	QQPQQPFPQ	262	203	225	383	72	18	272	148
γ-gliadin	DQ2.5-glia-g5	QQPFPEQPQ	QQPFPQQPQ	181	185	122	241	15	12	210	67
γ-gliadin	DQ2.5-glia-g1/DQ8.5-glia-g1	PQQSFPEQQ/PQQSFPEQE	PQQSFPQQQ	17	7	10	36	16	1	25	17
γ-gliadin	DQ2.5-glia-g3/DQ8-glia-g1b	QQPEQPYPQ/EQPQQPYPE	QQPQQPYPQ	7	5	3	15	8	0	14	8
γ-gliadin	DQ2.5-glia-g4a	SQPEQEFPQ	SQPQQQFPQ	5	0	5	8	1	0	3	5
γ-gliadin	DQ2.5-glia-g2	IQPEQPAQL	IQPQQPAQL	4	4	7	7	4	2	4	7
γ-gliadin	DQ2.5-glia-g4b	PQPEQEFPQ	PQPQQQFPQ	3	5	0	20	1	0	11	3
γ-gliadin	DQ2.5-glia-g4d	PQPEQPFCQ	PQPQQPFCQ	3	0	0	3	0	0	1	0
	Total γ-gliadin			**482**	**409**	**372**	**713**	**117**	**33**	**540**	**255**
α-gliadin	DQ2.5-glia-a2	PQPELPYPQ	PQPQLPYPQ	43	63	0	49	4	0	0	2
α-gliadin	DQ2.5-glia-a1b	PYPQPELPY	PYPQPQLPY	30	40	0	30	4	0	0	1
α-gliadin	DQ2.5-glia-a1a	PFPQPELPY	PFPQPQLPY	15	29	0	17	1	0	0	0
α-gliadin	DQ2.5-glia-a3	FRPEQPYPQ	FRPQQPYPQ	0	3	0	2	0	0	0	0
α-gliadin	DQ8-glia-a1	EGSFQPSQE	QGSFQPSQQ	4	13	0	7	0	0	0	0
	Total α-gliadin			**92**	**148**	**0**	**105**	**9**	**0**	**0**	**3**

FIG. 13

			Plasmid Combination	0	1	2	3	4	5	6	7
			Line	BW208	D623	I17	H754	D783	E82	H320	H811
Prolamin	Epitope	Deaminated sequence	Original sequence								
ω-gliadin	DQ2.5-glia-w1	PFPQPEQPF	PFPQPQQPF	17	8	11	14	2	0	13	3
ω-gliadin	DQ2.5-glia-w2	PQPEQPFPW	PQPQQPFPW	7	4	4	1	0	0	3	1
Total ω-gliadin				**24**	**12**	**15**	**15**	**2**	**0**	**16**	**4**
HMW-GS	**DQ8-glut-1**	**QGYYPTSPQ**	**QGYYPTSPQ**	**0**	**0**	**1**	**2**	**2**	**9**	**4**	**3**
α-gliadin	p31-43	PGQQQPFPPQQPY	PGQQQPFPPQQPY	13	14	0	3	3	0	0	0
α-gliadin	p31-43	LGQQQPFPPQQPY	LGQQQPFPPQQPY	8	6	0	6	3	1	0	0
Total α-gliadin p31-43				**21**	**20**	**0**	**9**	**6**	**1**	**0**	**0**
LMW-GS	DQ2.5-glut-L1	PFSEQEQPV	PFSQQQQPV	16	24	4	0	0	11	15	2
LMW-GS	DQ2.5-glut-L2	FSQQQESPF	FSQQQQSPF	0	1	0	0	0	1	0	0
Total LMW-GS				**16**	**25**	**4**	**0**	**0**	**12**	**15**	**2**
Hordein	DQ2.5-hor-2	PQPEQPFPQ	PQPQQPFPQ	10	1	4	5	1	0	3	2
Hordein	DQ2.5-hor-3	PIPEQPQPY	PIPQQPQPY	0	0	0	0	0	0	0	0
Total Hordein				**10**	**1**	**4**	**5**	**1**	**0**	**3**	**2**

FIG. 13 (cont.)