Chapter 23

Wheat Varieties Suitable for Celiac Patients

María J. Giménez[1], Javier Gil-Humanes[2], Carmen Victoria Ozuna[1], Francisco Barro[1]

[1]Institute for Sustainable Agriculture, CSIC, 14080 Córdoba, Spain.

[2]Department of Genetics, Cell Biology and Development and Center for Genome Engineering, University of Minnesota, Minneapolis, Minnesota, USA.

mjga06@ias.csic.es, javigil@ias.csic.es, cvozuna@ias.csic.es, fbarro@ias.csic.es

Doi: http://dx.doi.org/10.3926/oms.224

How to cite this chapter

Giménez MJ, Gil-Humanes J, Ozuna CV, Barro F. *Wheat Varieties Suitable for Celiac Patients.* In Rodrigo L and Peña AS, editors. *Celiac Disease and Non-Celiac Gluten Sensitivity.* Barcelona, Spain: OmniaScience; 2014. p. 461-475.

Abstract

Domesticated wheat is very complex genetically due to its origins in an ancestral diploid species, which underwent a process of natural hybridization and subsequent polyploidization. All cultivated wheat varieties and their wild relatives contain epitopes, which are toxic in relation to celiac disease (CD). RNAi is an excellent tool for silencing single genes or groups of them. Combining this technology with genetic manipulation the authors have down-regulated the toxic epitopes present in ω-, γ-, and α-gliadins of bread wheat. Monoclonal antibodies showed a decrease of almost 98% in the presence of toxic gluten. Protein extracts from those lines were assayed using specific T lymphocytes for DQ2 and DQ8 epitopes, showing that the new wheat lines were 100 times less reactive than their parental genotypes. These results represent a major breakthrough in achieving wheat types suitable for CD patients. The silencing of gliadins is a new breeding trait and can be transferred by crossbreeding with elite wheat varieties. A daily intake between 10 and 50 mg of gluten could be safe for most CD patients, suggesting that the transgenic lines reported here could be used in foodstuffs tolerated by many CD patients. Moreover, these lines could serve as a basis for treating other gluten pathologies such as wheat-dependent exercise-induced anaphylaxis and gluten sensitivity.

1. Introduction

Although celiac disease (CD) has been known since ancient times, the first references relating it to the intake of certain foods do not appear until the late nineteenth century. During the first half of the twentieth century the pernicious effect of bread in celiac patients was already known, but it was not until after World War II that Dicke, Weijers and Van De Kamer[1] observed that certain cereal grains, especially wheat and rye, were detrimental to children with CD, demonstrating the role of gluten as the agent responsible for triggering the disease. Since then, the gluten-free diet has been the only effective therapy for treating it and, during this time, great strides have been made in identifying the elements within gluten responsible for gluten enteropathy. At first glance, it might appear that, once known, toxic epitopes could be easily removed by plant breeding techniques and thus obtain varieties suitable for consumption by celiac patients. In fact, a similar process has taken place since the beginning of agriculture with other grains, whose domesticated varieties do not produce toxic substances (or do so to a lesser extent) which wild species often do have as, for example, antinutrients in legumes and glucosinolates in cabbages and more recently, erucic acid in rapeseed. In the case of wheat, the main gluten-bearing cereal, this is not a simple task due to the complexity of its genetics and of the proteins of which it is composed.

2. Wheat

The term "wheat" refers to the group of cereals, both domesticated and wild, belonging to the botanical genus *Triticum*, of the *Triticeae* tribe, belonging to the *Poiideae* subfamily of the grass family. Barley and rye are included in the same tribe as wheat, while oats belong to another tribe of the same subfamily. Other important grasses are maize and rice, which, along with wheat, are humanity's main cereal staples.[2]

Wheat grain is used to make flour, wheat meal, beer and a wide variety of food products, each world region making use of certain wheat types for specific ends. After the thousands of years elapsed since the beginning of its cultivation, each culture has developed characteristic habits and customs regarding wheat consumption.

From a genetic point of view, domesticated wheat is quite complex due to its origin in an ancestral diploid species, which has gone through natural hybridization processes and subsequent polyploidization. The main two species of agriculturally important wheat, durum (for pasta) and bread wheat (90% of all wheat produced in the world) are, respectively, tetraploid (two genomes, AABB) and hexaploid (three genomes, AABBDD) (Figure 1). The first originated naturally through the spontaneous hybridization of two diploid species between 0.5 and 2 million years ago, each one the donor of A and B genomes. Bread wheat (AABBDD) originated in the cultivated fields around 8,000 years ago, through spontaneous hybridization between durum wheat (AABB) and *A. tauschii*, a diploid species that donated the D genome (Figure 1). All wheat species have chromosome numbers in multiples of seven, including the diploid, tetraploid and hexaploid species with 14, 28 and 42 chromosomes, respectively. Wheat chromosomes are named using a number and a letter indicating from which genome it proceeds. Because of the close relationship between the donor species of the A, B and D genomes, for each pair of

homologous chromosomes from one of the genomes present in bread wheat, there is a pair of similar chromosomes (homeologous) in the other genomes. In practice, polyploid wheat composition implies that each one of its genes is encoded two (durum wheat) or three times (bread wheat), so that changing a character through genetic improvement implies more effort than that which would have to be performed for a diploid species.

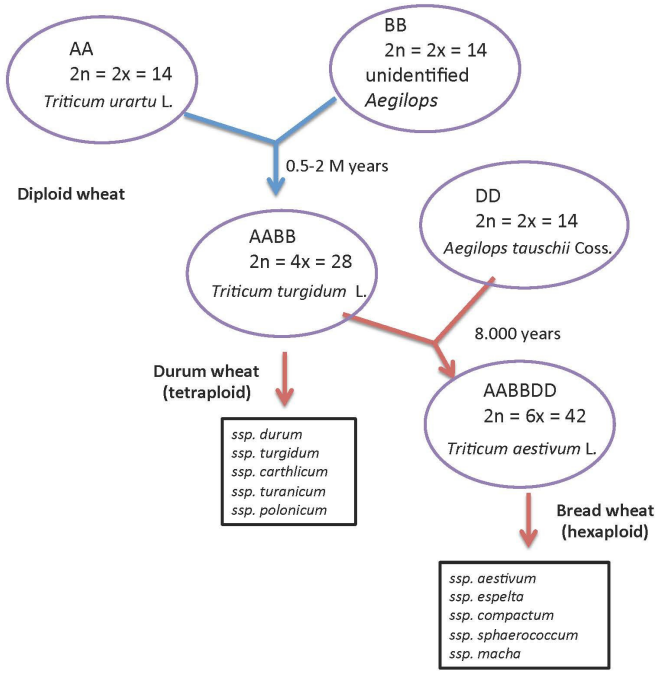


Figure 1. Origin of bread wheat (hexaploid) and pasta wheat (tetraploid) from diploid ancestors and subsequent polyploidization. Bread wheat, which comprises 90% of the wheat grown in the world, has a very recent origin.

3. Wheat Protein

Wheat grain is composed of proteins with a structural or metabolic function and by storage proteins (gluten).[3] The latter have the function of providing nutrients, such as amino acids, to the seedling in its early stages of development. According to Osborne's original classification[4] based on wheat grain protein solubility differences, these would be composed of albumins, globulins, prolamins (gliadin) and glutelins (glutenins). Gluten represents 80% of total grain protein and consists of gliadins and glutenins (Figure 2), which have different physicochemical properties due to their different ability to form polymers. While gliadins are monomeric, glutenins are assembled into polymers, stabilized by disulfide bonds, which remain physically, linked together, forming large aggregates of variable size. These proteins are the largest known in nature.

Figure 2. Wheat gluten composition. Gluten is a complex mixture of proteins belonging to two groups: glutenins and gliadins. While glutenin forms polymers, gliadin remains a monomer. Proteins in each group may be extracted and separated on SDS-PAGE gels (glutenin) and A-PAGE gels (Gliadins). Glutenin are divided into high (HMW) and low (LMW) molecular weight glutenins. Gliadins are formed by three structural groups: ω-, γ- and α-gliadins.

The classification of gluten proteins based on solubility has been offset today thanks to knowledge of their nature and genetics, so that the glutenins must also be considered to be prolamins since they are soluble in aqueous ethanol following interchain disulfide bonds reduction and since; in addition, they are closely related to gliadins in an evolutionary sense.[5] Within glutenins, two fractions are distinguished according to their separation by electrophoresis using polyacrylamide gels containing sodium dodecyl sulfate (SDS PAGE): low and high molecular weight glutenins (LMW and HMW, respectively), whereas gliadins are classified into three structural groups: ω-, γ- and α-gliadin according to their mobility in polyacrylamide gels with an acid pH (A-PAGE) (Figure 2).

Gluten is, therefore, a complex of proteins whose genetic regulation is also intricate. Diploid wheat species contain two closely linked HMW glutenin genes encoded in the *Glu-1* locus on the long arm of chromosome 1, and a group of LMW glutenin genes, also closely linked, encoded by

the *Glu-3* locus in short arm of chromosome 1. Gliadins occur in groups of linked genes (blocks) located on the short arm of chromosomes 1 and 6 (Figure 3). Most γ- and ω-gliadins are located in the *Gli-1* locus on the short arm of chromosome 1, near the *Glu-3* locus (LMW glutenin subunits), while the α-gliadins are controlled by the *Gli-2* locus on the short arm of chromosome 6. Other minor loci on the short arm of chromosome 1 regulate some gliadin and LMW glutenin. Each block includes a variable number of genes that are inherited as a locus, making it very difficult to separate one gliadin gene from another, within the same block, by genetic recombination. Since bread wheat possesses three genomes, its complement is three times larger: several hundred different proteins whose genes are inherited in blocks, most of them being gliadins and LMW glutenins.

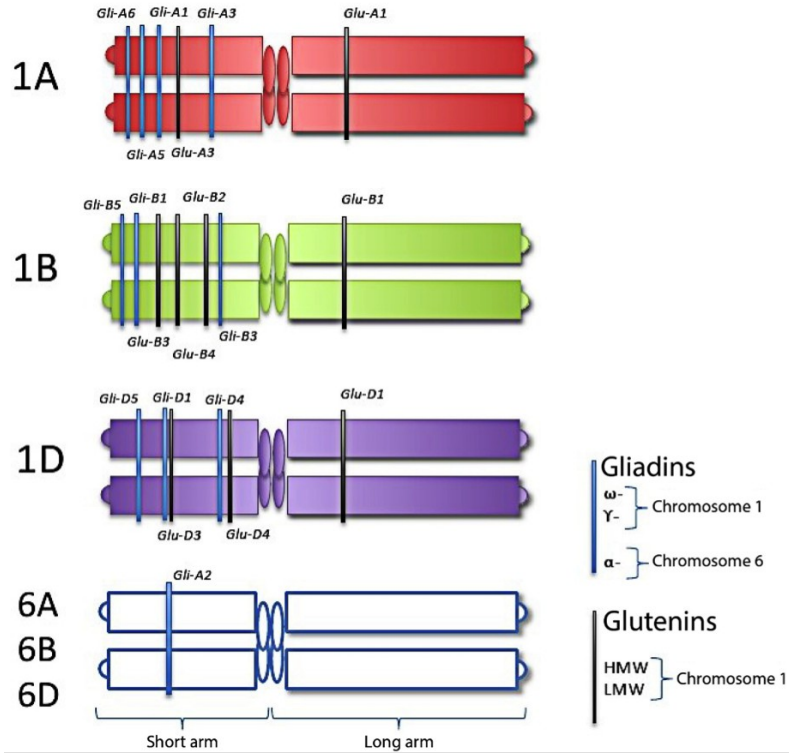


Figure 3. Chromosomal location of glutenin and gliadin loci in hexaploid wheat. The high molecular weight glutenins are located on the long arm of chromosome 1 group. ω- and γ-gliadins are located at various loci on the short arm of chromosome 1 group while the α-gliadins are located on chromosome 6. Low molecular weight glutenins are also located on the short arm of chromosome 1 group, closely linked to the gliadin loci.

4. The Gluten Toxic Fraction

The food codex standard on foodstuffs for special dietary destined for gluten-intolerant persons (CODEX STAN 118-1979) defines gluten as "a protein fraction from wheat, rye, barley, and oats or their crossbred varieties and derivatives the same, to which some people are intolerant and which is insoluble in water and 0.5 M NaCl." Moreover, the same codex defines "prolamins" equating them with "gliadins"; despite this last having been pointed out as incorrect, it had not been corrected in the latest revision of 2008. These definitions of gluten and prolamins can lead to confusion so it is important to make it clear that not all gluten proteins are toxic, and that all those which are toxic are not so to the same extent. The term *gluten* will be used in this chapter to refer to the entire prolamin fraction, not to be confused with the gluten related to food for celiac patients, which actually refers to the toxic portion thereof. The latter will be referred to as *toxic gluten*.

Wheat gluten proteins are rich in the amino acids proline (15%) and glutamine (30%) and have unusually low aspartic and glutamic acid content. The high proline amount is the cause that gluten proteins are digested by gastrointestinal proteases with great difficulty, resulting in relatively large peptides that accumulate in the small intestine.[6] These peptides are the perfect substrate for the glutamine residues deamidation in glutamate mediated by transglutaminase 2 (TG2), fundamental for the creation of the T lymphocyte-stimulating epitopes involved in CD.[7,8]

Gliadins are indubitably the main toxic component in gluten, especially α and γ-gliadins since most (DQ2 or DQ8)-specific CD4+ T lymphocytes[9-11] obtained from small intestinal biopsies from celiac patients seem to recognize this fraction. In recent years, based on their T-lymphocyte stimulation ability, immunotoxic epitopes have been identified in wheat gluten proteins and in other grasses. At the time of the writing of this chapter, and only taking into account bread wheat, in the IEDB epitope database (http://www.iedb.org/) 190 T-lymphocytes stimulating epitopes related to CD can be found. Of these, 94 are caused by α-gliadin molecules, 74 by γ-gliadin, 12 by ω-gliadin, 8 in LMW glutenin and 2 in HMW glutenin. Therefore, the gluten gliadin fraction is by far largely responsible for CD. Since immunogenic epitopes induce the autoimmune response that generates CD, the type and number of epitopes determine the toxicity of each gluten protein variant. A particular peptide, α-gliadin 33-mer (residues 57-89), which is highly resistant to proteolysis, contains 6 epitopes recognized by T lymphocytes, which makes it a major contributor to the gluten immunotoxicity.[12]

5. Are There any Non-Toxic Wheat Varieties?

Gluten confers unique viscoelastic properties to wheat dough, hence the huge variety of foods that can be made. Humankind, in the wheat-domestication process, has been selecting for this trait and at no time has there been a process of genetic selection considering toxicity in relation to CD. However, inside gluten, there is some variability regarding the relative content of each of the prolamin fractions: glutenin and gliadin, specifically as well as within the species.[13-15] This variability is the reason why 129 different α-gliadin sequences can be found in the GenBank protein database (http://www.ncbi.nlm.nih.gov/genbank/). Seventy-one of these variants were added in 2012. Great efforts are made to find non-toxic or low toxicity variants in wheat as well

as in related wild species tracing the presence of T lymphocyte-stimulating epitopes in gliadin gene sequences from different wheat species.[16] Gliadin gene sequence analysis has shown that simple changes in certain amino acids in the peptide toxins would be enough to make them lose their T lymphocyte-stimulatory nature and, since there are non-toxic natural variants of these peptides,[16] genetic selection has been suggested as a tool for obtaining varieties containing non-toxic epitopes.[17] However, due to the close linkage of the genes found in them, recombination within a locus is unlikely, and so far it seems doubtful that by crossbreeding and recombination non-toxic wheat varieties can be obtained. These studies have also found that *Aegilops tauschi* prolamin sequences, donor of one of the three bread wheat genomes (D genome) are richer in immunotoxic epitopes than those of other related species.[18] This could be one of the reasons why bread wheat is more toxic than the durum wheat: the latter lacks the D genome. However, when the gluten content in durum and bread wheat varieties are examined, even though there are differences between varieties,[19] these values are well above the maximum limit allowed for celiac patients (Figure 4). Consequently, gluten toxicity has become more a quantitative than a qualitative issue and the solution is to apply modern biotechnology techniques to develop less toxic wheat varieties, which can be tolerated by celiac patients.

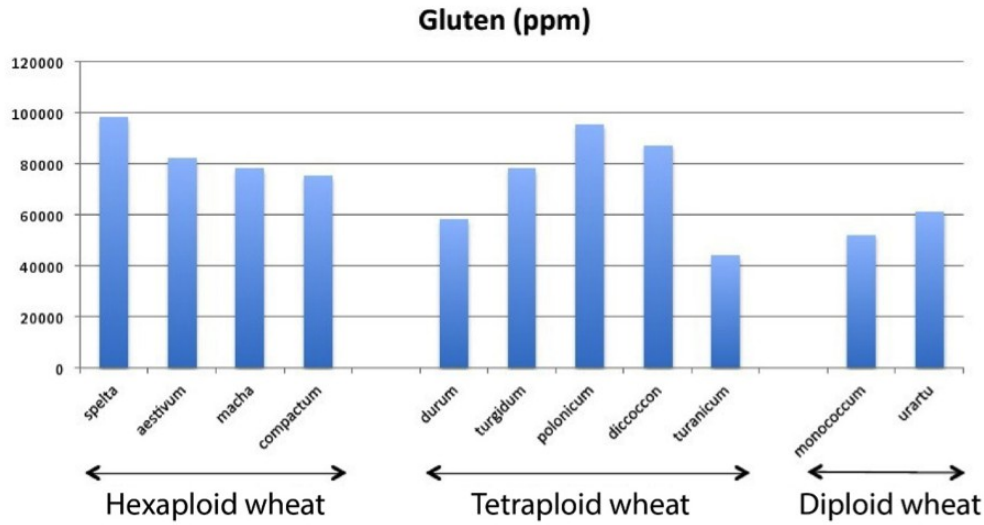


Figure 4. Gluten content in hexaploid, tetraploid and diploid genotypes. Gluten content was determined by ELISA assays with R5 antibodies. The values shown represent the mean of seven lines within each genotype.

6. Development of Wheat Varieties Suitable for Celiac Patients

The low toxic gluten content character is, as we have seen, extremely complex and its genetic regulation is insufficiently known. Biotechnological techniques based on specific gene silencing by RNA interference (RNAi) are the option which, to date, has been better explored. This technique involves a very specific post-transcriptional gene silencing mechanism by which small RNA molecules complementary to messenger RNA (mRNA) lead to its degradation, thereby preventing its translation into proteins.[20,21] The discovery of its mechanism earned researchers Andrew Fire and Craig Mello the Medicine Nobel prize in 2006, and they have already noted the potential this technique would have in medicine, since any gene whose sequence is known may be the target of a tailor-made interfering RNA and therefore, it can be turned off, thereby ending whatever adverse effect it may have.

In principle, the most direct approach is to specifically eliminate gliadin where toxic epitopes have been described, so that new varieties retain their properties for bread making. The use of this specific gliadin silencing technology in wheat grain implies a very precise knowledge of the synthesis of this protein group of in the grain[22] and the use of very specific promoters[22,23] which operate only in the grain, so that the silencing fragment may be successfully synchronized with the gliadin synthesis to be silenced. Thus, the α-gliadins in the "Florida" variety[24] and γ-gliadins in the "Bobwhite" variety (Figure 5)[25] have been successfully silenced. However, the reduction in the content of a specific group of gliadins has not led to varieties with toxicity levels that may be considered suitable for celiac patients (Figure 5B). One of the reasons for this lack of toxicity level reduction may be a prolamin synthesis offset[26] so that the decrease of a specific gliadin group is compensated with proteins from another gliadin group, which also contain toxic epitopes. However, wheat varieties with reduced levels of various toxic fractions could contribute to reducing the gluten burden for the entire population if introduced as parents in breeding programs seeking to obtain "less toxic" varieties by means of crossbreeding, genetic recombination and the selection of genotypes that contains the appropriate silencing.

To prevent this compensatory effect and obtain a more effective toxicity reduction in new wheat varieties, the best option is the use of chimeric RNA interference capable of silencing the genes of the three groups: ω-, γ- and α-gliadin. The construction of an RNAi chimeric fragment involves identifying highly conserved zones in the genes of each of the three gliadin groups and combining said sequences in a single silencing fragment. Gene silencing may be enhanced by using the same silencing fragment and a combination of promoters specific to the grain, but with different expression patterns. This strategy would allow the silencing fragment to run for longer stages of grain development.

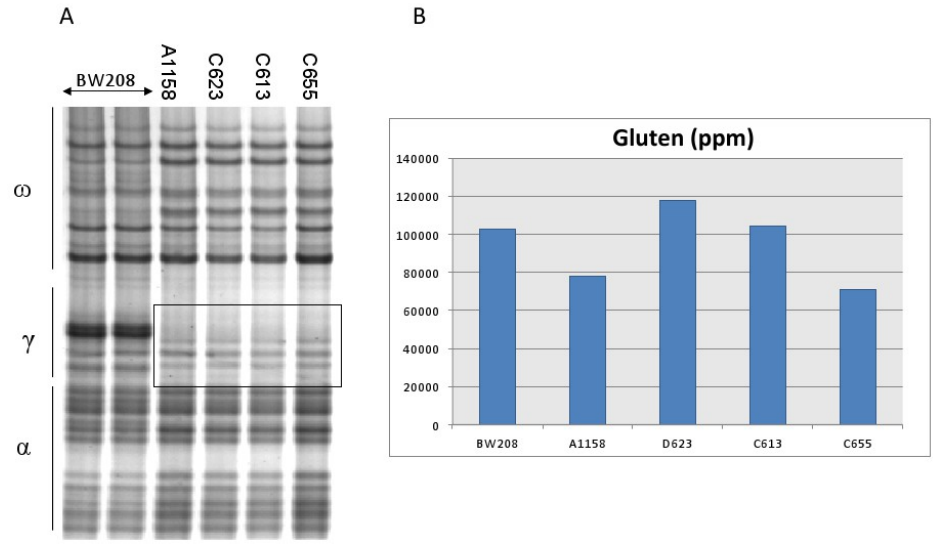
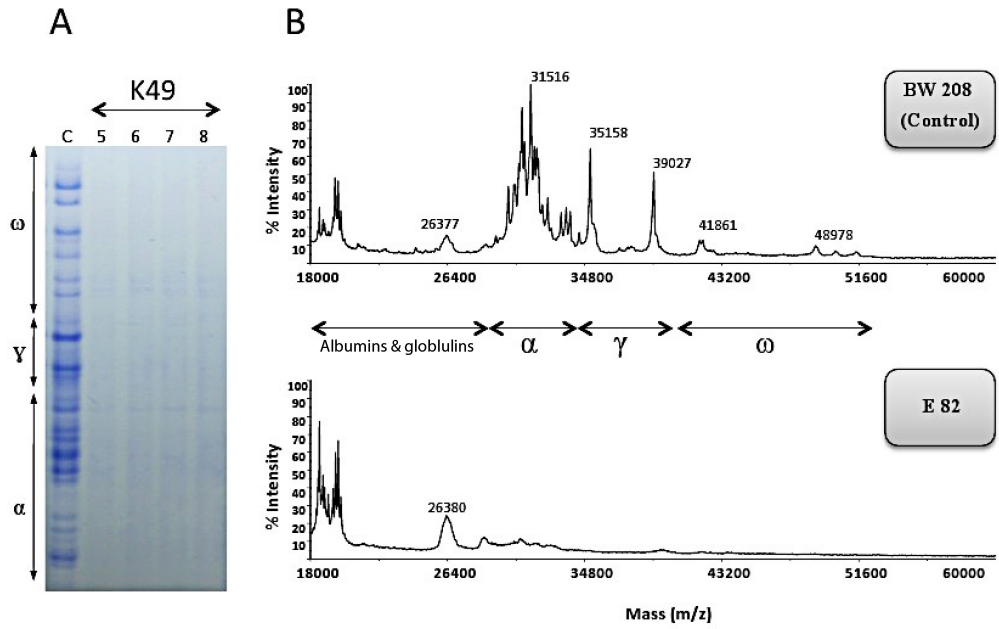


Figure 5. Specific silencing of γ-gliadins in wheat grain. A. The silencing vector expression is highly specific and only reduces γ-gliadin. B. Gluten content, as determined by ELISA R5, in several lines with γ-gliadin silenced.

With this technique we have produced a collection of more than 50 wheat lines of different varieties and, therefore, bearing different gliadin patterns. As shown in Figure 6A, the chimeric fragment used was effective in silencing genes belonging to the three gliadin groups. The use of different promoters apparently enhances the silencing effect and the two promoters used were equally effective in silencing the gliadins.[27] In addition, the chimeric RNAi fragment was also very effective in silencing gliadins from different wheat varieties.[24] The specificity of the RNAi fragment is shown in Figure 6B, showing silencing in the gliadin fraction but not in albumins and globulins. However, the specific gliadin silencing causes a compensating effect on other proteins such as glutenins[27,28] and also on the albumin and globulin fraction[28] so that there are no great differences in the total protein content between the gliadin-free and their respective gliadin-bearing controls.[27] ELISA sandwich quantification using R5 antibodies has shown that in some lines the gluten percentage has decreased by about 98%. Those tested with specific T lymphocytes specific to some particular highly stimulatory epitopes corroborated this data. The results of the quantification of the proliferation of T lymphocytes specific to the DQ2-α-II, DQ8-α-, DQ2-γ-VII and DQ8-γ-I epitopes in response to gluten from silenced lines, digested with pepsin and trypsin, were actually very large.[27] For some of these lines, the protein amounts required were a hundred times greater than those of their respective controls in order to obtain a response in the activation of T lymphocytes that recognize the DQ2-α-II[24] epitope situated in 33 mer, one of most immunotoxic peptides known.[13] The T lymphocyte clones specific for other epitopes (DQ2-γ-VII, DQ8-α-I and DQ8-γ-I) located in γ- and α-gliadins did not exceed the detection level for the highest evaluated protein extract concentrations.[27] Similar results were found with T lymphocyte clones that recognize the highly stimulatory epitopes present in ω-gliadins[29] which showed a very small proliferative response when compared to gliadin-bearing controls.[27]



C, control line with gliadins.

Figure 6. RNAi gene silencing in the three gliadin groups. A. A-PAGE gel which shows that the expression of a chimeric RNAi containing highly conserved sequences for the three gliadin groups causes an effective silencing of all gliadins in the grains of bread wheat. B. MALDI-TOF, which shows that silencing is specific for gliadin, while other fractions (such as albumins and globulins) are not reduced.

The wheat varieties described show a reduction in the three gliadin fractions, so that they might be suitable for other gluten related pathologies. For example, exercise-dependent anaphylaxis, which occurs in susceptible individuals after practicing sports, is triggered by genes encoded on the short arm of chromosome 1B of durum and bread wheat genes, ω-5-gliadins.[30,31] In the lines described, this protein fraction is highly reduced, so these flours could help to combat this grave pathology. Gluten sensitivity, a new gluten intolerance disease, which excludes celiac disease and allergy, affects 6% of the U.S. population[32] (whose treatment is a gluten-free diet) and could also benefit from the new varieties described this work.

An important issue is to conserve flour and baking quality in the new varieties without toxic gluten. Ideally, they could be widely used to produce bread and other food products for celiac patients and other gluten intolerances so that their organoleptic properties should be as close to normal wheat bread as possible.

HMW glutenin subunits are functionally very important, as they are the main determinants of gluten elasticity, a property that correlates directly with the baking qualities of flour. The baking quality of the lines was assessed using the SDS sedimentation test, since the obtained sedimentation volumes are correlated to the bread-making qualities.[33] Most of the gliadin-free lines showed SDS sedimentation values comparable to the control lines and only five lines had

significantly lower values than the control lines.[24] However, SDS sedimentation values of these five lines are still comparable to those of medium quality bread wheat varieties.

7. Conclusions

To date, various studies indicate that all varieties of cultivated wheat and their wild relatives are toxic and although there are differences between them, they are well above the limit tolerated by celiac patients. RNAi is an excellent tool for the specific silencing of T-cell stimulating-epitopes present in the three gliadin groups. These results are a major breakthrough in achieving wheat varieties suitable for most celiac patients. Moreover, these lines could serve as a basis for treating other gluten related pathologies such as exercise-dependent anaphylaxis and non-celiac gluten sensitivity. The "silencing" can be transferred by crossbreeding with other wheat varieties thus allowing the availability of enough genetic variability for the selection even less toxic lines than those already produced. In the case of the toxicity described for some glutenins, especially those of high molecular weight, varieties that carry nontoxic alleles could easily be selected and be used as parents in breeding programs.

References

1. Dicke WK, Weijers HA, Van De Kamer JH. *Coeliac disease. II. The presence in wheat of a factor having a deleterious effect in cases of coeliac disease.* Acta Paediatr. 1953; 42(1): 34-42. http://dx.doi.org/10.1111/j.1651-2227.1953.tb05563.x
2. FAOSTAT. Enero de 2013. http://faostat.fao.org/site/339/default.aspx
3. Shewry PR, Halford NG. *Cereal seed storage proteins: structures, properties and role in grain utilization.* J Exp Bot. 2002; 53(370): 947-58. http://dx.doi.org/10.1093/jexbot/53.370.947
4. Osborne TB. *The proteins of the wheat kernel.* Carnegie institution of Washington, 1907.
5. Shewry PR, Tatham AS, Forde J, Kreis M, Miflin BJ. *The classification and nomenclature of wheat gluten proteins: A reassessment.* J Cereal Sci. 1986; 4(2): 97-106. http://dx.doi.org/10.1016/S0733-5210(86)80012-1
6. Wieser H. *The precipitating factor in coeliac disease.* Baillière's Clin Gastroenterology. 1995; 9(2): 191-207. http://dx.doi.org/10.1016/0950-3528(95)90027-6
7. Wal Y van de, Kooy Y, Veelen P van, Peña S, Mearin L, Papadopoulos G, et al. *Cutting Edge: Selective deamidation by tissue transglutaminase strongly enhances gliadin-specific T cell reactivity.* J Immunol. 1998; 161(4): 1585-88.
8. Molberg O, Mcadam SN, Körner R, Quarsten H, Kristiansen C, Madsen L, et al. *Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease.* Nat Med. 1998; 4(6): 713-17. http://dx.doi.org/10.1038/nm0698-713
9. Lundin, KE, Scott H, Hansen T, Paulsen G, Halstensen TS, Fausa O, et al. *Gliadin-specific, HLA-DQ(alpha 1*0501,beta 1*0201) restricted T cells isolated from the small intestinal mucosa of celiac disease patients.* J Exp Med. 1993; 178(1): 187-96. http://dx.doi.org/10.1084/jem.178.1.187
10. Lundin KE, Scott H, Fausa O, Thorsby E, Sollid LM. *T cells from the small intestinal mucosa of a DR4, DQ7/DR4, DQ8 celiac disease patient preferentially recognize gliadin when presented by DQ8.* Hum Immunol. 1994; 41(4): 285-91. http://dx.doi.org/10.1016/0198-8859(94)90047-7
11. Arentz-Hansen H, Mcadam SN, Molberg Øyvind, Fleckenstein B, Lundin KEA, Jørgensen TJD, et al. *Celiac lesion T cells recognize epitopes that cluster in regions of gliadins rich in proline residues.* Gastroenterology. 2002; 123(3): 803-9. http://dx.doi.org/10.1053/gast.2002.35381
12. Shan L, Molberg O, Parrot I, Hausch F, Filiz F, Gray GM, et al. *Structural basis for gluten intolerance in celiac sprue.* Science. 2002; 297(5590): 2275-9. http://dx.doi.org/10.1126/science.1074129
13. Wieser H. *Comparative investigations of gluten proteins from different wheat species I. Qualitative and quantitative composition of gluten protein types.* Eur Food Res Technol. 2000; 211(4): 262-8. http://dx.doi.org/10.1007/s002170000165
14. Wieser H, Koehler P. *Is the calculation of the gluten content by multiplying the prolamin content by a factor of 2 valid?* Eur Food Res Technol. 2009; 229(1): 9-13. http://dx.doi.org/10.1007/s00217-009-1020-5
15. Žilić S, Barać M, Pešić M, Dodig D, Ignjatović-Micić D. *Characterization of proteins from grain of different bread and durum wheat genotypes.* Int J Mol Sci. 2011; 12(9): 5878-94. http://dx.doi.org/10.3390/ijms12095878

16. Spaenij-Dekking L, Kooy-Winkelaar Y, Van Veelen P, Wouter Drijfhout J, Jonker H, Van Soest L, et al. *Natural variation in toxicity of wheat: potential for selection of nontoxic varieties for celiac disease patients.* Gastroenterology. 2005; 129(3): 797-806. http://dx.doi.org/10.1053/j.gastro.2005.06.017
17. Mitea C, Salentijn EMJ, Van Veelen P, Goryunova SV, Van der Meer IM, Van den Broeck HC, et al. *A universal approach to eliminate antigenic properties of alpha-gliadin peptides in celiac disease.* PLoS One. 2010; 5(12). http://dx.doi.org/10.1371/journal.pone.0015637
18. Xie Z, Wang C, Wang K, Wang S, Li X, Zhang Z, et al. *Molecular characterization of the celiac disease epitope domains in α-gliadin genes in* Aegilops tauschii *and hexaploid wheats* (*Triticum aestivum L.*). Theor Appl Gene. 2010; 121(7): 1239-51. http://dx.doi.org/10.1007/s00122-010-1384-8
19. Molberg Ø, Uhlen AK, Jensen T, Flæte NS, Fleckenstein B, Arentz-Hansen H, et al. *Mapping of gluten T-cell epitopes in the bread wheat ancestors: Implications for celiac disease.* Gastroenterology. 2005; 128(2): 393-401. http://dx.doi.org/10.1053/j.gastro.2004.11.003
20. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. *Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans*. Nature. 1998; 391(6669): 806-11. http://dx.doi.org/10.1038/35888
21. Mello CC, Conte D. *Revealing the world of RNA interference.* Nature. 2004; 431(7006): 338-42. http://dx.doi.org/10.1038/nature02872
22. Pistón F, Marín S, Hernando A, Barro F. *Analysis of the activity of a γ-gliadin promoter in transgenic wheat and characterization of gliadin synthesis in wheat by MALDI-TOF during grain development.* Mol Breed. 2009; 23(4):655-67. http://dx.doi.org/10.1007/s11032-009-9263-1
23. Pistón F, León E, Lazzeri PA, Barro F. *Isolation of two storage protein promoters from Hordeum chilense and characterization of their expression patterns in transgenic wheat.* Euphytica. 2008; 162(3): 371-9. ttp://dx.doi.org/10.1007/s10681-007-9530-3
24. Osorio C, Wen N, Gemini R, Zemetra R, Wettstein D von, Rustgi S. *Targeted modification of wheat grain protein to reduce the content of celiac causing epitopes.* Funct Integr Genomics. 2012; 12(3): 417-38. http://dx.doi.org/10.1007/s10142-012-0287-y
25. Gil-Humanes J, Pistón F, Hernando A, Alvarez JB, Shewry PR, Barro F. *Silencing of γ-gliadins by RNA interference (RNAi) in bread wheat.* J Cereal Sci. 2008; 48(3): 565-8. http://dx.doi.org/10.1016/j.jcs.2008.03.005
26. Pistón F, Gil-Humanes J, Rodríguez-Quijano M, Barro F. *Down-regulating γ-gliadins in bread wheat leads to non-specific increases in other gluten proteins and has no major effect on dough gluten strength.* PLoS ONE. 2011; 6(9): e24754. http://dx.doi.org/10.1371/journal.pone.0024754
27. Gil-Humanes J, Pistón F, Tollefsen S, Sollid LM, Barro F. *Effective shutdown in the expression of celiac disease-related wheat gliadin T-cell epitopes by RNA interference.* Proc Natl Acad Sci. USA. 2010; 107(39): 17023-28. http://dx.doi.org/10.1073/pnas.1007773107
28. Gil-Humanes J, Pistón F, Shewry PR, Tosi P, Barro F. *Suppression of gliadins results in altered protein body morphology in wheat.* J Exp Bot. 2011; 62(12): 4203-13. http://dx.doi.org/10.1093/jxb/err119

29. Camarca A, Anderson RP, Mamone G, Fierro O, Facchiano A, Costantini S, et al. *Intestinal T cell responses to gluten peptides are largely heterogeneous: implications for a peptide-based therapy in celiac disease.* J Immunol. 2009; 182(7): 4158-66. http://dx.doi.org/10.4049/jimmunol.0803181
30. Morita E, Matsuo H, Chinuki Y, Takahashi H, Dahlström J, Tanaka A. *Food-dependent exercise-induced anaphylaxis -importance of omega-5 gliadin and HMW-glutenin as causative antigens for wheat-dependent exercise-induced anaphylaxis-.* Allergol Int 2009; 58(4): 493-8. http://dx.doi.org/10.2332/allergolint.09-RAI-0125
31. Palosuo K, Alenius H, Varjonen E, Koivuluhta M, Mikkola J, Keskinen H, et al. *A novel wheat gliadin as a cause of exercise-induced anaphylaxis.* J Allergy Clin Immunol. 1999; 103(5): 912-7. http://dx.doi.org/10.1016/S0091-6749(99)70438-0
32. Sapone A, Lammers KM, Casolaro V, Cammarota M, Giuliano MT, Rosa MD, et al. *Divergence of gut permeability and mucosal immune gene expression in two gluten-associated conditions: celiac disease and gluten sensitivity.* BMC Medicine. 2011; 9(1): 23. http://dx.doi.org/10.1186/1741-7015-9-23
33. Carter BP, Morris CF, Anderson JA. *Optimizing the SDS sedimentation test for end-use quality selection in a soft white and club wheat breeding program.* Cereal Chem. 1999; 76(6): 907-11. http://dx.doi.org/10.1094/CCHEM.1999.76.6.907