PERSPECTIVE

Breeding for Healthier Wheat

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Wheat in a Historical Context

Consumption of cereal grains by humans did not begin suddenly with the inception of agriculture after the last ice age (16). When moving from African forests into savanna areas some 6 million years ago, the diets of the first humanoids changed, with increased consumption of small, hard grass (cereal) seeds. Analysis of tooth enamel revealed that cereal grains were consumed by ancient *Homo* species living 1.5 million years ago, and this consumption has continued into the modern age. Some 12,000 years ago, the global climate rapidly changed, transitioning to a climate with higher temperatures and increased humidity. This change promoted the growth of plants and animals around the world, resulting in better living conditions for humans. Although most people continued to live a nomadic life, maintaining their hunting, gathering, and fishing habits, some people living in areas where food sources were more readily available became sedentary and began to establish permanent settlements situated at strategic sites in the landscape.

One of these areas was the Fertile Crescent in the Near East, where people built the first stone-based huts. The Fertile Crescent was the biotope of numerous ungulates that local humans hunted, including gazelles, wild goats, wild sheep, mouflon, wild swine, aurochs, red deer, and wild donkeys. These mammals thrived on a rich supply of vegetation that consisted, among others, of a wide variety of grass species. Humans living in this region developed a new focus on plant and animal food sources. Instead of gathering the seeds of cereals and pulses, they brought the plants to their houses and made these cereals and pulses the "founder crops" in their innovative activity—agriculture. At the same time, they domesticated sheep, goats, and aurochs and began the practice of herding animals. Thus, the first farmers appeared on the stage. They were no longer equivalent participants in the local ecosystem as both hunters and prey; they instead gradually placed themselves apart from their natural environment. Because of the success of this new sedentary "mixed farming" lifestyle centered on nearby cultivated and domesticated food sources, the human population in the Fertile Crescent rapidly increased and overpopulated the area. Migration was a consequence of this overpopulation, and the first farmers from the Near East crossed over into Europe between 8,500 and 7,500 years ago. This also marked the beginning of the Neolithic period. Migrating farmers took with them a complete Neolithic "food package" of plants and animals that gradually adapted to their new environments. Within 2,000 years, the descendants of the first farmers reached the northern and northwestern parts of Europe, becoming new neighbors who had a lifestyle that differed from that of the original European hunter/gatherer populations (8,22).

The cereal grains included in the Neolithic food package were the wheat species einkorn (*Triticum monococcum*), emmer (*T. dicoccoides*), and durum (*T. durum*) and barley (*Hordeum vulgare*). Einkorn is an ancient diploid wheat characterized by its AA genome, whereas emmer and durum are ancient tetraploid wheat species that have an AABB genome. Species known today as bread wheat and spelt were not present in the Neolithic food package. These wheat species are thought to have originated during early agricultural activities in the Fertile Crescent, where many wild *Triticum* species grew, including *T. tauschii*, which has a DD genome. Genetic analysis has confirmed that bread wheat (*T. aestivum* var. *aestivum*) and spelt (*T. aestivum* var. *spelta*) originated from natural hybridization between cultivated emmer and wild *T. tauschii* as a side effect of early agricultural activity some 8,000–9,000 years ago. Spelt was moved north of the Alps into Europe via later farmer migrations; bread wheat was spread into the Mediterranean area, where in Roman times it became a crop renowned for the production of fine viscoelastic doughs and flavorful white breads consumed by emperors and prosperous individuals. Roman bread bakers commanded great respect (24). From this time on, the focus of wheat agriculture was increasingly directed to bread wheat. While ancient einkorn, emmer, and spelt dominated the middle, western, and northern regions of Europe as the main cereal crops for many centuries, the rise of bread wheat cultivation and consumption during more recent centuries, especially after the 1950s, appeared unstoppable. Selection and breeding for better adaptation to climates in higher latitudes and environmental biotic and abiotic stresses provided bread wheat with an economic advantage over ancient wheat species. Today, more than 90% of wheat cultivation worldwide is bread wheat grown on 220 million ha that produce 700–750 million tons annually (11).

Role of Wheat in Health

The consumption of healthy foods may help substantially reduce health care costs and may increase life expectancy and well-being. "Let food be thy medicine and medicine be thy food" is a statement made by Hippocrates, the "father of medicine," 2,400 years ago. Today, the graduation ceremonies of many medical education programs include taking the Hippocratic Oath—the moral and ethical message of which has exhibited remarkable resiliency through the ages and remains valid (19). Whole grain wheat fits within this sage advice. Whole grain foods contain all parts of the grain: bran, starchy endosperm, and germ. Several large cohort studies have clearly shown that consumption of whole grain products (including whole grain wheat) reduces the risk of several chronic diseases. A significant inverse relationship has been found between whole grain intake and mortality, in general, and more specifically cardiovascular diseases and several forms of cancer (26,39). Government agencies in many countries, therefore, strongly advise their consumers to eat whole grain products, including whole grain breads (17)—the more the better (7).

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Despite its well-recognized nutritional and health benefits, wheat may also cause a variety of diseases associated with allergic responses, sensitivity, and intolerance. Allergic responses may be acute or chronic. Wheat allergy, which is in general caused by bread wheat (through consumption or inhalation of flour dust), is rare, with a prevalence of 0.25%. Symptoms include atopic eczema (dermatitis) and vomiting in children and wheat-dependent, exercise-induced anaphylaxis and occupational baker's asthma in adults (40). Several wheat proteins have been identified as allergenic, including lipid transfer protein (LTP), amylase trypsin inhibitor (ATI), and omega-5 gliadin. Baker's asthma, which has a relatively high negative economic impact, may best be prevented by adaptation of the working environment through the adjustment of air flows and further protection from inhalation of flour dust. Wheat food allergy prevention includes avoidance of wheat-containing foods that may cause allergic reactions (15).

During the last decade, avoidance of wheat consumption has increased to 10% or more of the Western population. This avoidance is greater among younger females (25–55 years of age) who have attained higher levels of education and live in urban areas (4,5,37). Avoidance of wheat is mainly based on self-diagnoses of symptoms that suggest a form of gluten sensitivity. Wheat as the only cause of such sensitivity is doubtful; however, a relationship with irritable bowel syndrome (IBS) is possible. Certain proteins and carbohydrates are suspects, but they are not limited to wheat and occur in several plant foods. According to medical experts, the prevalence of gluten sensitivity is estimated at about 1% of the population (5). With the goal of preventing gluten sensitivity, the potential causal factors are now being investigated in an international intervention study—the Well on Wheat project (www.wellonwheat.org).

More than 2,000 years ago (and ignorant of the direct cause), Arathaeus of Cappadocea described a food-related abdominal disease that has since been recognized as coeliac disease. Coeliac disease is a chronic inflammation of the small intestine in genetically predisposed humans. It is caused by gluten proteins from wheat, rye, and barley. Coeliac disease can give rise to malnutrition and may present with a variety of symptoms, ranging from bowel disorders to skin, bone, nerve, and muscle problems. The prevalence is estimated at about 1% of the world population and is increasing in different geographic areas, perhaps due to changes in gluten consumption and infant feeding habits (6). The only remedy for coeliac disease is strict, lifelong adherence to a gluten-free diet. Adhering to such a diet is challenging for individuals due to the presence of wheat, wheat ingredients, and gluten in many food products (1,18).

Gluten is the collective term for representatives of various protein families that function as seed storage proteins and can be subdivided into glutenins and gliadins. There are a number of well-defined small fragments (termed epitopes), mainly from the gliadins and some from the glutenins (30,38), that can activate specific immune cells (T cells) in the small intestine. These activated cells initiate the breakdown of the villi that are necessary for efficient uptake of food compounds. Thus, continuous consumption of wheat products leads to inefficient food uptake and chronic symptoms. Strict adherence to consumption of gluten-free foods generally results in complete recovery of the small intestine and improvement in coeliac disease (18).

Currently, coeliac disease is one of the best understood food intolerances, from both the human side (immunology and T-cell specificity) and the plant side (wheat genome complexity and epitope diversity). Using this detailed knowledge, strategies directed toward production of wheat varieties that are (more) safe for consumption by indviduals with coeliac disease can be developed (15,20,23,25,29). Such breeding-related strategies may include selection, reconstitution, mutation, and genetic modification, as well as combinations of these strategies.

Strategies for Production of Coeliac-Safe Wheat

Selection of Wheat Accessions and Varieties with Low Coeliac Disease Immunogenicity. Gene banks around the world maintain many thousands of wheat accessions that represent ancient (diploid, tetraploid, and hexaploid) wheat species, their land races and varieties, as well as modern wheat accessions obtained from more recent breeding. With regard to coeliac disease, many accessions have been analyzed at the genetic and genomic, protein (gluten and epitopes), and immune reactivity levels. Analyses have revealed that the vast majority of these wheat accessions do not have even slightly reduced coeliac disease immunogenic properties. A few diploid (einkorn) and tetraploid (durum) wheat landraces and varieties were identified (using epitope-specific monoclonal antibodies [MAbs] for detection) as having reduced coeliac disease immunogenic properties. Such wheat lines require more in-depth genetic and protein analyses and testing in food intervention studies to determine their safety for individuals with coeliac disease.

A recently developed technology that applies "quantitative proteomics" (counting the number of coeliac disease immunogenic epitopes) in wheat variety analysis might be useful for quantitatively determining the coeliac disease immunogenicity of wheat varieties. Interestingly, individual coeliac disease patients express specific sensitivity profiles toward different epitopes. Epitope sensitivity profiling of individual patients is not yet common, but good examples have been recorded (3,33). With such a profile, a match can be searched for between the absence of specific epitopes in wheat and a patient's specific sensitivity profile (35), enabling a personalized management approach.

Production of Reconstituted Hexaploid Wheat with Low Coeliac Disease Immunogenicity. Bread wheat (*T. aestivum*) is a relatively new species that was unintentionally created during the first stages of agricultural production (discussed earlier): *T. aestivum* contains the combined AABB genome of existing cultivated ancient wheat species and the DD genome of an incidental wild neighbor, *T. tauschii*. The presence of this DD genome in bread wheat provides additional baking quality, improving gluten proteins in the grain, but it also increases coeliac disease immunogenicity (38). Although the diversity of the DD genome as it occurs in bread wheat is very small, indicating hybridization with the AABB genome must have been a rare, single event, in nature the DD genome of wild *T. tauschii* demonstrates a very wide diversity (21). Current studies are directed toward selection of representatives from among the widely varying *T. tauschii* DD genome that have both high baking quality potential and reduced coeliac disease immunogenicity. The goal is to hybridze suitable line(s) with a selected low coeliac disease-immunogenic tetraploid wheat (discussed earlier) to constitute a new hexaploid wheat line with low coeliac disease immunogenicity, good baking quality, and competitive agronomic characteristics.

Mutation Breeding. Mutations are changes in DNA. These may occur naturally and are a driving factor in evolution. Mutations also can be induced chemically (e.g., ethyl methane sulfonate) and physically (e.g., ionizing gamma irradiation). Application of mutagenesis in wheat breeding might be used to create changes in the epitopes of gluten proteins, especially in gliadins, in such a way that their coeliac disease immunogenicity would be eliminated. Mutations can be easily detected using genome sequencing. However, because the number of gliadin genes in wheat is very high (up to 150 in hexaploid bread wheat) and the DNA composition of these genes is highly similar, identifying desired mutations is challenging, and requires analysis of very long DNA molecules (20).

Some advances have been made using gamma irradiation-induced deletions in gluten genes from barley. Using gamma irradiation, an ultra-low–gluten barley variety was produced that fulfilled the criterion of having a gluten content below 5 ppm, allowing a "gluten-free" classification (31). A gamma-irradiated population of mutant lines has been produced from the bread wheat variety Paragon, which is currently being screened for changes in their gluten protein patterns (20).

The main disadvantage of chemical and physical mutagenesis is the unpredictability of the mutations that occur in the DNA, making the application untargeted. Hundreds of induced mutant lines must be analyzed to find desired mutation(s) in a very few lines, as well as several unwanted mutations. This requires further back-crossings to maintain the desired mutations and eliminate unwanted mutations, which is laborious.

A biological approach to inducing large genome mutations, such as deletions of parts of or complete chromosomes, involves crossings to cause incomplete chromosome pairing during meiosis and chromosome disruption during separation after recombination during pollen formation or later during fertilization. This has been applied to progenies and back-crossing populations of the bread wheat cultivar Chinese Spring crossed with a species of *Aegilops* (a genus that is related to *Triticum*) (9). Interestingly the back-crossings resulted in deletion lines that lack a part of chromosome 6 of the D genome where many gliadin genes are located. These deletion lines showed a decreased presence of several relevant coeliac disease-immunogenic epitopes. These deletions also led to stiffer and less elastic dough, which is an improvement in the limited baking quality of Chinese Spring. For other lines that had a part of chromosome 1 of the D genome deleted, only some epitopes appeared to be absent, while dough characteristics remained intact (36). Crossings between both types of deletion lines resulted in a plant line with both chromosome parts deleted and with epitope content further reduced. This line might be useful in attempts to further lower coeliac disease-immunogenic epitopes through wheat breeding (34) but would also provide an indirect approach, compared with recently developed targeted approaches, to eliminate gluten genes and silence or attack epitopes directly.

Advanced Targeted Approaches. Advanced technology for targeted silencing of genes uses the natural biological process of RNA interference (RNAi). RNAi plays a role in plant protection against double-stranded RNA (ds-RNA) viruses through the cleavage of all ds-RNAs contained in the nucleus, which inhibits further replication. Genetic modification of a plant with a ds-RNA construct of a particular gene from that plant also will break down endogenous single-stranded RNA of that gene, silencing it. This technology has been successfully applied to silence a major allergen in apple (14) and to reduce gliadin epitope expression in bread wheat by 10- to 100-fold (2,13). No effects on dough strength or glutenin and starch properties were observed, and standard breads were produced from this genetically modified wheat (12). There are, however, several requirements for modification of such plants: 1) integration of the ds-RNAi construct into the plant genome should be stable; 2) agronomic and food-technology properties of the transformed plant should be comparable to its original counterpart; and 3) national and international regulations concerning genetic modifications (GM) should be followed.

Other recent technology enables very specific targeting of a particular gene or even of a well-defined gene DNA sequence. The CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9) method (28) is highlighted. The CRISPR-Cas9 system protects bacterial cells against viral infections. After its discovery and recognition as a potential tool in genome editing, it has been developed to induce mutations and deletions in both animal and plant cells resulting in improved phenotypes (27). The advantage of this system is that after its introduction into plant cells, for example, no foreign DNA is added to the genome of the resulting mutated organism. As a result, in the United States this technology is considered a non-GM mutation breeding technology (32). Whether this approach to gene editing is considered non-GM in Europe is still under debate. The introduction of the CRISPR-Cas9 construct in a plant cell follows a GM "process," according to EC Directive 2001/18 (10), even though the construct is removed by segregation from the offspring, which makes the "product" of the application of the technology equivalent to conventional mutagenesis through chemical treatment or irradiation. In this case, the voice of consumers, and more specifically coeliac disease patients, as prominent stakeholders should be heard.

CRISPR-Cas9 technology is currently being applied in wheat to eliminate the coeliac disease immunogenicity of specific, targeted epitopes, as well as to delete specific gluten genes. The resulting mutated plants and their seeds can be analyzed using conventional electrophoresis of the gluten proteins, but currently a new procedure is being followed: gluten gene enrichment and sequencing. This procedure is used to first enrich gluten gene genomic DNA from mutated lines of interest before further sequencing is performed as a first screening. Gene enrichment is followed by the application of advanced proteomics methods (such as LC-QTOF-MS/MS [liquid-chromatography–quadrupole time-of-flight–tandem mass spectrometry]) to quantify epitope levels and determine the occurrence of amino acid changes in targeted epitopes. Application of the CRISPR-Cas9 technology system specifically targeted to wheat gluten epitopes is considered to be the ultimate approach to development of wheat varieties that are completely safe for coeliac disease patients, without loss of any agronomic and food-technology characteristics (Jouanin et al., *unpublished*).

A wheat line that is safe for coeliac disease patients and that has good agronomic and baking qualities could be introduced in the market as a specialty product. Because the plants and their grains are phenotypically identical to the original variety, special care should be given to produce and process such a line in a guaranteed separated production chain to avoid any contamination with coeliac disease-immunogenic (i.e., traditional) wheat varieties. This will require extremely high quality control. When this scenario becomes common practice, a further adaptation will be needed to current regulations regarding the 20 ppm gluten threshold established for gluten-free products.

Perspective

Wheat consumption, and the consumption of grains in general, is much older than agriculture and has been linked with humanity since Paleolithic times. Wheat cultivation, selection, and processing started with agriculture and should be considered a gradually developing human phenomenon that intensified when the first human populations began building permanent settlements.

Wheat as a whole grain component of the daily diet can be considered healthy, as has been confirmed in large-scale studies. Allergies to wheat, and other cereal grains (15), are rare. Sensitivity to wheat may be part of a much broader phenomenon and likely related to IBS, with limited causality to wheat alone. Coeliac disease is a well-defined medical condition caused by the consumption of gluten from wheat, rye, and barley by genetically predisposed individuals. The gluten proteins from these grains contain specific fragments (epitopes) that are resistant to human digestion and can interact with specific cells (T cells) in the human immune system, causing degeneration and inflammation of the small intestinal mucosa. These epitopes have been clearly identified with regard to their amino acid sequences (30). This detailed knowledge of coeliac disease-associated epitopes enables targeted searches for wheat varieties with low or no gluten epitopes.

No bread wheat varieties have been identified as having low coeliac disease immunogenicity. A very small number of durum and einkorn lines have been identified using coeliac disease-specific MAbs as having smaller quanitites of certain coeliac disease epitopes. These lines need to be investigated in coeliac disease food intervention studies for their potential as lines that can be classified as "low in gluten" before further investigations can be conducted in wheat breeding programs and food production chains. Because classification as "low coeliac disease immunogenic" is considered the highest level for these lines, "coeliac disease safe" will not become a realistic target through the application of conventional breeding techniques. On the other hand, these low coeliac disease-immunogenic lines may be of interest with regard to their epitope profiles, because individual patients may have sensitivities to only specific epitopes, which might be absent in specific wheat lines. Thus, such wheat lines might be considered "coeliac disease safe" for specific patients.

The route for production of new hexaploid wheat varieties will inevitably be complex. First a *T. tauschii* D-genome representative that is coeliac disease safe must be identified followed by hybridization with a low coeliac disease-immunogenic tetraploid (durum or emmer) variety (which remains to be identified and confirmed in a coeliac disease food challenge). Further selection based on the agronomic and food-processing qualities of the resulting hybrid line would then be required before new varieties could be implemented in any food production chain.

The results from RNAi techniques, which have led to wheat lines with greatly reduced amounts of gluten and high baking quality, are of more interest and have better prognoses. However, such plants are considered GM, resulting in high costs for regulatory approval in countries that accept GM and rejection by countries (or regions) that prohibit GM foods.

The greatest potential is offered by a wheat line that has been made coeliac disease safe through application of CRISPR-Cas9 techniques and from which the mutation-inducing principle has been deleted, which could then be deemed non-GM if the end product, independent of the process, is considered. CRISPR-Cas9 is a very new technology with many potential innovative applications at the DNA, as well as the RNA, level expected (28). Here, attention should be given to the societal context of the application of these technologies regarding the goal of reducing the burden of coeliac disease for patients, with the involvement of consumers and patients as the most relevant stakeholders in discussions on acceptance.

Next-generation breeding strategies, as well as food processing strategies, can be applied to develop wheat- and gluten-containing products with low or no coeliac disease immunogenicity.

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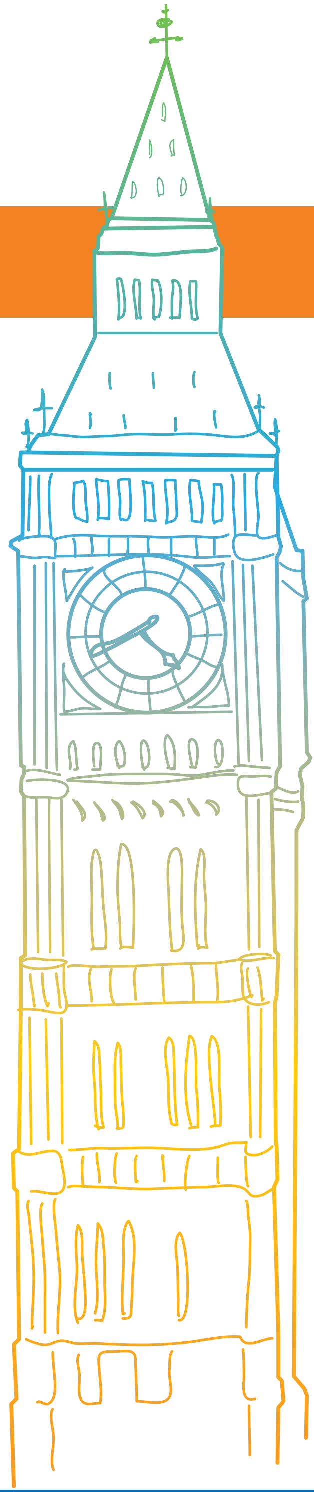

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