Comprehensive REVIEWS in Food Science and Food Safety

Wheat Seed Proteins: Factor's Influencing Their Content, Composition, and Technological Properties, and Strategies to Reduce Adverse Reactions

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Abstract: Wheat is the primary source of nutrition for many, especially those living in developing countries, and wheat proteins are among the most widely consumed dietary proteins in the world. However, concerns about disorders related to the consumption of wheat and/or wheat gluten proteins have increased sharply in the last 20 years. This review focuses on wheat gluten proteins and amylase trypsin inhibitors, which are considered to be responsible for eliciting most of the intestinal and extraintestinal symptoms experienced by susceptible individuals. Although several approaches have been proposed to reduce the exposure to gluten or immunogenic peptides resulting from its digestion, none have proven sufficiently effective for general use in coeliac-safe diets. Potential approaches to manipulate the content, composition, and technological properties of wheat proteins are therefore discussed, as well as the effects of using gluten isolates in various food systems. Finally, some aspects of the use of gluten-free commodities are discussed.

Keywords: coeliac disease, gluten, gluten technological properties, wheat, wheat proteins

Introduction

Wheat has been grown in temperate regions since antiquity, and is primarily used as food and to a lesser extent as feed (Orth & Shellenberger, 1988). However, various other uses have been developed, including as a binding agent in masonry and as a glue in woodwork and bookbinding. Not too long ago, wheat was also used as currency for trading (Åsmund, 2012). Therefore, it is not an overstatement to say that human life in many parts of the world depended on wheat in the past, and wheat-based products remain staples in many countries (IWGSC, 2018). Furthermore, reliance on a wheat-based diet is increasing in many countries where it is associated with urbanization and industrialization (Curtis, 2002; Gustafson, Raskina, Ma, & Nevo, 2009).

Wheat currently provides about one-quarter of the global annual demand for the plant proteins, carbohydrates, and dietary fiber (Langridge, 2017; Shewry & Hey, 2015). Wheat is also increasingly used for brewing, distilling, and the production of bioethanol and biodegradable plastics, while the vegetative parts may be used for pasture (Reitz, 1967; Xu, Wang, Koutinas, & Webb, 2010). However, wheat and wheat-derived products are also associated with a range of adverse effects on human health and well-being, notably coeliac disease, which was first reported in the 19th century but is now more readily and widely diagnosed (Lebwohl, Sanders, & Green, 2018; Wieser, Koehler, & Konitzer, 2014). In addition, recent attention has been focused on wheat allergy and noncoeliac (gluten) wheat sensitivity (NCWS).

The etiology, prevalence, diagnosis, and treatment of these conditions are discussed in the accompanying review in this journal, entitled: "Adverse reactions to wheat or wheat components" (Brouns, Van Rooy, Shewry, Rustgi, & Jonkers, 2019). However, to understand and evaluate the evidence for the role of wheat proteins in determining these conditions, and strategies for ameliorating them, it is necessary to provide an account of the putative causative proteins, focusing on features relevant to their activity. The present article, therefore, provides this information, focusing on gluten and amylase trypsin inhibitors (ATIs), which are considered to be responsible for eliciting most of the wheat-associated disorders in susceptible individuals. This knowledge also provides a basis for discussions of genetic and technological strategies to develop new types of wheat and wheat-derived foods that are tolerated better by mankind.

Wheat Grain Proteins

Wheat grain proteins have been studied for almost 300 years with a vast literature. However, most early studies focused on

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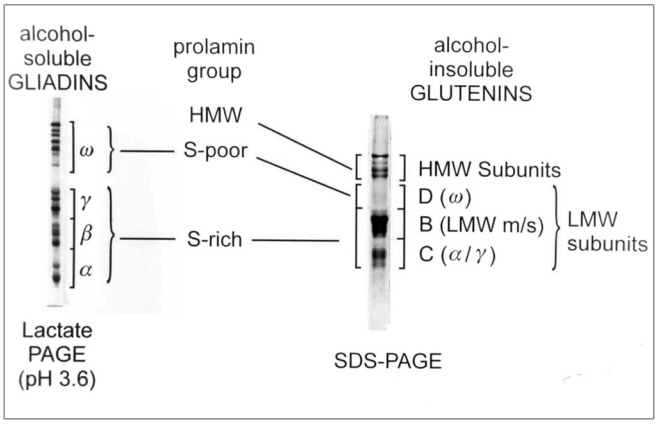

Figure 1—The classification and nomenclature of wheat gluten proteins separated by SDS-PAGE and electrophoresis at low pH. The D-type of LMW subunits are only minor components and are not clearly resolved in the separation shown. Taken from Shewry et al. (1999) with permission.

the gluten protein fraction and its role in determining processing quality. More recently, other proteins have become of interest, particularly the ATIs, in relation to impacts on health and well-being as well as on processing.

What is gluten?

Gluten is the cohesive mass that remains after dough made from wheat flour is washed to remove the starch and other particulate and soluble materials. It comprises about 75% protein on a dry weight basis, with most of the remainder being residual starch and lipids. Isolated gluten also contains small amounts of other proteins, which may be associated physically with the gluten proteins or entrapped in the protein network.

Gluten proteins account for up to 80% of the total grain nitrogen (Seilmeier, Belitz, & Wieser, 1991), although their quantity increases with total grain protein content, due to their role as storage components (Shewry, 2007). Gluten proteins are classified into two groups: the gliadins (classified as prolamins) and glutenins (classified as glutelins), based on their sequential extraction in a series of solvents, with prolamins being extractable in aqueous ethanol and glutelins in dilute acid or alkali (Osborne, 1924). However, we now know that these fractions comprise related proteins (as discussed below), which differ in being present as monomers (gliadins; ranging in mass from 30 to 60 kDa) or as components of polymeric complexes, which may range up to 20 MDa (Delcour et al., 2012). Hence, gliadins and glutenins are now considered as a single protein family, with the major difference being the ability of glutenin subunits to form the interchain disulfide (SS) bonds that stabilize the glutenin polymers (Shewry, Tatham, Forde, Kreis, & Miflin, 1986).

The gliadins and glutenins form the major storage proteins of wheat and are deposited in discrete protein bodies in the starchy endosperm cells of the developing grain. These bodies coalesce as the cells mature and die, leading to the formation of a protein matrix in which the starch granules are embedded. This network forms a basis for the continuous network formed when the protein contents of the individual cells are brought together in the dough.

Gluten protein types. Comparisons of extensive protein sequence data show that all gluten proteins are related but form a number of groups and subgroups, which can be separated by electrophoresis (as shown in Figure 1, which is taken from Shewry, Tatham, & Halford, 1999). Gliadins can be separated by electrophoresis at low pH (Figure 1) into four groups of bands called, in order of decreasing mobility, α-gliadins, β-gliadins (which together form a one group called α-gliadins), γ-gliadins, and ω-gliadins. The glutenin polymers are too large to be separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), but reduction of the interchain SS bonds releases the subunits that are separated by SDS-PAGE into two groups, called high molecular weight (HMW) and low molecular weight (LMW) subunits (Shewry et al., 1999).

More detailed comparisons have shown that the gliadins and glutenin subunits can be classified into three groups. The ω-gliadins and HMW subunits form distinct groups, which have been called sulfur-poor (to reflect their lack of cysteine residues) and HMW prolamins, respectively (Shewry et al., 1986). Most of the other gluten proteins, the α-gliadins, γ-gliadins, and the major group of LMW subunits (called B-type), are more closely related to each other and called sulfur-rich prolamins. However, two minor groups of LMW subunits, termed C-type and D-type, are forms of α-/γ-gliadins and ω-gliadins, respectively, in which mutations have led to the presence of additional cysteine residues which form interchain SS bonds.

Quantification of the proportions of gluten proteins remains a challenge, with no single widely accepted method (see, for example, Scherf & Poms, 2016). The two most widely used approaches are scanning of gel separations and reversed-phase high-performance liquid-chromatography (RP-HPLC), but both methods may give different results depending on the precise systems used. Nevertheless, there is broad agreement on the relative amounts of the major components. Glutenins are generally more abundant than gliadins, and LMW subunits more abundant than HMW subunits. Thus, Seilmeier et al. (1991) using RP-HPLC, reported that gluten proteins accounted for a mean of about 80% of total proteins in white flours of 17 European wheat cultivars, with gliadins and glutenin accounting for means of 39% and 61% of total gluten proteins, respectively. The LMW subunits and HMW subunits were present in a ratio of about 2:1, accounting for means of 41.5% and 19.7% of total gluten proteins, respectively. Within the gliadin fraction, the α-gliadins are most abundant, followed by γ-gliadins and ω-gliadins. For example, the mean proportions of these three groups in 24 flour samples were about 48%, 36%, and 15% of total gliadins, respectively (Wieser & Seilmeier, 1998). The proportions of gluten proteins calculated from the proteomic study reported by Dupont, Vensel, Tanaka, Hurkman, and Altenbach (2011) are broadly consistent except for a higher proportion of HMW subunits: 26% α-gliadins, 15.6% γ-gliadins, 13.4% ω-gliadins, 23% LMW subunits, and 21.9% HMW subunits.

The proportions of gluten protein groups are also affected by crop nutrition (see below). In particular, increases in the proportions of total gliadins and ω-gliadins occur when high levels of nitrogen are applied, which are greater in the absence of added sulfur (Godfrey, Hawkesford, Powers, Millar, & Shewry, 2010; Shewry, 2011). The proportions of gliadins and glutenins may also vary between wheat species, as reported by Geisslitz, Wieser, Scherf, and Koehler (2018), but further studies on a wider range of genotypes and environments are required to confirm this.

Even more problematic is the determination of the numbers of individual protein components within each group, partly because the numbers almost certainly vary between genotypes. The large number of gluten proteins in individual genotypes and the variation between genotypes may arise from both genetic variation and post-translational modification.

Genetic factors are the presence of three genomes, of multiple loci on these genomes, and multigene families at these loci. The genetics of wheat gluten proteins has been reviewed in detail by Shewry, Halford, and Lafiandra (2003) and only a summary is provided here. The three genomes of bread wheat, which are termed A, B, and D, are derived from related species, and hence, there is a high degree of similarity in their gene content. Only two of these genomes (A and B) are present in durum (pasta) wheat, while the three cultivated forms of "ancient" wheat are hexaploid (spelt, ABD genomes), tetraploid (emmer, AB genomes), and diploid (einkorn, A genome).

Major loci for gluten proteins are present on the group 1 (1A, 1B, 1D) and group 6 (6A, 6B, 6D) chromosomes of these species. Loci encoding α-type gliadins (called *Gli-A2* loci) are present on the short arms of the group 6 chromosomes and loci encoding the HMW subunits of glutenin (called *Glu-1*) on the long arms of the group 1 chromosomes. The other gluten proteins are encoded by loci on the short arms of the group 1 chromosomes. Linked major loci encode most of the γ-gliadins and ω-gliadins (*Gli-1*) and the LMW subunits of glutenin (*Glu-3*). Minor loci encoding these proteins have also reported on the same chromosome arms, although these may not be present on all three genomes. Hence, the situation is highly complex.

Finally, all of the major gluten protein loci discussed above comprise multiple genes. The simplest are the *Gli-1* loci, which comprise only two genes (Payne, Holt, Hutchinson, & Bennet, 1984). By contrast, Huo et al. (2018) reported a total of 47 α-gliadin genes of which 26 encoded intact full-length protein products, while Qi et al. (2009) reported the sequences of 29 putatively functional γ-gliadin genes in a single genotype. Furthermore, it is likely the numbers of active genes at these highly complex loci also vary between genotypes.

Further variation may arise from post-translational modifications, particularly deamidation of glutamine residues, which comprise up to 50% of the total amino acids (in some ω-gliadins). However, whether these modifications take place *in vivo* or *in vitro* has not been established. For example, the best-understood group of gluten protein constituents is the HMW subunits, where genotypes of bread wheat contain six genes, of which only four or five are expressed (reviewed by Shewry et al., 2009). However, Dupont et al. (2011) reported the presence of HMW subunit sequences in 43 spots separated by 2D electrophoresis, presumably due to post-translational modifications. The total number of other "gluten protein spots" identified in the same study was 87, making 130 spots in total.

Allowing for the effects of post-translational modification, we would suggest that the number of gluten proteins present in significant amounts is between 50 and 100. This number is consistent with the proteomic study of Bromilow et al. (2017b) who identified 63 gluten proteins in a single cultivar, using mass spectroscopy and a curated sequence database (Bromilow et al., 2017a). These comprised four ω-gliadins, 14 α-gliadins, eight γ-gliadins, 29 LMW subunits, and eight HMW subunits. However, the number of HMW subunit sequences identified (8) was still greater than the number of HMW subunit genes, which were known to be expressed in the cultivar (4).

Irrespective of origin, the vast variation in both the amounts and allelic forms of the different types of gliadins and glutenin subunits is a major factor determining the functional properties of the grain, including the quality for bread making (Veraverbeke & Delcour, 2002).

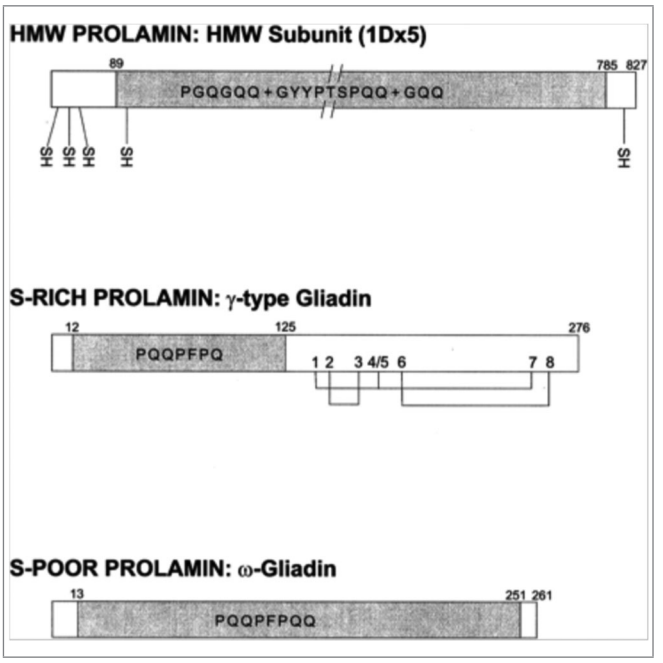

Figure 2–Schematic structures of a typical HMW subunit of glutenin, γ-gliadin, and ω-gliadin. The figure is taken from Shewry and Halford (2002), which gives references for the sequences. Repetitive domains are shaded and the positions of cysteine residues (SH) and interchain disulfide (SS) bonds are shown. Standard single letter abbreviations are used for amino acids: F, phenylalanine; G, glycine; P, proline; Q, glutamine; S, serine; T, threonine; Y, tyrosine.

Amino acid sequences of gluten proteins. A vast number of sequences of gluten proteins are available, based on the sequences of complementary DNA and genomic DNA. For example, Bromilow et al. (2017a) retrieved over 24000 sequences from the UniProt database and used these to assemble a curated database of 630 sequences. These sequences validate the broad classification into the three groups (S-rich, S-poor, and HMW prolamins) and major subgroups of gliadins and LMW glutenin subunits discussed above. They also show that all of the proteins have clearly defined domain structures, with repetitive and nonrepetitive domains.

The domain structures of typical S-rich (γ-gliadin), S-poor (ω-gliadin), and HMW (HMW subunit) prolamins are shown schematically in Figure 2 (Shewry & Halford, 2002). All have central repetitive domains based on repeats of one or more short peptide motifs. These domains are flanked by nonrepetitive domains, but the relative sizes of these domains vary widely. Notably, whereas the nonrepetitive domains are reduced to a few amino acids in the ω-gliadins, the S-rich prolamins contain extensive nonrepetitive N-terminal domains. The sequences of the nonrepetitive domains of the three protein groups are clearly related, and may have been derived from a common ancestral protein by insertion of blocks of repeated peptide sequences.

The nonrepetitive domains also contain most, and often all, of the cysteine residues, which is important because all of the cysteine residues in gluten proteins form SS bonds. The α/β- and γ-gliadins form only intramolecular SS bonds (6 and 8 in α- and γ-gliadins, respectively), which are inaccessible for SH/SS exchange reactions at room temperature (for example, during dough mixing) (Muller & Wieser, 1995, 1997). However, during heat treatments, they can rearrange to form intermolecular SS bonds (see below).

The B-type LMW subunits form intrachain SS bonds, as in the related gliadins, but also form interchain SS bonds. Similarly, all of the HMW subunits form intermolecular SS bonds, stabilizing the high molecular mass polymers, which form the core of the glutenin network in mature grain. In addition, at least some HMW subunits also form one intrachain SS bond. C-type and D-type LMW subunits have additional unpaired cysteine residues that form interchain bonds. The locations of cysteine residues are also shown in Figure 2.

However, of the most interest concerning the potential to elicit adverse reactions in the human body are the repeated sequences. These are based on repeats of short peptide motifs, ranging in length from three to about 10 amino acid residues, and may be repeated in tandem or interspersed with a second motif. The repetitive sequences of some prolamins are highly conserved, making it easy to identify "consensus" motifs: PQQPFPQ, PQQPFPQQ, and PQGPQQ+GYYPTSPQQ+GQQ in the three examples shown in Figure 2 (letters are single letter abbreviations for amino acids, which are listed in the footnote to Figure 2). However, in the LMW subunits, the repeats are degenerate and clear consensus motifs are difficult to define. These sequences are discussed in detail by Shewry et al. (2009), who also provide alignments.

The repetitive domains determine the solubility properties of the gliadins and glutenin subunits and also result in the unusual amino acid compositions of the whole proteins: notably, glutamine (Q) accounts for between 30% and 50% of the total amino acid residues (the proportion relating to the extent of the repetitive domains and their peptide motifs). The numbers of glutamine residues and their sequence contexts also define the ability of gluten proteins to elicit a response in coeliac disease, as discussed in the associated article (Brouns, Van Rooy, Shewry, Rustgi, & Jonkers, 2019—accompanying review).

The prolamin superfamily: amylase trypsin inhibitors

Wheat gluten proteins are defined as prolamins based on the properties (solubility and amino acid composition) conferred by their repetitive domains. However, wheat grains also contain several types of proteins whose sequences are related to the nonrepetitive domains of prolamins, particularly the presence of conserved cysteine residues. They are therefore classified with prolamins as the "prolamin superfamily" and account for most of the water-soluble components with molecular masses between about 15000 and 25000 on SDS-PAGE.

The major, and most widely studied of these groups of proteins, comprises inhibitors of α-amylase and/or trypsin (ATIs). These were first described by Kneen and Sandstedt (1946) and account for up to two-thirds of the total albumins. Different nomenclatures have been used, based either on their electrophoretic mobility at alkaline pH (0.19, 0.28, 0.53) or on their selective extraction in chloroform-methanol mixtures (CM1, CM2, and so on). However, the availability of full sequences has allowed the identification of 11 subunits that form monomeric, dimeric, and tetrameric structures, and their genes to be assigned to chromosomes. They have been described in detail by Carbonero and Garcia-Olmedo (1999) and reviewed by Shewry et al. (2009). Further information on the diversity and relative abundances of ATIs comes from two recent proteomic studies (Altenbach, Vensel, & Dupont, 2011; Dupont et al., 2011). Although most ATIs are inhibitory to human α-amylases, their relative activities vary (Salcedo et al., 2004).

As discussed above, ATIs make up a small but significant (about 4%) portion of the wheat proteins (Altenbach et al., 2011). However, the intake of ATIs may have increased with higher consumption of wheat in some communities and is estimated at 0.5 to 1.5 g/day. In common with other members of the prolamin superfamily (Jenkins, Griffiths-Jones, Shewry, Breiteneder, & Mills, 2005; Mills, Jenkins, & Shewry, 2004; Shewry, Jenkins, Beaudoin, & Mills, 2004), ATIs have conserved cysteine residues that form intrachain disulfide bonds stabilizing a compact form, which is stable to heating and resistant to proteolytic digestion in the human gastrointestinal tract. Consequently, ATIs in cooked wheat (5 min at 100 °C) are still able to trigger an allergic response (Pastorello et al., 2007). Zevallos et al. (2017) suggested a role of ATIs in inducing inflammation and eliciting an innate immune response, both of which have implications in the coeliac disease. Additionally, it has long been known that ATIs play a role in bakers' asthma and food allergy to wheat (Pastorello et al., 2007; Tatham & Shewry, 2008). In recent years, there is a growing consensus that ATIs may trigger NCWS (Reig-Otero, Mañes, & Manyes, 2018; Schuppan & Zevallos, 2015; Verbeke, 2018). These studies collectively suggest that ATIs are not only relevant to food allergies and coeliac disease but probably also in NCWS, as well as perhaps in other gastrointestinal disorders.

Technological Properties of Gluten Proteins

The input of *mechanical energy* in the presence of an appropriate level of water results in the formation of a viscoelastic dough from wheat flour. During mixing, the gluten proteins become hydrated and start to interact to form the gluten protein network. This network has unique viscoelastic properties, with the glutenin components being considered to determine dough cohesiveness and elasticity and entanglement of molecules leading to the continuity of the dough. Hydrogen bonds play an important role in stabilizing the network (Delcour et al., 2012).

Different models have been proposed to explain gluten viscoelasticity. A much cited one is that of Belton (Belton, 1999), which provides a basis for explaining both small and large deformations (Delcour et al., 2012). It views the gluten network as a combination of trains and loops. Trains are interacting protein sequences, where glutamine plays an important role by forming interchain hydrogen bonds. The loops are formed by a single chain, where the amino acids interact with water molecules only. Gliadins interfere with this process and are believed to act as plasticizers and to affect the viscosity of dough. The ratio of glutenin to gliadin also affects the balance between dough viscosity and elasticity (Veraverbeke & Delcour, 2002). Applying too much energy can result in overmixing, with the breakage of SS bonds into radicals, which can further react with other protein fragments to reform crosslinks or with other constituents. In the latter case, the net result is a breakdown of the network (Delcour & Hoseney, 2010).

The input of *thermal energy* sets the protein network. At temperatures exceeding 50 °C, SS crosslinks form (Domenek, Morel, Redl, & Guilbert, 2003). Glutenin reacts first (Guerrieri, Alberti, Lavelli, & Cerletti, 1996) but upon further heating to about 90 °C, α- and γ-gliadins also become involved (Lagrain, Thewissen, Brijs, & Delcour, 2008; Schofield, Bottomley, Timms, & Booth, 1983). The two main mechanisms are sulfhydryl (SH) oxidation (initially glutenin only) and SH/SS exchange reactions (Lagrain et al., 2008). Although dityrosine cross-linking may also occur, less than 0.1% of the tyrosyl residues in bread dough are involved in such cross-links and they are considered to have little, if any, impact on bread making quality (Rombouts, Lagrain, Brijs, & Delcour, 2012). Other types of crosslinks (covalent bonds) can be formed, for example, at high temperatures (for example, in

bread crust) or high pH (for example, during pretzel preparation) (Delcour et al., 2012; Rombouts et al., 2012).

Food applications of wheat and isolated vital wheat gluten

Wheat is the raw material for many types of food products, the most important of which are discussed below. In some cases, industrially isolated vital gluten is also used.

Wheat and gluten-free bread. The gluten proteins are present in dough as a continuous cohesive network that surrounds the starch granules (Singh & MacRitchie, 2001). This network is important for bread quality, affecting loaf volume, crumb structure, and initial texture (Delcour et al., 2012). A recent study used a thermoactive peptidase to show that the gluten network contributes mainly to the coherence of the crumb and less to the texture and the final bread volume (Verbauwhede et al., 2018).

The viscoelastic properties of gluten stabilize the liquid foam structure in dough and the capacity to hold gas during fermentation and oven rise (Campbell, 2003; Kasarda, 1989). When dough elasticity is too low, gas cell expansion is restricted, resulting in a low bread loaf volume (Kasarda, 1989). During baking, the gluten protein network is further set. Initially, SH oxidation and SH/SS exchange reactions take place between glutenins (Lagrain, Brijs, & Delcour, 2008). Later in the process, exchange reactions with gliadin also occur (Lagrain, Thewissen, Brijs, & Delcour, 2007). Vital gluten produced by industrial separation processes (Van Der Borght, Goesaert, Veraverbeke, & Delcour, 2005) is often included in wheat bread recipes in order to meet the quality requirements for products, such as wholemeal bread, multigrain bread, and hamburger buns (Delcour & Hoseney, 2010).

Given the crucial role of gluten proteins in determining bread quality, it is not surprising that the list of possible ingredients for gluten-free bread making is very long (Masure, Fierens, & Delcour, 2016). Recipes are based on flours, from cereals (rice, oat, corn, sorghum, millet, or teff), pseudocereals (amaranth, buckwheat, and quinoa), tubers (cassava and potato), or pulses (soy and chickpea), and starches, which are usually from rice but may be from maize and potato too.

However, as stated above, gluten plays a crucial role in bakery products and different additives are needed to improve the quality of gluten-free products. Protein powders are frequently added, mainly from egg white, whey, maize zein, or soy (Masure et al., 2016; Zannini, Jones, Renzetti, & Arendt, 2012). Crosslinking enzymes (for example, transglutaminase, glucose oxidase, tyrosinase, and laccase) are also often used to enhance the network-forming abilities of the proteins (El Khoury, Balfour-Ducharme, & Joye, 2018), while hydrocolloids, such as hydroxypropyl methylcellulose, carboxymethylcellulose, and methylcellulose, but also guar gum, xanthan gum, locust bean gum, and pectin, are often used to improve the viscosity of the batter and/or gel of the baked product in order to enhance loaf volume, crumb porosity, and/or retard staling. Further additives include amylases, emulsifiers, and ascorbic acid. The processing technology, including type of fermentation, also varies (Masure et al., 2016; Zannini et al., 2012) with high hydrostatic pressure and sourdough technologies (see below) being increasingly used (Zannini et al., 2012). Detailed overviews of gluten-free bread recipes are provided by Zannini et al. (2012) and Masure et al. (2016).

Durum wheat pasta. Gluten also plays a key role in pasta, which is made from durum wheat (*Triticum durum* Desf.) (Delcour et al., 2012). Pasta dough has a lower moisture content than dough produced from bread wheat (*Triticum aestivum* L.), but a protein network is also formed during mixing and sheeting (Zweifel, Handschin, Escher, & Conde-Petit, 2003). Whereas the quality of fresh pasta is strongly determined by the amounts and types of glutenin and gliadin proteins (D'Ovidio & Masci, 2004), the drying process is of greater importance in determining the quality of dried pasta (Delcour et al., 2012). Pasta drying is a delicate process which under the correct conditions (for example, time, temperature, and moisture levels) results in the required content of SS crosslinks (Lamacchia et al., 2007). During boiling of dry pasta in excess water, further protein polymerization takes place. However, if the network formed during drying is too rigid, it hinders starch swelling during boiling and results in poor quality (Bruneel, Pareyt, Brijs, & Delcour, 2010). By contrast, if the network formed during drying is too weak, it cannot retain the starch during boiling and leads to unacceptable cooking losses and poor texture (Pagani, Gallant, Bouchet, & Resmini, 1986; Resmini & Pagani, 1983). It is therefore crucial that drying results in the formation of a dense, continuous network that encapsulates (parts of) the starch granules (Bruneel et al., 2010; Zweifel et al., 2003). Proteins (such as those in egg white) and starch can be used to strengthen or weaken the protein network. Other additives used to improve quality include monoacyl glycerols to improve cooking stability and enzymes, such as transglutaminase and lipase. The former increases the strength of the protein network strength, while the latter leads to different lipid–starch interactions and improves firmness and cooking tolerance (Li, Zhu, Guo, Brijs, & Zhou, 2014).

Gluten proteins are crucial for the volume and crumb structure of pastry products (Ooms et al., 2018). During mixing, a pastry predough is formed, which is comparable to bread dough (Ooms, Pareyt, Brijs, & Delcour, 2016). While this predough can be considered as "undermixed" (Cauvain & Young, 2009), further dough development occurs during lamination (Ooms et al., 2016). During lamination, bakery fat is folded into the dough and alternating sheeting and folding steps result in a multilayered dough-fat-system (Baardseth, Naes, & Vogt, 1995). The laminated dough thus consists of individual layers containing three-dimensional gluten networks (Ooms et al., 2016). After lamination, the gluten networks are oriented in the (final) sheeting direction (Ooms et al., 2017). During baking, glutenin polymerization occurs within the dough layers together with incorporation of gliadin. In addition to the connections within the different layers (intralayer), disulfide bonds between the different dough layers (interlayer) also need to be formed. The formation of too few of these connections leads to collapse by "sliding" of the different layers upon melting of fat, while too many connections lead to a bread-like instead of the desired honeycomb crumb structure (Ooms et al., 2018). The additives that are most commonly used to modify the protein network are enzymes, surfactants, and redox agents (Ooms et al., 2016; 2017). Transglutaminase can be used to obtain a high volume and desirable crumb structure and surfactants to increase crumb softness and/or improve crumb structure (Ooms et al., 2016). Ascorbic acid is a commonly used redox agent that increases dough strength and tolerance and results in a highly specific volume and a desirable crumb structure (Ooms et al., 2017).

Cookies (biscuits). Gluten has a strong effect on the final dimensions of (sugar snap) cookies (Pareyt, Brijs, & Delcour, 2009; Pareyt, Wilderjans, Goesaert, Brijs, & Delcour, 2008). No gluten network is formed during mixing due to the high sucrose and low water concentrations (Baltsavias, Jurgens, & van Vliet, 1999; Gaines, 1990). Although the gluten proteins are below their glass transition temperatures and thus immobile, they do provide viscosity during mixing (Doescher & Hoseney, 1985; Miller, Mathew, &

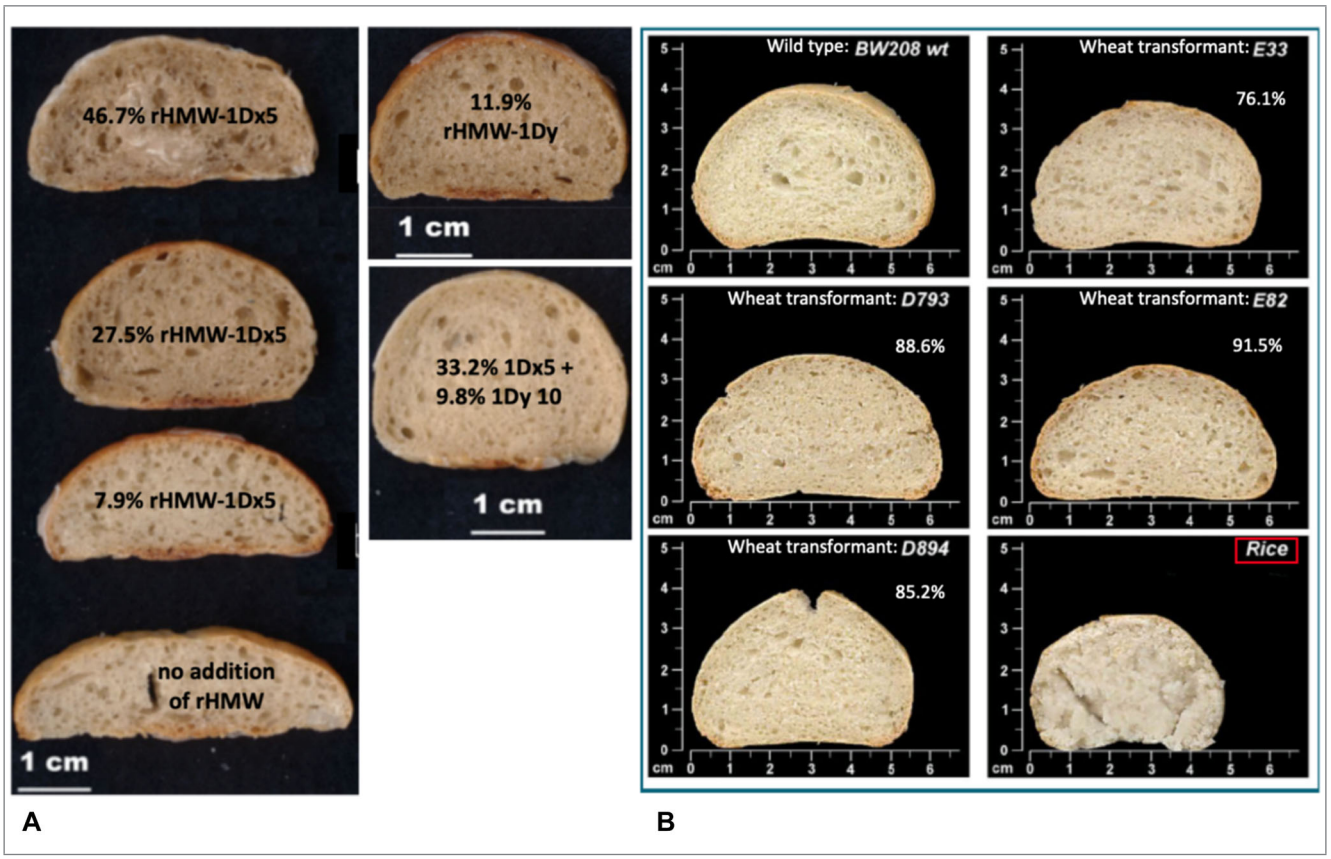

Figure 3–Physical properties of breads baked from the (A) dough prepared with wheat flour washed to remove all gliadins and glutenins (modified from Kieffer et al., 2007), and (B) flour derived from gliadin-deficient transformants of bread wheat (modified from Gil-Humanes et al., 2014).

Hoseney, 1996) and cross-linking between gliadins and glutenins plays a crucial role in network formation (Pareyt et al., 2008; Pareyt, Van Steertegem, Brijs, Lagrain, & Delcour, 2010).

Batter-type cakes. The protein network in batter-type cakes contributes to the springiness and cohesiveness of the crumb and the volume of pound cake (Deleu et al., 2015; Wilderjans, Pareyt, Goesaert, Brijs, & Delcour, 2008). However, in addition to gluten proteins, egg white and egg yolk proteins also participate in this mixed network (Lambrecht, Deleu, Rombouts, & Delcour, 2018). During mixing, a batter is formed, not a dough. At this stage, no protein network is formed, probably due to the high sugar and low protein concentrations and/or the high level of lipids (Deleu, Wilderjans, Van Haesendonck, Brijs, & Delcour, 2017). Gluten proteins contribute to the viscosity of the system. During baking, a network is formed above 78 °C. This initially comprises mainly egg protein, that is, yolk proteins and some egg white proteins with low denaturation temperature. At higher temperatures (approximately 84 °C), ovalbumin (the most abundant protein in egg white that has four free cysteine SH groups for each 385 amino acids) starts to participate and reacts with other proteins, for example, gliadin to form a mixed network (Deleu et al., 2015; Deleu, Wilderjans, Van Haesendonck, Brijs, & Delcour, 2016). Glutenin is also thought to participate in the protein network (Deleu et al., 2015; Wilderjans, Luyts, Goesaert, Brijs, & Delcour, 2010).

Effects of manipulating gluten protein content and composition on technological properties

Many of the proteins discussed above contribute to the unique textural and organoleptic properties of wheat products. Their exclusion or replacement with alternative proteins by processing or crop modification or their enzymic degradation would therefore be expected to affect the quality of products made from all market classes of wheat (for detailed discussion on this subject, see El Khoury et al., 2018; Foschia, Horstmann, Arendt, & Zannini, 2017; Zannini, Pontonio, Waters, & Arendt, 2012).

Several studies have been carried out to determine the consequences of excluding specific glutenins and gliadins on the end-uses of different market classes of wheat. A few examples are given below.

Kieffer and coworkers demonstrated that it is possible to make bread with reasonable crumb and crust structures after replacing gluten by HMW subunits. They made bread from a mixture of washed-out wheat flour residues (containing starch, soluble protein, fat, fiber, and minerals) and known amounts of recombinant HMW subunits (1Dx5 and 1Dy10). The doughs showed good elasticity and after baking resulted in bread rolls with reasonable volume and internal structure (Kieffer, Wieser, Bauer, Hoffmann, & Meuser, 2007) (Figure 3A). Similar conclusions were reached when flour derived from wheat transformants lacking one or more families of gluten proteins were baked into normal looking loaves with acceptable organoleptic properties (Gil-Humanes et al., 2014) (Figure 3B). Although it is unlikely that these lines could be used for large-scale commercial bread making, the work nevertheless indicates that it should be possible to produce acceptable bread for specialist requirements with the use of appropriate additives.

More recently, four near-isogenic lines differing in the numbers and allelic forms of HMW subunits encoded by the A and D genomes of bread wheat were compared. Deletion of the HMW

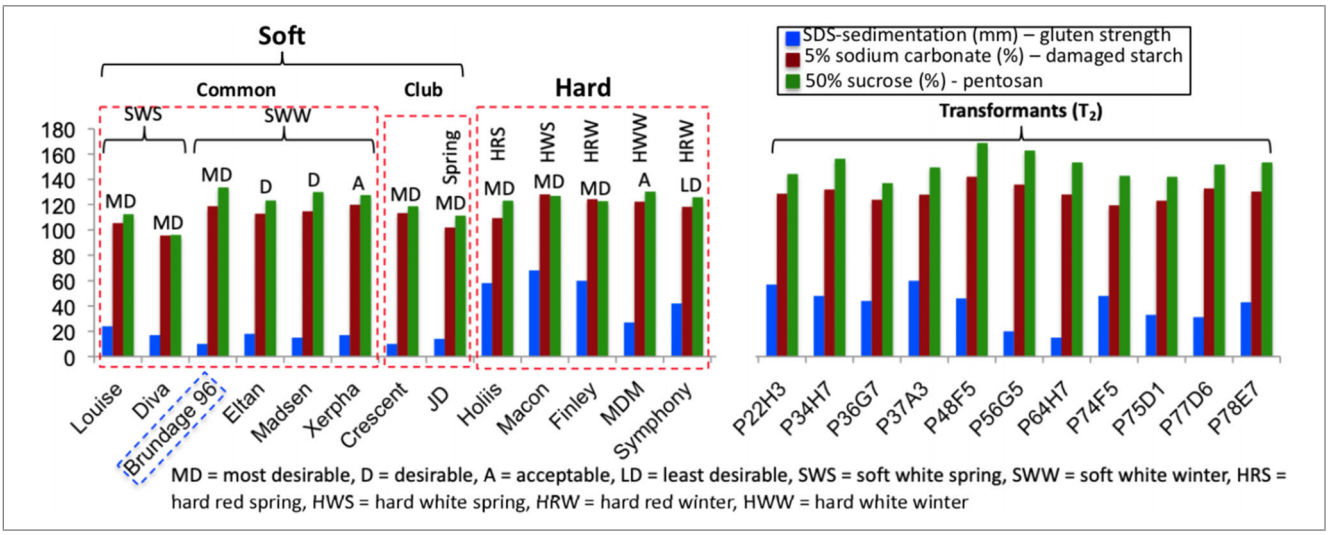

Figure 4–Preliminary assessment of the technological properties of the wheat *DEMETER* transformants using sodium dodecyl sulfate-sedimentation and microsolvent retention capacity tests.

subunits encoded by the D genome resulted in much weaker cookie dough than the absence of A genome subunits or in the control line. However, both double null genotypes lacking the A and D genome subunits and a single D genome null line showed significantly higher cookie diameter, crispness, and lower cookie height in comparison with the A genome null line and the control line. This study indicates that the elimination of the HMW subunits encoded by both the A and D genomes can be tolerated for cookie making (Zhang et al., 2018). In another study, a line of Chinese Spring wheat with a deletion of the A genome LMW subunit genes was used to study the effect of deleting an LMW subunit allele encoded by the A genome. It was concluded that the deletion of this LMW subunit allele significantly reduced the dough strength and bread making quality of the deletion line compared to the control (Zhen et al., 2014).

In a similar study, the contributions of individual gluten protein loci to the technological properties of flour were determined using a set of deletion lines of wheat cultivar Chinese Spring. The results showed that deleting the α-gliadin locus from the short arm of chromosome 6 of the D genome resulted in a substantial loss of technological quality measured by dough mixing and rheology. However, deleting the ω-gliadin, γ-gliadin, and LMW glutenin subunit loci from the short arm of chromosome 1D had little effect on technological properties (van den Broeck et al., 2009). In line with these observations, Altenbach and coworkers reported the improvement in mixing time and tolerance in wheat transformant lacking ω1,2-gliadins and reduction in mixing properties in wheat transformant lacking most gluten proteins (Altenbach et al., 2019). Recent research on the silencing of wheat *DEMETER* gene homoeologues, which have effects on the accumulation of gliadins and LMW glutenin subunits, in a soft white winter wheat background resulted in lines with similar dough mixing properties and bread quality to hard red wheat lines (Figure 4) (unpublished results of R. Brew-Appiah, and D. von Wettstein of WSU Pullman and S. Rustgi of CU).

Collective, these studies suggest that the deletion of genes encoding gluten proteins may still allow the production of bread and/or cookies with reasonable textural and organoleptic properties. And, further improvement in the functional properties of such wheat flours could be achieved by blending them with the proteins from other cereals or other species (van den Broeck, Gilissen, Smulders, van der Meer, & Hamer, 2011).

Approaches to Reduce Gluten-Exposure in Sensitive Individuals

Coeliac patients may be sensitive to different gluten proteins (Koning, 2012). Despite extensive efforts, the repertoire of epitopes may still be incomplete. So far, 356 genes with known epitopes and an additional 472 potential allergen genes have been assigned to the wheat genome, of which, 226 belong to the prolamin gene superfamily. Of all the epitopes with known immune responses (determined based on the IFNγ-ELISpot assay), only 25 epitopes mapped to the HMW glutenins, and only one of these epitopes showed a medium immune response (spot-forming units value between 10 and 20). The other epitopes were reported to have weak immune reactions (spot-forming unit values of less than 10). Similarly, all epitopes that mapped to LMW glutenin sequences were shown to elicit a weak immune response. This suggests that all families of gliadins (α-, γ-, and ω-gliadins) are highly immunoreactive and especially those mapping to the A and D genomes of wheat or related cereals. Within the gliadin sequences, the epitopes mapping to the repetitive domain were more immunoreactive than those mapping to the C-terminal nonrepetitive domain. The epitopes rarely mapped to the N-terminal nonrepetitive domains of prolamin sequences (Juhász et al., 2018).

Screening the wheat gene pool for reduced-gluten genotypes

In theory, any gluten peptide comprising more than nine amino acids can potentially elicit an immune reaction in susceptible individuals (Osorio et al., 2012). Since no wheat genotypes are entirely gluten-free, no recent or old wheat varieties, landraces, or related diploid and tetraploid species or progenitors can be considered "coeliac-safe" (Brouns, Van Rooy, Shewry, Rustgi, & Jonkers, 2019; Brouns, van Buul, & Shewry, 2013; Goryunova et al., 2012; Mitea et al., 2010; Shewry, 2018—accompanying review).

Support for this conclusion comes from wide genetic screens of wheat and related species for their immunogenic potential. This screening has been performed using immunological and nonimmunological methods. Immunological methods used are

enzyme-linked immunosorbent assays (ELISA) based on first- or second-generation antibodies (the former has a broad spectrum and targets one or more prolamin families, while the latter has a narrow spectrum and targets specific epitopes) and T-cell assays. Nonimmunological methods used are based on sequence analysis, gene/transcript sequencing, and gluten profiling (cf. Gilissen, van der Meer, & Smulders, 2014; Rosella, Barro, Sousa, & Mena, 2014).

These studies showed that certain putative "ancient" tetraploid wheat types, such as Graziella Ra, Khorasan, and Kamut wheats, have even higher amounts of total gliadin than modern accessions of tetraploid pasta wheat (Brouns et al., 2013; Brouns, Van Rooy, Shewry, Rustgi, & Jonkers, 2019; Colomba & Gregorini, 2012, accompanying review) and are therefore unsuitable for coeliac patients (Gregorini, Colomba, Ellis, & Ciclitira, 2009; Shewry, 2018). Furthermore, whereas Pizzuti et al. (2006) proposed that diploid einkorn wheat (*Triticum monococcum*) is nontoxic for coeliac patients, this was based on limited data and later studies revealed that it is not suitable (Gianfrani et al., 2012) (Table 1; Kasarda, 2007; Vaccino, Becker, Brandolini, Salamini, & Kilian, 2009). Similarly, extensive analyses of 103 tetraploid durum wheat accessions for two α-gliadin epitopes by immunoblotting and 61 durum wheat cultivars for three α-gliadin epitopes using 454 RNA-amplicon sequencing revealed that none of these genotypes are free of coeliac disease epitopes (Salentijn et al., 2013; van den Broeck et al., 2010b).

Wide screens of hexaploid wheat genotypes for coeliac-safe genotypes were carried out by the Coeliac Disease Consortium in the Netherlands (Gilissen et al., 2014; Molberg et al., 2005; van den Broeck et al., 2010a; van Herpen et al., 2006). Although no genotypes were suitable for general use by coeliac patients, a few "low-toxicity" wheat genotypes, which are devoid of specific epitopes or gluten proteins, were identified (Table 1). These genotypes could therefore be suitable for consumption by coeliac patients, who have sensitivities to specific gluten proteins/protein types, but not for all coeliac patients. However, it is not currently possible to define the full spectrum of epitopes recognized by individual patients. Therefore, it can be concluded that all types of wheats, including hexaploid spelt (*Triticum spelta* L.), tetraploid wheats, such as Khorasan, Kamut, and "2ab" durum (all forms of *Triticum turgidum* L.), diploid einkorn (*T. monococcum*), and related species, such as barley, rye, triticale, and tritordeum, are immunogenic and should be avoided by coeliac patients.

Screening of genetic stocks of wheat for reduced-gluten genotypes

Modified forms of the wheat genotype Chinese Spring, which lack specific chromosomes or chromosome segments, have been screened. These lines showed low reactions with antibodies to gliadins or secalins (rye prolamins) or in T-cell assays, which were related to the absence of specific gliadin loci (Ciclitira, Hunter, & Lennox, 1980; Ciclitira, Lennox, & Hunter, 1980; Frisoni et al., 1995; van den Broeck et al., 2009, van den Broeck et al., 2011) (Table 1). Similarly, wheat mutants lacking α/β-, γ-, and/or ω-gliadins and/or showing reduced accumulation to complete elimination of specific gliadins and/or LMW glutenin subunits have been screened (Table 1). Due to their unique protein profiles (lacking specific prolamins or prolamin groups), some of these mutant lines were suggested to be suitable for individuals with wheat allergy or gluten sensitivity (Waga & Skoczowski, 2014), but these claims need to be substantiated by clinical studies. Later research performed by the same group using the ω-gliadins free and a control wheat line with the sear from seven individuals with different wheat allergy symptoms also emphasized that different fractions of wheat seed storage proteins (including both gluten and nongluten proteins) trigger unique allergic responses in susceptible individuals (Skoczowski et al., 2017). Therefore, the same wheat genotype may trigger different reactions in individuals, and the conclusions for the use of such wheat genotypes cannot be generalized (see the discussion on food labeling in the companion review by Brouns, Van Rooy, Shewry, Rustgi, & Jonkers, 2019).

However, given the large number of gliadin genes in wheat, and their presence in complex multigenic loci, the possibility of pyramiding all low toxicity gliadin genes in a single wheat variety is a formidable task through conventional breeding (Koning, 2012). There are also logistical issues associated with the release of individual low-toxicity wheat lines, such as determining their suitability for individual coeliac patients and their labeling.

Use of genetic modification to develop coeliac-safe wheat genotypes

Several research groups have used genetic engineering to develop "coeliac-safe" wheat genotypes. These approaches mainly fall into two categories: the elimination of gluten proteins and the detoxification of gluten proteins. Following the former approach, Becker and coworkers produced a series of transgenic lines, where α-gliadin genes were downregulated using RNA interference (RNAi). In these lines, α-gliadins were reduced by over 60% compared to the control cultivar with compensatory increases in albumins, globulins, other gliadins, and LMW subunits (Becker et al., 2012; Becker & Folck, 2006; Becker, Folck, Knies, Lörz, & Wieser, 2006; Wieser, Koehler, Folck, & Becker, 2006). Using a similar approach, Altenbach and coworkers reported silencing of the ω5-gliadins (Altenbach & Allen, 2011; Altenbach, Tanaka, & Seabourn, 2014) and ω1,2-gliadins (Altenbach et al., 2019). In the second set of transformants expressing a hairpin construct targeting the wheat ω1,2-gliadins, authors recovered two transgenic lines, one lacking ω1,2-gliadins and the other showing near-complete elimination of all gliadins and LMW glutenin subunits. Similar to the earlier reports, a significant increase in the content of HMW glutenin subunits and nongluten wheat proteins, such as triticins, purinins, globulins, serpins, and alpha-amylase/protease inhibitors, was reported (Altenbach et al., 2019).

More extensive studies were carried out by Barro and coworkers (Table 2). This work has been reviewed in detail (García-Molina, Giménez, Sánchez-León, & Barro, 2019; Gilissen et al., 2014; Jouanin, Boyd, Visser, & Smulders, 2018; Ribeiro et al., 2018; Rosella et al., 2014; Shewry & Tatham, 2016) and is therefore discussed only briefly here. In summary, they generated two series of lines, with downregulation of only γ-gliadins (Gil-Humanes et al., 2008; Piston, Gil-Humanes, Rodríguez-Quijano, & Barro, 2011) or all gliadins (α/β-, γ-, and ω-) and LMW subunits (Gil-Humanes, Piston, Shewry, Tosi, & Barro, 2011; Gil-Humanes, Piston, Tollefsen, Sollid, & Barro, 2010). The γ-gliadin RNAi lines showed between 65% and 97% reduction in the target proteins. Another series of lines showed 60% and 88% reduction in their contents of all gliadins. Assays of these genotypes with intestinal T-cell clones derived from the biopsy samples of coeliac patients showed almost complete suppression of disease-related T-cell epitopes (Gil-Humanes et al., 2010). More recently, the same group used the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) gene editing technology to target conserved regions adjacent to the coding sequence of the 33-mer peptide in the α2-gliadin genes

Table 1–A list of reduced-gluten wheat genotypes identified from the germplasm screen or developed using conventional breeding methods.

Target genes	Genotype	Method	Reduction of target proteins (%)	Assay	Response	Reference
α-gliadins	Wheat var. Chinese Spring, nullisomics 6A tetrasomic 6B/6D	Chromosome engineering	6A-specific α-gliadins are missing	Feeding trail with celiac patients followed by the estimation of fecal fat and D-xylose excretion	No adverse reactions reported	(Kasarda, Qualset, Mecham, Goodenberger, & Strober, 1978)
α-gliadins	Wheat var. Chinese Spring, nullisomics 6A tetrasomic 6B/6D	Chromosome engineering	6A-specific α-gliadins are missing	Feeding trail with celiac patients followed by the estimation of fecal fat, D-xylose excretion, and jejunal biopsy	Malabsorption and jejunal mucosal damage	(Ciclitira et al., 1980; Ciclitira et al., 1980)
α/β-gliadins (*Gli-A2*) and/or γ- and ω-gliadins (*Gli-D1*)	Wheat cv. Raeder (-Gli-A2) and line C173 (-Gli-A2, -Gli-D1)	Natural mutation	6A-specific α/β-gliadins and 1D γ- and ω-gliadins are missing	*In vitro* organ culture	No negative effects on enterocyte height	(Frisoni et al., 1995; Lafiandra, Colaprico, Kasarda, & Porceddu, 1987)
α/β-gliadins (*Gli-A2*) and γ- and ω-gliadins (*Gli-D1*)	Wheat line C173 (-Gli-A2, -Gli-D1)	Cross breeding	6A-specific α/β-gliadins and 1D γ- and ω-gliadins are missing	Duodenal mucosa biopsies and immunological markers	Lowers direct toxicity but activates an immunologic reaction of the duodenal mucosa	(Carroccio et al., 2011)
γ- and ω-gliadins (*Gli-D1*)	Wheat cv. Darius	Natural mutation	1D-specific ω-gliadins are lacking	–	–	(Autran, 1975; Branlard, Dardevet, Amiour, & Igrejas, 2003; Payne et al., 1984)
β-, γ-, and ω-gliadins (*Gli-B1*) and LMW glutenins (*Glu-B3*)	Progeny of (Hope x Cappelle-Desprez) x Highbury	Cross breeding	1B-specific β-, γ-, and ω-gliadins and LMW glutenins are lacking	–	–	(Payne, Holt, Jackson, & Law, 1984)
β-, γ-, and ω-gliadins (*Gli-B1*)	Wheat cv. S. Pastore	Natural mutation	1B-specific β-, γ-, and ω-gliadins are lacking	–	–	(Pogna, Dal Belin Peruffo, & Mellini, 1985)
ω-5 gliadin	Wheat var. Chinese Spring, deletion line 1BS-18	Chromosome engineering	1B-specific ω-gliadins are lacking	Guinea pig feeding trial	Severity of symptoms was much less	(Kohno et al., 2016)
ω-gliadins (*Gli-B1*, *Gli-A1*, and *Gli-D1*)	Wheat lines B1N (-Gli B1 and -one ω-5 gliadin) and Nep1 (-Gli A1 and -Gli D1) and 3xN (-Gli-B1, -Gli-A1, and -Gli-D1)	Cross breeding	1A-, 1B-, and/or 1D-specific ω-gliadins are lacking	Sandwich ELISA using sera of patients allergic to gluten	Considerable decrease (approximately 30%) of gliadin immunoreactivity	(Waga & Skoczowski, 2014)
α/β-, γ-, or ω-gliadins (*Gli-A1*, *Gli-A2*, *Gli-B1*, *Gli-B2*, *Gli-D1*, and *Gli-D2*)	Wheat cv. Saratovskaja 29 biotypes (null mutants)	Natural mutation	1A-, 1B-, 1D-, 6A-, 6B-, or 6D-specific gliadins are lacking	–	–	(Metakovsky, Davidov, Chernakov, & Upelniek, 1993; Redaelli, Metakovsky, Davydov, & Pogna, 1994)
β-, γ-, and ω-gliadins (Gli-B1)	Wheat cv. Spada	Natural mutation	1B-specific gliadins are lacking	–	–	(Lafiandra, D'Ovidio, Tanzarella, Ciaffi, & Margiotta, 1990)

(*Continued*)

Table 1–Continued.

Target genes	Genotype	Method	Reduction of target proteins (%)	Assay	Response	Reference
α/β-, γ-, or ω-gliadins (*Gli-B1*, *Gli-D1*, *Gli-A2* and, *Gli-D2*)	–	Cross breeding	1A-, 1B-, 6A-, and 6D-specific gliadins are lacking	–	–	(Pogna, Monari, Cacciatori, Redaelli, & Ng, 1998)
γ-gliadins	Wheat cv. Paragon	γ-irradiation	–	Acid-PAGE	Deletion of the γ-gliadin locus	(Jouanin et al., 2018; Jouanin et al., 2019)
α/β-gliadins (*Gli-A2*, *Gli-D2*, and *Gli-A2/Gli-D2*)	Wheat cv. Pegaso (-Gli-A2, -Gli-D2, or -Gli-A2/Gli-D2)	Cross breeding	6A- and/or 6D-specific gliadins are lacking	–	Did not contain 33-mer peptide	(Camerlengo et al., 2017)
α/β-, γ-, or ω-gliadins (*Gli-A1*, *Gli-A2*, *Gli-B1*, *Gli-B2*, *Gli-D1*, and *Gli-D2*)	Wheat var. Chinese Spring, deletion line 1AS, 1BS, 1DS, 6AS, 6BS, and 6DS	Chromosome engineering	1A-, 1B-, 1D-, 6A-, 6B-, or 6D-specific gliadins are lacking	mAbs specific for T-cell stimulatory epitopes	Removal of the α-gliadin locus from 6DS or 1DS resulted in a significant decrease in the T-cell stimulatory epitopes	(van den Broeck et al., 2009)
α/β-gliadins (*Gli-A2*, *Gli-B2*, or *Gli-D2*)	Wheat var. Chinese Spring, ditelosomic (Dt 6 AL, Dt 6 BL, and Dt 6 DL), and deletion lines (6AS-1, 6BS-1, 6DS-2, 6DS-4, and 6DS-6) and diploid and tetraploid wheat progenitors	Chromosome engineering or wild wheat genotypes	6A-, 6B-, or 6D-specific gliadins are lacking	T-cell proliferation assays	33mer epitope is encoded by α-gliadin genes on 6D and it is absent from diploid einkorn and certain tetraploid wheats	(Molberg et al., 2005)
α/β-, γ-, or ω-gliadins (*Gli-B1*, *Gli-B2*, *Gli-D1*, and *Gli-D2*)	*Tricicum monococcum*	Natural genotype	B and D subgenome-specific gliadins and glutenins are lacking	*In vitro* organ culture of distal duodenum biopsies	Lack of toxicity of *T. monococcum* gliadin	(Pizzuti et al., 2006)
α/β-, γ-, or ω-gliadins (Gli-B1, *Gli-B2*, *Gli-D1*, and *Gli-D2*)	*Tricicum monococcum* cv. Monlis and ID331	Natural genotype	B and D subgenome-specific gliadins and glutenins are lacking	*In vitro* organ culture of small intestinal biopsies	Activated CD T-cell response	(Gianfrani et al., 2012)
				Urinary lactulose/rhamnose ratio (L/R ratio measured by high-pressure liquid chromatography	Well tolerated by all patients	(Zanini et al., 2013)
α/β-, γ-, or ω-gliadins (*Gli-D1* and *Gli-D2*)	*T. aestivum* CGN08006, *T. turgidum durum* 84866, *T. turgidum dicoccon* CGN08339	Natural genotype	D subgenome-specific gliadins and glutenins are lacking	Immunoblotting with monoclonal antibodies	Significantly reduced levels of Glia-α9 and Glia-α20 epitopes	(van den Broeck et al., 2010b)
α/β-, γ-, or ω-gliadins (*Gli-D1* and *Gli-D2*)	*Tritordeum*	Cross breeding	Reductions in the numbers of immunogenic epitopes: 78% for α-gliadins, 57% for γ-gliadins, and 93% for ω-gliadins	Feeding trail with first-degree relatives of CD sufferers	Not suitable for celiac disease sufferers	(Vaquero et al., 2018)

(*Continued*)

Table 1–Continued.

Target genes	Genotype	Method	Reduction of target proteins (%)	Assay	Response	Reference
WPBF gene (5AL, 5BL, and 5DL)	Wheat var. Express (*wpbf_A, wpbf_B,* and *wpbf_D*)	TILLING	50% to 60% lowered gliadin and glutenin	–	–	(Moehs et al., 2019)
DEMETER gene (5AL, 5BL, and 5DL)	Common wheat var. Express (*dme_A, dme_B,* or *dme_D*), Durum wheat var. Kronos (*dme_A* or *dme_B*)	TILLING	Reduced accumulation of prolamins	–	–	(Rustgi et al., 2014)
Dre2 gene (2AL and 2BL)	Durum wheat var. Kronos *(dre2_A or dre2_B)*	TILLING	–	–	–	Rustgi unpublished data
–	Wheat cv. Chinese Spring, WARM1 and WARM6 (ABA hypersensitive); var. Scarlet ScABI1b, ScABI1c, and ScABI5 (ABA insensitive); var. Brevor 144-29A (ABA insensitive)	Induced mutagenesis (EMS)	Reduced accumulation of prolamins	–	–	Rustgi unpublished data
lys3a (5HS)	Spring malt barley, cv. Bomi, Risø -1508 mutant	Induced mutagenesis (ethylenimine)	66% reduction in prolamin fraction (C-hordeins null and decreased B- and D-hordein), 44% lysine increase	Immunological markers interferon-gamma (IFN-γ), tumor necrosis factor (TNF), and interleukin-8 (IL-8)	RG barley diet might be used for the partial improvement of gluten-induced disease	(Ingversen, Koie, & Doll, 1973; Sestak et al., 2015, 2016)
lys3b (5HS)	Spring malt barley, cv. Bomi, Risø -18 mutant	Induced mutagenesis (sodium azide)	Reduced in prolamin content and high lysine	–	–	(Cook et al., 2018; Munck, 1992)
lys3c (5HS)	Spring malt barley, cv. Bomi, Risø -19 mutant	Induced mutagenesis (sodium azide)	Reduced in prolamin content and high lysine	–	–	(Cook et al., 2018; Munck, 1992)
lys3m (5HS)	Barley cv. Minerva, Risø -1460 mutant	Induced mutagenesis (sodium azide)	Reduced in prolamin content and high lysine	–	–	(Cook et al., 2018; Munck, 1992)
lys1 (5HL)	Barley accession CI3947, Hiproly mutant	Natural mutation	Reduced in prolamin content and high lysine	–	–	(Munck, 1972; Munck, Karlsson, Hagberg, & Eggum, 1970)
hor-2-ca (1H)	Barley cv. Carlsberg II, Risø -56 mutant	Induced mutagenesis (X-rays)	B hordein null	–	–	(Doll, 1976)
lys3a (5HS), *hor-2-ca* (1H), and *Hor3* (1H)	Carlsberg II Risø 56 x Bomi Risø 1508 x D-null Sloop	Cross breeding	C-, B-, and D-hordeins null (below 5 ppm gluten)	ELISA and mass spectrometry	Grain has application in the preparation of food and beverages for coeliacs	(Tanner, Blundell, Colgrave, & Howitt, 2016)
lys1 (5HL) and *lys3a* (5HS)	DH lines (Risø 1508 x Hiproly)	Cross breeding	Reduced in prolamin content and high lysine	–	–	(Tallberg, 1981a, 1982)
–	Spring malt barley, cv. Bomi, Risø -7	Induced mutagenesis (fast neutrons)	Reduced in prolamin content and high lysine	–	–	(Doll, 1976)
–	Barley var. NP113, Notch-1, Notch-2 mutants	Induced mutagenesis (EMS)	Reduced in prolamin content and high lysine	–	–	(Balaravi, Bansal, Eggum, & Bhaskaran, 1976)
–	Risø 1508 x Risø 7	Cross breeding	Reduced in prolamin content and high lysine	–	–	(Tallberg, 1981b, 1982)

Table 2–List of reduced-gluten wheat genotypes developed using genetic engineering procedures.

Target genes	Genotype	Method	Gluten proteins downregulated	Reduction of target proteins (%)	References
Gluten elimination					
α-gliadins	Bread wheat cv "Florida"	hpRNAi	α-gliadins	63	(Becker et al., 2012; Becker, Folck, Knies, Lörz, & Wieser, 2006; Wieser et al., 2006)
C-hordeins	Barley cv "Golden Promise"	Antisense	C-hordeins	29 to 40	(Hansen et al., 2007; Lange, Vincze, Wieser, Schjoerring, & Holm, 2007)
γ-gliadins	Bread wheat cv "Bobwhite BW2003," "Bobwhite BW208"	hpRNAi	γ-gliadins	65 to 97	(Gil-Humanes et al., 2008; Piston et al., 2011)
ω-5-gliadins	Bread wheat cv "Butte 86"	hpRNAi	ω-5-gliadins	–	(Altenbach & Allen, 2011)
ω-1,2-gliadins	Bread wheat cv "Butte 86"	hpRNAi	ω-1,2-gliadins	6.9 and 45.7	(Altenbach et al., 2019)
α/β-, γ-, and ω-gliadins	Bread wheat cv "Bobwhite BW2003," "Bobwhite BW208"	hpRNAi	α/β-, γ-, and ω-gliadins and LMW-GS	60 to 88	(Gil-Humanes et al., 2010; Gil-Humanes et al., 2011; Gil-Humanes, Piston, Barro, & Rosell, 2014; Gil-Humanes et al., 2014; Gil-Humanes, Piston, Gimenez, Martin, & Barro, 2012; Gil-Humanes, Piston, Rosell, & Barro, 2012)
Wheat DEMETER	Bread wheat cv "Brundage 96"	hpRNAi	α/β-, γ-, and ω-gliadins and LMW-GS	45 to 76	(Rustgi et al., 2014; Wen et al., 2012)
	Bread wheat cv "Brundage 96"	amiRNA	α/β-, γ-, and ω-gliadins and LMW-GS	54 to 88	(Brew-Appiah, 2014)
Wheat DEMETER and Dre2	Bread wheat cv WB926	CRISPR/Cas9 (Dre2) with DME TALE repressor (donor)	α/β-, γ-, and ω-gliadins and LMW-GS		(Rustgi, Kashyap, Ankrah, & von Wettstein, 2019)
Barley DEMETER	Barley cv	TALEN	B-, C-, and γ-hordeins		(Wen, 2014)
α/β-, γ-, and ω-gliadins and LMW-GS	Bread wheat cv "Brundage 96"	Chimeric hpRNA	α/β-, γ-, and ω-gliadins and LMW-GS		(Brew-Appiah, 2014)
α/β-, γ-, and ω-gliadins	Bread wheat cv "Bobwhite BW208"	hpRNAi	α/β-, γ-, and/or ω-gliadins and/or LMW-GS	90	(Barro et al., 2016; Piston, Gil-Humanes, & Barro, 2013)
α- and γ-gliadins	Bread wheat cv "Fielder"	CRISPR/Cas9	γ-gliadins	–	(Jouanin et al., 2018; Jouanin et al., 2019)
33-mer in the α-gliadin genes	Bread wheat cv. BW208 and THA53, and durum wheat line, cv Don Pedro	CRISPR/Cas9	α-gliadins	85	(Sánchez-León et al., 2018)
Gluten detoxification					
EP-B2 and Fm-PEP	Bread wheat cv "Brundage 96"	Ectopic expression linear and circular constructs	α/β-, γ-, and ω-gliadins, LMW-GS and HMW-GS	Near complete detoxification	(Osorio et al., 2019)
EP-B2 and PfuPEP	Bread wheat cv "Brundage 96"	Ectopic expression linear and circular constructs	α/β-, γ-, and ω-gliadins, LMW-GS and HMW-GS	Near complete detoxification	(Osorio et al., 2019)

(Sánchez-León et al., 2018) leading to 85% reduction in immunoreactivity of mutant lines. This study showed that CRISPR/Cas9 technology can be used to precisely and efficiently reduce the amounts of coeliac-causing epitopes.

Taking a different approach, Rustgi and coworkers have attempted to silence the wheat *DEMETER* (*DME*) genes, which are master regulators of the accumulation of gluten proteins (except HMW glutenin subunits) in grains. To achieve *DME* suppression, two series of transgenic lines were produced, one with DME-specific hairpin RNA and the other with DME-specific artificial micro RNA (amiRNA). Seven plants produced using the hairpin construct exhibited 45% to 76% reductions in the content of immunogenic prolamins (Rustgi et al., 2014; Wen et al., 2012). However, since hairpin constructs can lead to off targeting of genes, three different amiRNAs were expressed in the second series of lines to silence the wheat *DME* gene. Two amiRNAs were designed from the active site and one from the N-terminal region of the wheat *DME* gene. Genetic transformation with amiRNA constructs resulted in 12 lines showing 54% to 88% reduction in prolamin content (Brew-Appiah, 2014; Rustgi et al., 2014).

In order to "detoxify" gluten, Rustgi and coworkers expressed proteinases that are able to digest gluten ("glutenases") in wheat endosperm. A prolyl endopeptidase from *Flavobacterium meningosepticum* and a glutamine-specific endoprotease from barley (EP-B2) were selected based on parameters, such as target specificity, substrate length, optimum pH, and site of action (Osorio et al., 2012, 2019). Several transformants expressing these glutenases exhibited significant reductions in the amounts of indigestible gluten peptides separated by Tricine SDS-PAGE gels and RP-HPLC.

Development of reduced-gluten wheat products using processing

It is possible to produce flour with reduced immunogenicity from regular wheat genotypes by applying specific processing procedures, such as milling and twin-screw extrusion techniques.

Due to the differences in the distributions of proteins in the grain, with the gliadins and glutenins being expressed only in starchy endosperm cells, and other members of the prolamins superfamily, including ATIs proteins, being enriched in the aleurone and transfer cell layers, it may be possible to reduce the levels of specific proteins by milling (Juhász et al., 2018).

In conventional food extrusion, the material is moistened, heated under pressure, and mechanically sheared as it is conveyed. Extrusion is used in the production of breakfast cereals, some biscuits (Alam, Kaur, Khaira, & Gupta, 2016; Delcour et al., 2012; Hager, Zannini, & Arendt, 2012), and some meat analogues, but also in nonfood applications, such as the production of bioplastics. In extrusion, the formulation is generally subjected to elevated temperature, pressure, and shear. The elevated temperature can induce polymerization (Fischer, 2004; Padalino, Conte, & Del Nobile, 2016; Pietsch, Emin, & Schuchmann, 2017) and different types of wheat protein undergo varying degrees of heat-induced polymerization. Shomer, Lookhart, Salomon, Vasiliver, and Bean (1995) reported that albumins and globulins are more heat stable than HMW glutenins, while Lagrain et al. (2008) reported that γ- and α-gliadins are more heat labile than ω-gliadins. Protein polymerization is dominated by SS bond formation and mainly takes place in the extruder itself (Alam et al., 2016; Fischer, 2004; Pietsch, Emin, & Schuchmann, 2017). For meat analogues, the polymerization of wheat gluten largely determines end product quality (Pietsch, Emin, & Schuchmann, 2017). With extrusion, wide variation in sizes, shapes, texture, and taste can be obtained (Alam et al., 2016). Of course, finding the right settings is critical for end product quality (Hager et al., 2012; Padalino et al., 2016) and digestibility (Meijun Zhu, personal communication).

Recently, Lamacchia and coworkers proposed the use of microwaves to remove antigenic properties of wheat gluten proteins and named the procedure "Gluten Friendly" (Landriscina et al., 2017). The authors claimed the method reduces antigenicity by 99% (Lamacchia, Landriscina, & D'Agnello, 2016), largely based on R5 ELISA assays. However, Gianfrani and coworkers rejected the original claims based on G12 antibody-based ELISA, mass spectrometry-based proteomics, and *in vitro* assay with T cells (Gianfrani et al., 2017). Lamacchia and coworkers then demonstrated positive effects of Gluten-Friendly bread on mucus production and gut-barrier function in human intestinal goblet cells (Lamacchia et al., 2018). However, the products need to be tested in more detail to prove the utility of this method for coeliac patients (Boukid, Mejri, Pellegrini, Sforza, & Prandi, 2017).

The use of grain sprouting (germination) to reduce or eliminate immunogenic gluten peptides has been evaluated, including exploiting peptidases extracted from sprouted wheat, barley, and rye grains. Although cereal endopeptidases synthesized during sprouting can efficiently hydrolyze gluten (Hartmann, Koehler, & Wieser, 2006), other research showed that using peptidases from sprouted wheat to digest gliadin did not result in food safe for coeliac patients (Stenman et al., 2009). Whereas these studies show that immunogenicity can be decreased but not eliminated by sprouting (Lemmens et al., 2019), it has been suggested that complete elimination can be achieved by optimization of the sprouting conditions together with the type of cereal and the cultivar used (Lemmens et al., 2019; Scherf, Wieser, & Koehler, 2018).

Sourdough fermentation has also been proposed as a strategy to develop coeliac-safe products (Zannini et al., 2012). However, bakery products produced by fermentation with lactobacilli in the presence of fungal peptidases from *Aspergillus oryzae* and *Aspergillus niger* showed nontoxicity in coeliac patients, it was concluded that such foods should not be recommended for patients with coeliac disease without formal testing (Cagno et al., 2010).

Problems associated with the use of the gluten-free commodities

There are several issues associated with the use of the gluten-free commodities available in the market:

(1) Unintended contamination of allegedly "gluten-free" products (Thompson, Lee, & Grace, 2010) can take place at any level from the field to shelf due to the widespread use of gluten or gluten-containing grains.
(2) Recent research provided compelling evidence that strict adherence to a diet devoid of gluten-containing grains, or based on foods manufactured for coeliac patients, results in deteriorating gut health due to negative influence on the gut microbiota. It has also been shown that this type of diet increases the risk of colon cancer, due to the reduced contents of dietary fiber and bioactive compounds (De Palma, Nadal, Carmen Collado, Sanz, & Collado, 2009; Gil-Humanes, Piston, Barro, & Rosell, 2014 and references cited therein).
(3) Adaption to a gluten-free diet may initially improve the condition of the patients, but long-term adherence results in multiple deficiencies and changes in body mass index (BMI), which increase vulnerability to other disorders (Theethira, Dennis, & Leffler, 2014). As most gluten-free foods are made with starches or refined flours with low fiber content (see above), this means that coeliac patients consume more energy than required (Martin, Geisel, Maresch, Krieger, & Stein, 2013). Consequently, individuals who have coeliac disease and lower BMI (which may result from impaired nutrient absorption) than the regional population on diagnosis, their BMI may increase on transfer to a gluten-free diet, especially those who adhere strictly to it (Kabbani et al., 2012; Sonti & Green, 2012).

Conclusion

There has been increased concern over the past two decades on the relationship between the consumption of wheat, and particularly wheat gluten, and adverse effects on health. The recent availability of the reference wheat genome sequence provides knowledge of the complete gene complement of bread wheat, including cis-regulatory elements, which will facilitate analysis of the transcriptional regulation of the complex gene families encoding allergenic and antigenic proteins. This knowledge is also expected to facilitate the identification of previously uncharacterized epitopes and the development of novel approaches to produce wheat genotypes safe for all consumers without compromising the organoleptic properties and the end-use quality. The major feat of developing allergen- and antigen-free coeliac-safe wheat could be achieved by a combination of technologies, including genome-editing, genotype and tissue-culture independent genetic transformation procedures, advances in biochemical and immunological detection procedures, and improved (more sensitive and accurate) noninvasive phenotyping methods. All of these approaches are currently being developed and used: the challenge will be to bring them together.

Wheat sensitivity is an umbrella term used to represent a heterogeneous group of disorders, which are alleviated by transfer to a gluten-free diet or a wheat exclusion diet. Current research using animal models (as proxies for coeliac disease or NCWS in

humans) and double-blind placebo-controlled human trials with pure wheat grain components are providing a detailed understanding of the factors contributing to NCWS and identifying the wheat component(s) that trigger specific responses. This knowledge will facilitate the development of therapies for NCWS and also of new types of wheat, which can be tolerated by those with sensitivity to wheat.

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Authors' Contributions

Sachin Rustgi wrote the base manuscript and obtained critical input and complementary paragraphs by Peter Shewry, Lomme Deleu, Jan Delcour, and Fred Brouns. Sachin Rustgi, Fred Brouns, Peter Shewry, and Jan Delcour were involved in final review and editing. All authors contributed to locating and interpreting the literature sources.

List of Abbreviations

amiRNA	artificial micro RNA
ATIs	amylase trypsin inhibitors
BMI	body mass index
Cas9	CRISPR-associated protein 9
CRISPR	clustered regularly interspaced short palindromic repeats
DME	DEMETER
Dre2	derepressed for ribosomal protein S14 expression 2
ELISA	enzyme-linked immunosorbent assays
ELISpot	enzyme-linked immunospot
EP-B2	barley endoprotease B2
HMW	high molecular weight
IFNγ	interferon-gamma
LMW	low molecular weight
RNAi	RNA interference
RP-HPLC	reversed-phase high-performance liquid-chromatography
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH	sulfhydryl
SS	disulfide
TALE	transcription activator-like effector

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