CHAPTER 6

Gluten quantity and quality in wheat and in wheat-derived products

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6.1 Introduction

Triticeae and *Homo*, two taxa that together have changed the appearance of the world dramatically. In a process of some millions of years, unaware of and initially indifferent to each other but linked through the grass-eating animals *Homo* hunted upon, both gradually drifted into each other's living sphere and gradually developed a kind of symbiosis, first exploring and unengaged but tight and obliging later on. Today, the family *Triticeae* [notably the genera *Triticum* (wheat) and *Hordeum* (barley)] and the genus *Homo* are unbreakably connected and together exert significant influence on world's daily life.

In this chapter, we will follow this route from coexistence to codomestication to cohabitation by explaining the increasingly important role of *Triticeae*, and notably wheat, in the development of human society. We start with the history of cereal consumption by early humans throughout their development and dispersal around the world, with special focus on the development of the Neolithic food package. Prior to the onset of agriculture, this already included several cereal species. Starting from this package, the diversity dynamics of the major wheat species during the last millennia will be analyzed: einkorn, emmer, durum, spelt, and bread wheat [1].

We also describe the complex and reticulate genetics within the genus *Triticum* and related genera, not in the last place due to the frequent occurrence of promiscuity resulting into a wide diversity in the wild, and occasionally (with the help of agriculture) into new *Triticum* species that did not exist in the wild before. We further describe the extreme versatility and flexibility of wheat in agriculture, breeding, and food processing as major drivers in the mutual dependence, with an important role for gluten. And finally, we will address the issue of human health: wheat as a healthy food for most but as a problematic food for some.

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6.2 Wheat in the Neolithic package

Some 6 million years ago, a gradual drying caused by the elevation of the East-African continent transformed part of the forest there into a savanna where grasses became the dominant vegetation, with ungulates feeding on. Some humanoids followed this change and started to hunt the animals and to consume the small, hard grass (cereal) seeds. Tooth enamel analysis of ancient *Homo* species evolved from these humanoids and living 1.5 million years ago in this savanna area has revealed cereal grain consumption. Some populations of one of these *Homo* species, *Homo erectus*, left Africa northwards maybe already 2 million years ago and spread eastward via the Near East over Asia. *Homo neanderthalensis* that probably evolved from *H. erectus* 1 million years later also left the African continent and moved westward into the European peninsula and lived there between 0.5 million and 30 thousand years ago. There is direct evidence, based on the starch grain identification of skeletal dental calculus, that Neanderthals consumed a variety of plant foods including *Triticeae* seeds, almost 50 thousand years ago. Starch with damages typical for cooking was found in the western European area (Spa, Belgium) as well as in the Near East area [2], where Neanderthals and *Homo sapiens* shared the same habitat for a significant period of time [3]. This shows that about 50 thousand years ago cooking of cereal materials (and plant food in general) to convert these into better digestible foodstuffs was an overall sophistication in Neanderthal dietary regimes. A grinding tool from 32 thousand years, found in a South-Italian cave, revealed that early *H. sapiens* collected and processed various plants: analysis of the starch grains attributable to *Avena* (oat) caryopses on the surface of the grinding stone, also suggested the use of thermal treatment (similar to present-day oat kilning) prior to grinding. Flour production during the Paleolithic in Europe and the Mediterranean Basin point at a food processing tradition persisting up to the present [4].

How revolutionary was the onset of agriculture? Many ancient *Homo* beings living in small population groups often searched for sheltered places, like caves, to protect themselves against physical and biological violation, to use these places as bases for their hunting and plant food collection activities, and to process their food. The Cro-Magnon, for instance, admired for their impressive grotto paintings of 40- to 30-thousand years ago, were cave-dwellers, but these early humans are also known from their first steps toward horticulture: they made and used tools for horticultural applications already 40 thousand years ago. They prepared soils for plant cultivation with bone digging sticks as shovel and pick-like mattocks for tillage. The awareness of cultivating wild plants at small scale in the neighborhood of settlements may be considered early horticulture as a relevant preceding development toward later agriculture [5]. Domestication of wild plants some 20 thousand years ago was a slow trial-and-error evolution with a long history, showing that agriculture was not a sudden revolution [6].

The Fertile Crescent in the Near East 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. A large camp site of 19 thousand years old in this area covered almost 2 hectares and was occupied for 12 hundred years; an advanced cultural and technological complexity and graves were found there ([7]; www.cam.ac.uk/research/news/from-foraging-to-farming). When 12 thousand years ago the global climate rapidly changed with higher temperatures and increased humidity, humans living in this region had acquired during a long history of a hundred of thousands years all necessary knowledge and tools, technologies, and cultural practices to generate a new focus on plant and animal food sources and on their way of living. In the Mesopotamia delta, clay enabled building stone houses, but clay was also a fertile soil type for grass and other plant species. The first permanent stone urban settlements were established there and, instead of gathering plants and seeds from the wild, these were brought to their living place and cultivatedsss, that is, they were domesticated. This way, cereals (and pulses) became the "founder crops" in people's changing strategies of food acquisition and production: the first steps toward modern agriculture had been made.

Cereals such as wild barley, einkorn, and emmer wheat were the first crops with domestication potential, due to their large durable seeds with prolonged availability in spikelets on the ground for collection during summer, and their apparent germination in autumn [8]. Simultaneously, sheep, goats, and aurochs became domesticated about 11 thousand years ago and herding practice began [9]. Thus the first modern mixed farms developed, and the first farmers appeared on the stage. They were no longer equivalent participants in the local ecosystem as both hunter and prey; they instead gradually placed themselves together with their acquired new lifestyle and domesticated plants and animals apart from their natural environment. Step by step, Neolithic economies developed and became successful. This resulted in overpopulation of the area and gradual migration waves have been seen during the next four thousand years from the Mesopotamia area over the Mediterranean Basin by seafaring colonists as well as northwest movements of farmers over land into the European continent together with their domesticated cattle and crops, making up the Neolithic food package [9,10].

6.3 Diversity of wheat species

The cereal grains included in the Neolithic food package were the wheat species einkorn (*Triticum monococcum*) and emmer (*Triticum turgidum*) on, and barley (*Hordeum vulgare*). Einkorn is an ancient diploid wheat species characterized by its AA genome, whereas emmer is an ancient tetraploid species with an AABB genome that resulted less than 0.5 million years ago after polyploidization and allotetraploid hybrid speciation from an AA lineage (*Triticum urartu*) and a BB progenitor wheat species (from an *Aegilops speltoides* lineage) [11–13]. Wild emmer (*T. turgidum*) evolved into domesticated emmer (*Triticum*

turgidum ssp *dicoccon*) that underwent considerable varietal diversification in response to selection as a result of agroecological and cultivation factors. One of these traits selected for is free-threshing (see Box 6.1), resulting in several free-threshing tetraploid subspecies: *durum*, *turgidum*, *polonicum*, and *turanicum*.

In early traditional agricultural practice, mixed cultivation of, initially, diploid and tetraploid wheats was common in the Middle East and Transcaucasia. Part of the mixed harvest is used as sowing seed in the following year. This will result in a gradual, steady selection toward higher producing genotypes that are better adapted to the local environment: this describes the development of a landrace. Landrace cultivations at that time often occurred in fields surrounded by local wild wheat species populations. Occasionally, along the borders and also within such mixed landrace cultivations, hybrid swarms may occur. A hybrid swarm can be defined as an interbreeding hybrid population including parental species, offspring of crosses, and later generation individuals backcrossed to one or both parental species and (occasional) wild local wheat species.

Bread wheat is thought to have originated within such hybrid swarm during early agricultural activities near the Caspian Sea, where many wild *Triticeae* species grew, including *Aegilops tauschii* with its DD genome [17,18]. Here, hybridization between a free-threshing AABB wheat and *A. tauschii* (DD) resulted in the new hexaploid species bread wheat (*Triticum aestivum*; AABBDD). *T. aestivum* diversified further into several subspecies: *aestivum*, *compactum*, and *sphaerococcum*. Of spelt (*T. aestivum* ssp. *spelta*; AABBDD), two landrace groups are known, one hulled (European spelt) and one free-threshing (Iranian spelt) group; both likely developed independently from an

BOX 6.1 Threshability: from *q* to *Q*.

"Free-threshing" in wheat means that the grains separate easily from the spikelet, which forms the chaff, and are not hulled in glumes [14]. This trait is controlled by the *Tg* (tenacious glume) gene on the short arm of chromosome 2B and 2D, and the *Q* (square-head spike, glume shape and toughness) gene on the long arm of chromosome 5A. *Tg* inhibits the expression of *Q*. A free-threshing phenotype requires both gene mutations from *Tg* to *tg* and from *q* to *Q*. The *Q* and the *q* allele differ in only one amino acid. Comparative sequence analyses among *Triticum* species revealed that the *q* to *Q* mutation occurred just once in the evolution of *Triticum*. The *Q* allele probably first appeared in tetraploid wheats ($QQ^{5A}tgtg^{2B}$) as most archaeological free-threshing wheats are suggested to be tetraploids. Hence, the tetraploid parent of hexaploid bread wheat is suggested to be a free-threshing form of tetraploid wheat [15]. Spelt wheat ($QQ^{5A}TgTg^{2B}tgtg^{2D}$), which is hulled, may be the result of an introgression event from a hulled emmer ($QQ^{5A}TgTg^{2B}$) into free-threshing bread wheat ($QQ^{5A}tgtg^{2B}tgtg^{2D}$), maybe *Triticum aestivum* ssp. *compactum* with regard to the European spelt; free-threshing Iranian spelt with the Tg^{2D} allele is suggested to be originated independently from another hexaploid wheat ([16] and references therein; [15]).

introgression of a cultivated tetraploid emmer into a hexaploid *T. aestivum* (although the origin of the Iranian spelt is still elusive) (Box 6.1). Other introgressions of wild emmer wheat into *T. aestivum* may have resulted into hulled Macha wheat (ssp. *macha*; AABBDD). Tetraploid *T. turgidum* ssp. *carthlicum*, which has morphological similarities with *T. aestivum*, is suggested to be an admixed (introgression) form of wild and domesticated emmer lines [19]. See for overviews Table 6.1 and Refs. [13,15,16]. Introgressions in hybrid swarms have contributed significantly to the diversification of wheat species and have enriched their gene pool [16]. Introgressions of large blocks of genes are easily detected, but also small or "cryptic" introgressions will have occurred. To date, it is likely that alleles have been introgressed from more than 50 species from 13 genera [20] highlighting the complex genomic pattern of bread wheat [21].

A recent complete list of wild and cultivated *Triticum* wheats can be obtained from http://www.k-state.edu/wgrc/. This list includes the species and subspecies with their genome constitution in the *Triticum* sections Monococcon (AA), Dicoccoidea (AABB and AAGG), and the section Triticum (AABBDD and AAAAGG) [22]). Table 6.1 lists the most relevant representatives that are cultivated in present-day agriculture. The tetraploid *Triticum*

Table 6.1: *Triticum* wheat species and subspecies with their genome constitution and threshability.

Section	*Triticum* species and subspecies (common name)	Genome constitution	Free-treshing
Monococcon	*T. monococcum*	AA	
	ssp. *aegilopoides* (wild einkorn)		−
	ssp. *monococcum* (cultivated einkorn)		−
	T. urartu	AA	−
Dicoccoidea	*T. turgidum*	AABB	
	ssp. *dicoccoides* (wild emmer)		−
	ssp. *dicoccon* (cultivated emmer)		−
	ssp. *durum* (durum wheat)		+
	ssp. *polonicum* (Polish wheat)		+
	ssp. *turanicum* (Khorasan wheat)		+
	ssp. *turgidum* (rivet wheat)		+
	ssp. *carthlicum* (Persian wheat)		+
	ssp. *paleocolchicum* (Georgian wheat)		−
Triticum	*T. aestivum* (common wheat)	AABBDD	
	ssp. *aestivum* (bread wheat)		+
	spp. *compactum* (club wheat)		+
	ssp. *sphaerococcum* (Indian dwarf wheat)		+
	ssp. *macha* (macha)		+
	ssp. *spelta* (European spelt; Iranian spelt)		−; +

Sources: After Matsuoka Y. Evolution of polyploid Triticum *wheats under cultivation: the role of domestication, natural hybridization and alloploid speciation in their diversification. Plant Cell Physiol 2011;52:750–64. https://doi.org/10.1093/pcp/pcr/pcr018 [16]; Van Slageren MW. Wild wheats: a monograph of Aegilops L. and Amblyopyrum (Jaub. & Spach) Eig (Poaceae). Wageningen Agricultural University papers, The Netherlands; 1994, p. 1–512 [22].*

timopheevii (AAGG) with ssp. *armeniacum* (wild timopheevii) and ssp. *timopheevii* (cultivated *thimopheevii*), and the hexaploid *Triticum zhukovskyi* (AAAAGG) have not been considered in this chapter.

6.4 Domestication of wheat species

Domestication (literally: bringing home) includes transfer, take care of, feed, adapt, disperse, and propagate living organisms (specific species of plants, animals, fungi, microorganisms) by human beings for a specific purpose (added value). This ceates a mutual dependence.

In grain crops, the domestic syndrome includes several characteristics [23]. First, reduced seed dispersal, which is necessary for effective harvest. In *Triticeae*, this relates to nonshattering and nondisarticulation of the rachis and especially free-threshing of the seeds as most important domestication traits. Genes involved are *Tg*, *Q* (both regulating free-threshing; see Box 6.1), *Br* (for rachis brittleness), and *C* (for spike compactness). Other selection targets in domestication are seed size and grain yield (both affected by many genes). Further, lodging is a problem in wheat harvesting and yield, making plant height a relevant characteristic: several *Rht* (reduced height) genes in wheat are involved in gibberellic acid signaling; mutant alleles confer dwarfism. Shorter height also means that the plant can invest more in grain production. Since the Green Revolution in the 1960s these alleles are being applied in all worldwide breeding programs. Grain hardness is a grain quality trait relevant to milling and baking with the involvement of the *Ha* (hardness) locus with three tightly linked genes *Pina* and *Pinb* (related to the production of puroindoline a and b, respectively) and *Gsp1* [for grain softness protein (GSP) synthesis]. Another trait is tillering that is coded for by a single gene (*til*, tiller inhibition gene), which in recessive form is responsible for fewer and sterile tillers, a larger grain size, and thus greater harvest index. Next, reduced seed dormancy and control of flowering time are genetically complex traits that enable rapid seed germination in autumn and flowering in springtime after sufficient cold (vernalization) in winter wheat types. Vernalization involves three *VRN* genes. We find loss of function or deletion of *vnr* in spring type wheats, which are sown after the winter and which will flower with no requirement for cold. Flowering time fine-tuning is also regulated by the earliness per se (*Eps*) gene ([23] and references therein).

Domestication significantly influenced *Triticum* species in many characteristics/traits on which accidentally or deliberately has been selected for by farmers and, later, also by breeders. Good adaptability to new environments (due to its excessive and redundant genetic composition of the tetraploid and especially the hexaploid species) made wheat the excellent companion of the Neolithic farmers migrating out of Anatolia into Europe and Asia. This way numerous landraces have been developed. The free-threshing state and the

brittleness of the rachis were very important in the competitive power of tetraploid durum wheat and hexaploid bread wheat, already in Roman times [24]. The high variability in genotypes and phenotypes also enabled selecting and breeding for many other traits, such as seed size, kernel row type, plant height, grain hardness, tillering, seed dormancy, photoperiod, vernalization, and heading date [23].

The "domestication bottleneck" concept refers to the small initial population of plants with desired traits taken from the wild population upon domestication, which led to a drastic reduction of genetic diversity in the selected (early domesticated) population of many crop species. However, einkorn appears to be devoid of such a bottleneck; it has been rediscovered as a useful source of genetic variation for wheat breeding. Similarly, in emmer wheat, the domestication bottleneck appeared relatively low, as 95% of the diversity in wild emmer *T. turgidum* ssp. *dicoccoides* was found in the domesticated emmer ssp. *dicoccon* ([23] and references therein). In addition, studying archaeological genomes of several wheat species shows only a limited reduction of genetic diversity, which may necessitate a reevaluation of the "domestication bottleneck concept" when considering the current understanding of the evolutionary process of domestication of wheat [25].

6.4.1 Einkorn [T. monococcum, *AA genome*]

The diploid einkorn wheat has been considered one of the first domesticated crops. Wild einkorn grains were harvested already about 24,000 years ago [26]. Cultivated einkorn is known from 12,000 years ago and was a popular cultivated crop during the Neolithic and Bronze Age. Domestication occurred probably in the presently southeast Turkey area (Karacadağ core area) from the progenitor species *Triticum boeoticum*. Einkorn was the staple crop of the Sumer populations. Domestication of einkorn occurred within the "core area" at several places connected with each other through the use of the same A race material and the same human culture. In these early times, humans in the region were familiar with the harvest of wild seeds both in natural habits and in cultivated fields. This has been resulted in the spread of an A^b race population, characterized by its large genetic variation, later followed by the independent development of domesticated lines at several separate localities far beyond the "core region". These domesticated lines all maintained the large genetic variation of the original A race, without domestication bottleneck [23].

Today, einkorn is cultivated only on a very small scale as feed for poultry and swine in some isolated mountainous villages in southern Europe and Turkey. It has recently been rediscovered as a source of genetic variation for wheat breeding and in the European niche food industry ([23,27,28] and references therein). Based on DNA marker analysis of einkorn, cultivars still show a very high similarity to wild einkorn. The wild and cultivated einkorn are differentiated by the brittleness of the rachis: brittle in the wild and less fragile in the cultivated einkorn.

6.4.2 *Emmer* [T. turgidum *ssp.* dicoccoides *(wild), ssp.* dicoccon *(domesticated); AABB genome*]

Wild emmer, growing in the western and central parts of the Fertile Crescent, was domesticated in southeast Turkey, similarly to einkorn in the Karacadağ core area. Two different races of *T. dicoccoides* exist, one western and one central-eastern, of which the central-eastern race has been considered the progenitor of the domesticated germplasm. From the core area, emmer cultivation expanded across Asia, Europe, and Africa. The southwestern expansion of domesticated emmer generated sympatry with the southern *T. dicoccoides* population and gave rise to a secondary diversity center. As a result, a northern and a southern subpopulation of emmer developed. The domestication bottleneck appeared relatively low, showing 95% similarity between ssp. *dicoccon* and ssp. *dicoccoides*. This rate is similar to that in einkorn ([23] and references therein). In the northeastern expanding subpopulation of domesticated emmer, the mutation of q to Q will have been taken place, resulting in a free-threshing form that evolved further into *Triticum durum*, with its subspecies *durum*, *turgidum*, *polonicum*, and *turanicum* (Table 6.1).

Emmer was consumed by the earliest civilizations already almost 20,000 years ago as porridge, prior to bread. In ancient Egypt, emmer was the favorite crop for breadmaking. Cultivated emmer dispersion and application exceeded that of einkorn. The additional BB genome enabled cultivate emmer to grow in a wider range of environments and climates compared with einkorn, and emmer became the dominant wheat throughout the Near and Far East (areas of Pakistan and India), Europe (gradually from Anatolia toward France, United Kingdom, and southwest Scandinavia areas between 6000 and 4000 years ago), and Northern Africa (Egypt, 7000 years ago; Morocco via the Iberic peninsula, 6000 years ago), from the Neolithic through the Bronze Age until 3000 years ago when it became gradually replaced by barley and naked (free-threshing) tetraploid durum wheat [27,29].

Presently, emmer cultivation has declined to 1% of the total wheat cultivation area, limited mainly to some traditional farming communities in Russia, and in Ethiopia where it remained as a relevant crop taking 7% of the total wheat cultivation area. In European Alpine countries, emmer is presently cultivated as a minor crop in organic agriculture. Due to long-term cultivation in a large range of ecogeographical conditions, a wide variety of emmer forms are known. Most emmer wheat landraces are spring types, except some from Western Europe. Several diversity studies have been carried out on cultivated accessions from various origins and agronomic characters, all studies mentioning a wide range of diversity [29]. Even three subspecies could be distinguished: ssp. *asiaticum*, ssp. *abyssinicum*, and ssp. *serbicum* [30]. In general, presently three emmer forms are distinguished according to the color of their ears: brown, black, and white emmer. Gene bank accessions prove to be highly heterogeneous [24].

6.4.3 *Durum* [T. durum*; AABB genome*]

T. durum (naked, free-threshing, tetraploid hard wheat) likely originated from domesticated *T. durum* ssp. *dicoccon* (hulled emmer) ([23] and references therein; [29]). Durum (Latin) means hard: durum grains are the hardest of all wheat grains requiring special milling technology. Even though it has a high protein content relative to other wheat species, this gives less strength to dough. The first naked wheats appeared in the archaeological record already about 10 thousand years ago. The free-threshing character (the mutation from q to Q, Box 6.1) must have taken place early in this domestication-related diversification process, most likely in the Caucasian region. Being part of the earliest food package, the free-threshing wheats quickly replaced hulled wheats in many regions, especially in the Mediterranean region. In particular, *T. durum* ssp. *durum* may have been further domesticated in the eastern Mediterranean region and spread westward into the Mediterranean Basin [19,23], where it is widely cultivated for pasta production. Pasta is known from the 10th century onwards. The yellow endosperm of durum grains gives pasta its color. Nowadays durum is the second most cultivated wheat species, representing about 5% of the total global wheat production. The European Union (EU), China, India, and Russia are the biggest durum producers.

6.4.4 *Common wheat* [T. aestivum *ssp.* aestivum *(bread wheat) and ssp.* spelta *(spelt wheat); AABBDD genome]*

A free-threshing domesticated *T. durum* came into contact with the wild population of *A. tauschii* in the Caucasus/Caspian Sea region, resulting in the hexaploid wheat *T. aestivum* [17,18]. It was suggested [15,16] that spelt wheat is the result of a new hybridization between this free-threshing hexaploid bread wheat (homozygous for *Tg* and *Q*) and a hulled emmer type (homozygous for *Tg* and *q*). This implicates that spelt was not ancestral to, but resulted from *T. aestivum* (maybe ssp. *compactum*) as parent in this new hybridization. European and Asian spelt are genetically different and are suggested to have a separate origin, but their common D genome points at a common hexaploid ancestor (Box 6.1; [15] and references therein).

Overall, the evolution of polyploid *Triticum* wheats under cultivation can be considered a diversification continuum, with wild emmer (*T. turgidum*) at the one end, and common wheat (*T. aestivum*) at the other end of the spectrum. Domestication of emmer wheat initiated the process of continuous diversification through occurrence of hybridizations and allopolyploid speciation in hybrid swarms in agricultural fields [16]. These diversification events and genetic redundancy made polyploid wheat *Triticum* a very versatile crop that was able to adapt to many different environmental conditions resulting in the development of many landraces and that carried ample possibilities for further breeding and application

in food, feed, and nonfood. Genetic redundancy masks the effects of recessive alleles and allows high densities of mutations [31].

Bread wheat was probably cultivated for centuries mixed with other wheat species. It spread across the Mediterranean Basin area but only in Roman times it became a separate crop renowned for the production of fine elastic doughs and flavorful white breads consumed by emperors and prosperous individuals. Roman bread bakers commanded great respect [24,32].

During the Middle Ages, cereals were the main dish in Europe. The rural population had cereal-based foods on their plates: rye (dark sourdough rye bread, which was preferred in the north and east; wheat in the west; spelt, often harvested green and then dried: "grunkern" in the southwest). Grain foods were served in a variety of forms, ranging from dark gruel eaten by the poor to fine white bread for the rich. The diversity of bread can still be found in today's supermarkets, although presently the social classification is almost reversed. All crops, including the cereal crops, were cultivated as mixed landraces or varieties, which was considered and experienced as a kind of insurance against plagues and diseases to the crops [33].

While the different wheat species have been cultivated along each other for centuries, most have been pushed aside after World War II by bread wheat, which is widely grown today (with about 95% of the total wheat cultivation). This led to a loss of the overall genetic diversity of wheat in the fields [32,34,35].

Nowadays, wheat is an important crop worldwide for many purposes. This is reflected in the number of wild and cultivated accessions that are stored in gene banks: ~470,000 accessions in all; in Mexico (at CIMMYT) 142,300 accessions; in the United States 62,000; in Lebanon 44,000; in Australia 43,000; and in Russia 34,000. This number of accessions stored worldwide surmounts that of rice (252,000), barley (225,000), and maize (107,000). Most wheat accessions concern bread wheat (344,000) and durum wheat (96,000); the bread wheat accessions include landraces (139,000), economically important cultivars (98,000), and breeding material (97,000 accessions) (www.genesys-pgr.org).

6.5 Wheat breeding

Until the 1950s many farmers preferred wheat landraces: wheat is a self-pollinating crop maintaining its quality over many generations, making cultivation simple by sowing seeds saved from the preceding year. This way, landraces gradually adapt highly to local environmental conditions, but limit the improvement of end-use quality. This situation changed when starting at small scale in the early 20th century "genetics" became involved in wheat breeding and pure line selection and breeding more and more put the focus on new

and individual quality trait improvements. This culminated in the introduction of dwarf genes in wheat during the Green Revolution in the 1960s, contributing to marked modifications in the gene pool over the world (Box 6.2).

Recently, 11 groups have been identified in the bread wheat germplasm, of which 7 belong to the Eastern European and Mediterranean pool, 2 to the Asian pool, and 2 to the Western European pool. Most modern cultivars are related to the landraces from Southeast Europe, the Iberian Peninsula, and the Mediterranean Basin; Northwest European landraces contributed only to Western Europe modern germplasm; Asian landraces have been scarcely used in modern breeding programs. Further, the introduction of wild relatives in crop improvement has resulted in much alien DNA in many wheat cultivars: some genes are shared between all cultivars while other genes may be absent in various cultivars. www.genesys-pgr.org

Initially "wheat quality" meant "bread quality." Then, when the relationship of bread quality with the gluten content was discovered, and additionally the all-steel roller mill was developed, the attention became focused on gluten quantity and quality, and on milling quality (flour quality). Cereal chemistry was gradually established. Breeding aims became mainly focused on yield (largely starch) and gluten quality (for baking applications). Agronomical breeding aims (including adaptation to the biotic and abiotic environment) were supportive (Box 6.3).

6.6 Gluten and other prolamins in wheat

Gluten are part of the wheat grain proteins of the prolamin superfamily. Three main types of gluten proteins can be distinguished: (1) the HMW prolamins (HMW glutenins of 65−90 kDa, taking 6%−10% of the total gluten fraction), (2) the sulfur-rich prolamins (with alpha- and gamma-gliadins, and B- and C-type LMW glutenins of 30−45 kDa, comprising 70%−80% of the total gluten fraction), and (3) the sulfur-poor prolamins (including omega-gliadins and D-type LMW glutenins of 30−75 kDa, representing 10%−20% of the total gluten fraction) (Fig. 6.1). Relationships between the different proteins within each group become clear from amino acid sequence comparison, showing for example the C-type LMW glutenins to be modified alpha- or gamma-gliadins, and the D-types modified omega-gliadins [53].

The gamma-gliadins are considered the oldest of the gliadin family in *Aegilops/Triticum*. In the hexaploid bread wheat, the genes are on the homoeologous *Gli-1* loci of the short arm of the homoeologous chromosomes 1 (*Gli-A1*, *Gli-B1*, *Gli-D1*). Cloning 59 gamma-gliadin genes from eight diploid *Aegilops* and *Triticum* species revealed early gene duplications (presumably two rounds of duplication of the locus) and further diversifications (including duplication, pseudogenization, and deletion). Overall, six groups of gamma-gliadins were

BOX 6.2 Applying the genetic flexibility of wheat in advanced breeding.

Introgression
Introgression uses crosses of closely related plant species, not to combine complete genomes, but to transfer chromosome fragments (blocks of genes), which are usually translocated to distal chromosome regions of the receptor genome. Tracking and tracing of translocations is nowadays done with molecular markers. In wheat, this technology is often applied to introduce disease-resistant (R) genes from wild relatives into the crop. Translocations in wheat are also a naturally occurring process that accidentally took place within traditional cultivations of mixed *Triticum* species (e.g., of different ploidy levels) and/or with wild relatives from the local environment.

Doubled haploids
Doubled haploids (DH) can be created in in vitro culture from anthers and microspores (haploid pollen in development) and have the potential to rapidly produce homozygous diploid inbred lines for further inclusion in breeding programs. When making homozygous lines, deleterious recessive alleles become unmasked. DH technology is generally applied in wheat breeding.

Synthetic hexaploid wheat
In agricultural history, bread wheat resulted from an alloploid hybridization between domesticated emmer (*Triticum turgidum* ssp. *dicoccon*; AABB) and wild goatgrass (*Aegilops tauschii*; DD) as the male partner. This process can be repeated: durum wheat (*Triticum turgidum* ssp. *durum*; AABB) (that historically developed from wild emmer), or (wild/domesticated) emmer itself, can be fertilized by pollen of *A. tauschii*. The triploid F1 hybrid (ABD) requires chromosome doubling to become a fertile synthetic hexaploid (AABBDD). The process includes embryo rescue for efficient development of the interspecific hybrid. The method enables adding genes from the wide diversity of the D genome into the synthetic hexaploid, which then can be used further in breeding programs. CIMMYT generated this technology in the 1980s with many successful breeding results in developing countries. NIAB uses the technology to serve European breeding programs (www.wheatisp.org).

Interspecific hybrids *Triticale* and *Tritordeum*
Promiscuity in the grass family (*Poaceae*) is also visible in the form of hybrids between wheat (*Triticum*) as mother and rye (*Secale*, R genome) as pollen donor, which also requires chromosome doubling of the F1 to create a fertile hybrid: *Triticale*. Tetraploid (AARR), hexaploid (AABBRR), and even octoploid (AABBDDRR) triticale hybrids have been created, of which especially the hexaploid became commercially successful, mostly as fodder of forage crop. These hybrids can be considered as new species, and *Triticale* as a new genus. Overall, triticale combines the yield potential and grain quality of wheat with the disease resistance and environmental tolerance of rye. The oldest triticale were produced in Scotland and Germany during the late 19th century. In 2014, 17.1 MMT (million metric ton) triticale was harvested in 37 countries (faostat.fao.org; https://en.wikipedia.org/wiki/Triticale, accessed June 2020).

Another example of the possibility of wide hybridization in wheat is *Tritordeum*, a hybrid of durum wheat with wild barley (*Hordeum chilense*, H genome) resulting in a new fertile hexaploid (AABBHH). Hundreds of different hybrids have been produced showing huge genetic variability. Their baking quality is similar to bread wheat [36]. Interestingly, tritordeum is low

(*Continued*)

BOX 6.2 Applying the genetic flexibility of wheat in advanced breeding. (Continued)

in celiac disease immunogenic peptides compared with wheat: 78% lower for alpha-gliadins, 57% for gamma-gliadins, and 93% for omega-gliadins. Although not celiac-safe, tritordeum may be a food option for those who want to reduce their intake of gluten [37].

Transgenics

Transgenesis in wheat has been used to introduce new individual genes or traits or for testing the function of genes (e.g., with gene silencing through RNA interference technology). Because of the genetically modified organism (GMO) status, transgenic plants or foods have not yet been introduced on the market. In the near future, gene editing using "clustered regularly interspaced short palindromic repeats and associated protein 9" (CRISPR/Cas9) will be used to target double-stranded DNA breaks and their repair actions that will occasionally result in desired mutations. The transgene construct can be removed by segregation in further generations, thus leaving only the mutation. The method can also be applied transiently. This gene editing technology has enormous potential, also in the area of wheat quality traits based on complex gene (quantitative trait loci) systems ([38] and references therein). The GMO status of gene editing is under discussion in the European Union [39], but in other continents plants derived from gene editing are not being considered as genetically modified when no added gene or construct is present and the selected mutation can also occur naturally [40,41].

The application of CRISPR/Cas9 for the reduction of celiac immunogenicity of wheat will be elaborated in another chapter of this book (Chapter 9, Engineering Wheat for Gluten Safe).

identified; the distribution of presence or absence in the different species analyzed enabled to draw an evolutionary model for the different species. Most genes encoded proteins with eight (sulfur-containing) cysteines. Some had an additional cysteine and may refer to C-type LMW subunits; proteins with unequal numbers of cysteines can covalently bind to a network of HMW subunits, which is relevant in dough quality [54].

The alpha-gliadins form another multigene protein family with the *Gli-A2*, *Gli-B2*, and *Gli-D2* genes located on the short arms of the homoeologous chromosomes 6A, 6B, and 6D, respectively. Each *Gli-2* locus is complex, containing clusters of alpha-gliadin gene copies, up to many tens, several of which have become pseudogenes. Large differences in the relative expression levels [measured by quantitative pyrosequencing and analysis of expressed sequence tag (EST) sequences] of the alpha-gliadin genes from the homoeologous loci among different wheat genotypes were found: expression of *Gli-A2* in durum was 41%, and expression was highly variable (12%–58%) in individual genotypes derived from landraces; the alpha-gliadin genes of the *Gli-B2* locus were hardly expressed in some advanced hexaploid bread wheat cultivars, but appeared more than 40% in others [55].

BOX 6.3 How to measure gluten diversity in wheat.

The study of gluten proteins became increasingly relevant with the development of a series of technologies for gluten identification and characterization [38].

Protein analysis

All protein analysis technologies require protein extraction, which should capture the most comprehensive repertoire and abundance without degradation or modification: sample preparation in proteomics is absolutely crucial [42]. In historic perspective, this development of wheat proteomics can be described according to the following milestone technologies.

- Several types of electrophoresis for gluten separation (moving boundary electrophoresis in 1959; starch gel electrophoresis in 1961; polyacrylamide gel electrophoresis in 1963; isoelectric focusing in 1968; SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) in 1972, later extended to the ELISA (enzyme-linked immunosorbent assay) for rapid screening of large numbers of samples).
- The development of the Payne score (in 1987), assigning a quality evaluation metric to high-molecular-weight (HMW) glutenin, which later enabled genetic selection showing the Glu-A1 and the Glu-D1 loci as highly relevant and the Glu-B1 locus as less important to bread quality.
- 2D (two-dimensional) electrophoresis (in 1973) expanded gluten protein research resulting in separation and identification of gliadins and in the localization of their genes on the chromosomes 1A, 1B, 1D, and 6A, 6B, 6D, and to separate low-molecular-weight (LMW) and HMW glutenin subunits, and further enabled characterization of individual wheat varieties.
- Capillary electrophoresis (in 1981) circumvents the application of slab gels and is useful in wheat variety differentiation.
- Liquid chromatography (LC) (in 1988, and especially the later development of high-performance reversed phase RP-HPLC) allows to easily separate gliadins, LMWs and HMWs and on size, charge, pH, and hydrophobicity and to study wheat quality in detail.
- Mass spectrometry (especially tandem mass spectrometry; MS/MS) (in 2010) enabled gluten identification at the peptide level and to correlate these to the corresponding genes in specific wheat varieties. An extension of this method involves coupling of LC to MS in a multiple reaction monitoring mode (LC-MRM-MS) enabling targeted comparative analysis of the gluten proteins present in different wheat varieties with regard to specific celiac disease immunogenic peptides (epitopes) [43]. Detection and differentiation of gluten proteins in complex food matrices was carried out using discovery proteomics with multiplexed LC-MS applying cereal-specific peptide markers [44]. In a further developmental step, LC-MRM-MS data could be linked to comprehensive sequence analysis, annotation, chromosomal locations, and epitope mapping to monitor and quantify immunostimulatory proteins, which enables breeding and selection of desired (e.g., low immunotoxic) genotypes (cultivars) [45,46].
- Nuclear magnetic resonance and X-ray crystallography are well-established technologies enabling high-resolution protein structural analysis in highly purified liquid and crystalline

(*Continued*)

BOX 6.3 How to measure gluten diversity in wheat. (Continued)

samples, respectively. In contrast to MS, these technologies study the protein in its intact form.

Genetic analysis

Advances in technologies for molecular biological (genomics) analysis, breeding assistance (genetic markers), and breeding techniques (genetic modification and gene editing) have broadened the view on the crop, and the possibilities and speed of breeding and crop management. Note that genetically modified wheat has not entered the consumer market.

- DNA sequencing (Sanger technology) (in 1977) for genetic identification of wheat varieties was followed by the development of genetic markers together with the polymerase chain reaction (PCR), including restriction fragment length polymorphism (RFLP), simple sequence repeats (SSR), AFLP, and diversity arrays technology markers.
- The markers of choice nowadays are single nucleotide polymorphism (SNP) markers, as these point mutations are abundant and their detection can be automated, for example, on SNP arrays). They can also be determined from sequence reads produced by next-generation sequencing techniques.
- DNA markers have been essential for the discovery of a long list of quantitative trait loci (QTLs) for various traits in wheat, including flour SDS sedimentation, softness equivalent/kernel texture, flour yield, flour protein, mixo peak time and height, cookie diameter, loaf volume, and grain protein content. Markers associated with QTLs or traits enable marker-assisted selection (MAS) that has been applied successfully for the improvement of several quality traits such as kernel texture and grain protein content, in the employment of numerous QTLs, for example, in the detection of mutations in granule-bound starch synthesis II and in quality studies of glutenins and gliadins related to gluten strength.
- Sequencing of the genomes of representative wheat varieties, accessions, and species using next-generation sequencing will increase the understanding of the complexity of the wheat genomes and the ability to breed better wheat varieties. This is particularly so for complex gene families such as gluten. The genome sequence of Chinese Spring [47] can be considered as a culmination of a long process, following the genome sequences of *Triticum urartu* [48], *Aegilops tauschii* [49,50], and wild emmer [51]. At the same time, it marks a start, as the combination of short-read and long-read sequencing technologies will make it possible to produce more complete and intact genome sequences of multiple individual accessions and varieties, at reduced costs. For example, the National Institute of Agricultural Botany (NIAB) Biotechnology and Biological Sciences Research Council (BBSRC) funded pangenome project aims to produce a high-quality reference sequence of 10 bread wheat accessions, assess millions of sequence variants in a further 94 accessions, and map the inheritance of sequence variants in 1000 offspring (https://gtr.ukri.org/projects?ref = BB%2FP010741%2F1).
- Genomic selection can be considered an extension of phenotypic selection and MAS through the use of well-defined genotyped and phenotyped "training populations" as selection model: the larger the training population and more refined the derived model, the higher the accuracy and prediction power to the test population for a given trait.

(*Continued*)

BOX 6.3 How to measure gluten diversity in wheat. (Continued)

- Speed breeding protocols using extended photoperiods and controlled temperatures to reduce the generation times of wheat and other grains during a breeding program by more than half [42] will speed up the process of developing superior wheat varieties needed to feed a population of 10 billion by the middle of this century.
- All these technologies and their further future developments have brought a profound insight in the diversity of the *Triticeae* family and are of great help in wheat breeding. Especially at the last breeding stages of line advancement and prior to varietal release and market introduction, phenotyping of end-use quality in a broad sense remains indispensable and irreplaceable. At this phenotypic level, also the interaction between genetics and environment will be visible. Regarding wheat quality, phenotypes such as gluten index, flour SDS sedimentation, and gluten viscoelasticity are relevant characteristics and breeding aims.

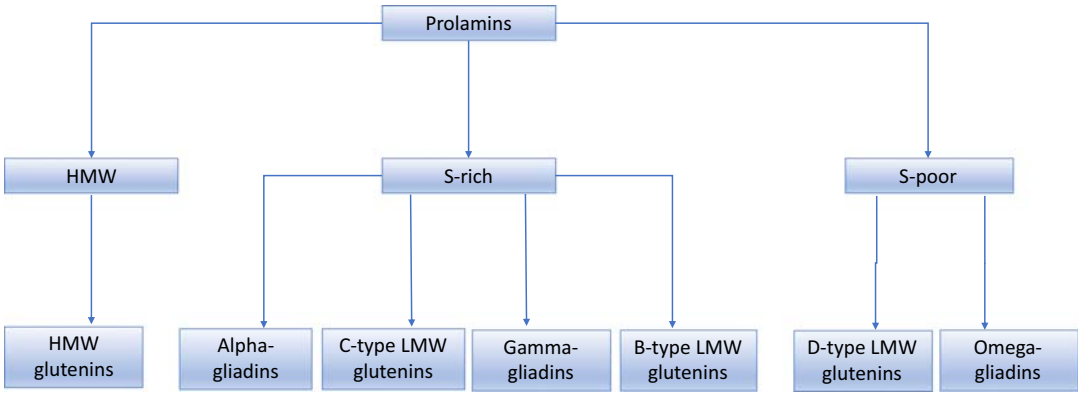

Figure 6.1
Gluten gene families in wheat. *HMW*, high-molecular weight; *LMW*, low-molecular weight.
Source: After Shewry PR, Lookhart GL. Wheat gluten protein analysis. St Paul, MN: American Association of Cereal Chemists (AACC); 2003 [1].

With regard to glutenin HMW subunit genes, these are located on the long arm of chromosome 1 per haploid (A, B, D) genome: the six loci each in hexaploid wheat include two genes encoding an x-type and a y-type HMW subunit. HMW has high relevance in baking quality and has been subject for research for many decades resulting in defining alleles and allelic pairs of subunits at all three loci [53].

A single bread wheat variety may harbor over 90 different gluten proteins as was shown by comparison of mass spectrometry-analyzed gluten peptides with a curated gluten sequence database ([53] and references therein). Identification of gluten genes in the variety

Chinese Spring after complete and accurate sequence annotation in the TGACv1 assembly counts for gliadins: in Chinese Spring, 29 alpha-gliadin genes, 18 gamma-gliadin genes, 10 omega-gliadin genes, 6 HMW glutenins, and 16 LMW glutenins. In addition, there are 12 prolamin genes with similarity to avenins (from oat), farinins (from wheat), and hordeins (from barley [11]). Variation in the sequences of encoded proteins and variation in the quantity of expressed genes determine the polymorphism in gluten protein composition observed between genotypes [53].

Other gluten-related wheat prolamins, which are often coextracted with the glutenins and the gliadins, are alpha-amylase/trypsin inhibitors (ATIs) (12−16 kDa; 4% of the total gluten fraction, contributing to pasta-making quality), next to the less abundant farinins (17−30 kDa; with a role in dough mixing properties), purinins (LMW gliadins; 17−19 kDa; these behave like gliadins), puroindolines a and b (13 kDa; determining grain softness), GSP (15 kDa; with effect on grain softness), arabinogalactan peptide (23 kDa), and nonspecific lipid-transfer protein (LTP1 of 9 kDa and and LTP2 of 7 kDa [53]).

6.7 Wheat gluten and end-use quality

End-use quality of wheat is largely determined by the gluten proteins through their ability to form a viscoelastic network that provides cohesiveness required for processing properties of wheat flour to make leavened breads and other baked products and to produce pasta and noodles. In addition, vital gluten, as a side product from the starch industry, is increasingly used as ingredient in processed food products.

The amount of total gluten as well as the amount of individual gluten types may vary largely between individual wheat genotypes, based on genetic composition but to some extent also on environmental conditions during cultivation. Comparing gluten (gliadins and glutenins) protein contents of 15 cultivars each of common wheat, spelt, durum, emmer, and einkorn, cultivated at four locations in Germany in the same year, revealed that the total protein (gluten) content was equally influenced by location and wheat species but that the specific gluten contents (gliadin, glutenin) were more species-related. In general, spelt and durum had the highest gluten content, whereas the gluten content of common wheat was in general the lowest. Regarding the gliadin/glutenin ratio, which is a predictor for baking quality (the lower this ratio, the higher the baking quality), this was as expected the lowest in common wheat (mean value at all locations of ~2.5), followed by spelt (~3.5), durum (~4.0), emmer (~5.0), and einkorn (~7.0) [56].

Domestication and breeding appeared to have selected for reduced overall protein (gluten) content, especially in the tetraploid (durum) and hexaploid (bread wheat) species, with a relative reduction of the gliadin content lowering the gliadin/glutenin ratio and increasing baking quality [57]. Breadmaking quality combines several characteristics like rheological

dough properties, dough fermentation, gas retention, crumb texture, loaf volume, and crust color. Using indirect tests, some progress was made in genetic improvement of cultivars after the 1950s, but since the 1980s, breeding for breadmaking quality accelerated with the genetic analysis of wheat storage proteins, especially gliadins and glutenins. The gluten proteins became among the first for which genetic markers were employed in breeding for bread wheat and durum wheat quality [58].

Heritability describes the amount of genetic variance relative to the total variance observed in the field (the sum of genetic and environmental variance for the measured trait, in this case the contents of protein, gluten, gliadin, glutenin, and the gliadin/glutenin ration as separate factors). Based on the data obtained from the 15 cultivars of each wheat species grown at four locations, heritability of all factors appeared high (between 0.68 and 0.94 on a scale from 0 to 1), enabling selection of cultivars with good predicted baking performance, also from wheat species with generally low baking quality such as spelt and emmer [56]. Remarkably, lower rainfall during the grain filling period in one of the two crop seasons was related to overall higher expression of HMW subunits and omega-gliadins in old and modern durum wheat genotypes [59]. Also, the effects of temperature and availability of nutrients (e.g., nitrogen and sulfur) have been described: the amounts of gliadins increase when nitrogen availability is high, and the amount of (sulfur-poor) omega-gliadins will increase at high nitrogen but low sulfur supply ([53] and references therein).

6.8 Gluten diversity related to celiac disease

Ingestion of gluten proteins (gliadins and glutenins) from wheat, barley, and rye can induce celiac disease (CD) in genetically predisposed individuals. This disease is triggered by a number of peptides derived from gluten proteins that are recognized by $CD4^+$ T cells in the human body. Gluten proteins are rich in glutamine (Q) and proline (P), which make them relatively resistant to proteolytic degradation in the intestine.

On the surface of these T cells, human leukocyte antigen (HLA)-DQ receptors bind the epitopes. From biopsies of patients, epitope-specific T cells can be obtained and cultured in vitro as T-cell clones. The majority is DQ2.5 restricted and can recognize epitopes from alpha-gliadins (three major and one minor epitopes) and gamma-gliadins (eight major epitopes), and minor epitopes from omega-gliadins (two) and LMW glutenins (two). One minor LMW-glutenin epitope is DQ2.2 restricted. Three minor epitopes are DQ8 (DQ8.5) restricted: one from alpha-gliadin, one from gamma-gliadin, and one from HMW glutenin. The binding motif of all epitopes is a 9-amino acid core sequence, although a few flanking amino acids may influence the binding as well [60]. Since 2012 [61], only a few minor new epitopes have been identified and published [62]. Importantly, all CD-immunogenic epitopes contain one or more glutamate (E) residues that are formed in the intestine by sequence motif-specific transglutaminase 2-mediated deamidation of glutamine (Q); this

deamidation from Q to E is crucial for increased affinity to the epitope-specific T-cell receptors. For example, the DQ2.5-glia-alpha1a epitope is the amino acid peptide PFPQP**Q**LPY in the food product and becomes immunogenically active only after deamidation into PFPQP**E**LPY.

The high diversity of gluten proteins between wheat species and between wheat genotypes within the same species, together with the diversity of CD-immunogenic gluten epitopes within a single variety, make this issue complex. Many attempts have been undertaken and many approaches have been followed to get more insight in this matter and to make further steps toward celiac-safe solutions [63,64]. Some examples of this quest are given here, and because of space limitations they were chosen notably from Dutch research in CD. The examples are listed chronologically showing some evolution in research strategies.

- *Genome specificity of epitopes*. With a subset of four identified epitope gene sequences, 230 distinct alpha-gliadin gene sequences isolated from diploid wheat species representing the A, B, and D genome in bread wheat were analyzed and compared to gliadin sequences of bread wheat in the public UniProt database after translation into protein sequences. Interestingly, *T. monococcum* (einkorn; A genome representative) and the bread wheat A genome contained the same two of the four epitopes; *Triticum speltoides* (B genome representative) and the bread wheat B genome did not contain any of the four epitopes; *Triticum tauschii* (D genome representative) and the bread wheat D genome contained all four epitopes [65].
- *Epitope variation and immunogenic activity*. In another study, more than 3000 expressed alpha-gliadin sequences from 11 bread wheat cultivars were analyzed for the presence of epitopes and epitope variants. All identified expressed variants (7 for DQ2-Glia-alpha1, 12 for DQ2-Glia-alpha2, 11 for DQ2-Glia-alpha3, and 8 for DQ8-Glia-alpha1) could be traced back to the A, B, or D genome. All were synthesized as deamidated peptides and tested for binding to HLA-DQ2 and -DQ8 receptor molecules and for recognition by patient-derived alpha-gliadin-specific T-cell clones. Several epitope variants with one to three amino acid substitutions at specific epitope positions appeared to have lost the immunogenic capacity [66]. This research provided basic information to design safe gluten genes, and for further gene editing toward inactivation of specific epitopes (see also Refs. [67,68]).
- *Mass screening of tetraploid and hexaploid varieties*. Further, gluten protein extracts from 36 modern hexaploid European bread wheat varieties and 50 wheat landraces (including bread wheat, spelt, and compactum wheat) were analyzed for the presence of and the variation in the major DQ2.5-glia-alpha1 epitope (and in the minor DQ2.5-glia-alpha3 as technical reference) by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and immunoblotting using epitope-specific monoclonal antibodies (mAbs): a low number of varieties and landraces could indeed be identified with reduced epitope contents [69]. Similarly, 103 tetraploid accessions

(*T. turgidum*, mainly ssp. *durum*, *dicoccon*, and *carthlicum*, all omitting the D genome) were analyzed for the presence of the DQ2.5-glia-alpha1 epitope, with three accessions showing a significantly reduced epitope content [70]. Such varieties and accessions might serve as a start to breed wheat for the introduction of "low CD immunogenicity" as a new breeding trait.

- *Gliadin transcriptome analysis*. Gamma-gliadins and their epitopes have been analyzed in hexaploid wheat, also in the context of the sequences flanking the core epitope region. To this purpose, the variation of T-cell epitopes (with main focus on epitope DQ2-gamma 1) has been examined in gamma-gliadin transcripts of developing bread wheat grains. Specific variations could be assigned to one of the three genomic gamma-gliadin loci (*Gli-A1*, *Gli-B1*, or *Gli-D1*). The highest frequency of variation was found on the Gli-D1 locus (average 10.1 CD epitopes per transcript), followed by Gli-A1 (8.6 epitopes), and Gl1-B1 (5.4 epitopes). Natural variants were obtained from the major DQ2-gamma 1 epitopes. These variants showed different in vitro T-cell reactivities related to the deamidation pattern (position of Q to E deamidation) and the flanking amino acids. This approach enabled direct quantification of the celiac immunogenicity from protein-coding mRNA sequences [60]. In a follow-up study, 454 RNA-amplicon sequencing was developed for alpha-gliadin transcripts. Expression profiles have been produced from 77 durum wheat plants representing 61 different accessions. According to the main unique alpha-gliadin protein fragments with known epitope sequences, comparative expression profiles of large sets of plants were generated. Profiles were clustered into profile types that enabled to trace back individual accessions to their geographical origin or dispersal [71].
- *Spelt versus bread wheat*. Another research focused on the diversity of spelt wheat with regard to celiac immunogenicity. Based on genotypic data from 85 spelt accessions obtained with 19 SSR markers, 11 contrasted accessions were selected, from which 446 full open reading frame alpha-gliadin genes were cloned and sequenced, revealing high allelic diversity in the composition of the alpha-gliadin T-cell epitopes. Remarkably, one accession had a specifically high proportion of alpha-gliadins from the B genome and accordingly (see Ref. [65]) a low immunogenic profile [72].
- *Quantification of gluten proteins and their epitopes*. From the entire *Triticeae* tribe, in total 245 representative accessions of 16 diploid, tetraploid, and hexaploid wheat species including diploid *Aegilops* species, and of 2 barley and 2 rye accessions, gluten proteins were studied [57] for their quantification by reversed-phase high-performance liquid chromatography and "in silico" epitope identification using the National Center for Biotechnology Information (NCBI) database. Clear tendencies were observed toward lower contents of alpha-, gamma-, and omega-gliadins and higher LMW and HMW glutenins in the cultivated species (bread wheat, durum wheat) compared with the wild ancestors. The DQ2.5-glia-alpha and DQ2.5-glia-gamma epitopes (described in Ref. [61]) could accurately be quantified per species. The major DQ2.5-glia-alpha1b and DQ2.5-glia-alpha2 epitopes were specific for *A. tauschii* and the D genome of bread wheat and spelt

wheat. The DQ2.5-glia-gamma4c epitope was the most abundant gamma-gliadin epitope in all species. The number of epitopes per sequence of bread wheat and spelt wheat were similar, but the absolute total gluten content was higher in spelt [57].

The last decade showed an enormous increase in advanced technologies and deep knowledge about the ancestry and reticulate relationships, genomes and proteomes, and the enormous diversity of wheat species, also including the detailed interactions of specific gluten fragments with the human autoimmune system. Together, this paves the way for further steps in the mutual domestication of wheat and human through the application of next-generation breeding technologies toward the development of (celiac-) safe and more healthy wheat varieties by targeted gene editing [including clustered regularly interspaced short palindromic repeats and associated protein 9 (CRISPR/Cas9) and related technologies [67,73]].

6.9 What we eat: diversity in wheat-derived products

6.9.1 Products for the average consumer

Several studies have looked at the gluten composition and protein content of wheat species and varieties, generally finding: (1) few or no differences between old and modern varieties, (2) an effect of the environment during cultivation, and (3) some differences between species [with einkorn (*T. monococcum*) as the lowest with regard to stimulation immune cells; see e.g., Ref. [74]]. However, we do not eat food produced from separate wheat varieties, but products made from flour of mixtures of varieties are customized each year so that their technological quality is constant, even though the varieties cultivated, their relative amounts, and their properties vary between years.

Of the ~700–750 million metric ton (MMT) wheat annually produced worldwide, China, India, and Russia are the top three producers with an annual production of approximately 135, 100, and 85 MMT, respectively [58]. The EU market annually produces about 165 MMT and imports another 5–6 MMT (main data from 2015 to 2020). Wheat includes bread wheat and durum wheat (representing only 3% of total wheat). Of this total supply, 30 MMT is exported to third countries, a bit more than 5 MMT is used as sowing seed, 1 MMT will be lost, and 18 MMT will be stored (ending stock). The remaining 120 MMT is used domestically: for human consumption (55 MMT), animal feed (55 MMT), and industrial application (11 MMT, of which 5 MMT for bioethanol/biofuel production). The total EU cereal balance sheet calculates 320 MMT, which is almost double that of wheat alone. The other grains include barley (20% of the total grains) and maize (20%), and some 10% for oats, triticale, rye, and minor cereals [75].

The European starch industry is big and produces annually 17 MMT from cereals (maize and wheat). Wheat starch has many applications, mostly in food (67%: as modified starch to improve rheology and processing, as emulsifier in foods, as binder in soups and sauces, to

improve storage performance in foods, as sweetener in beverages), feed (20%: added to milk powder, as piglet starter feed, in aquaculture feed pellets), seed coating (7%), and industrial (6%: as adhesive in paper industry, in laundry sizing, in green chemistry for fermentation, in bioplastics, in ethanol production) [58]. Wheat starch, when not highly purified, may contain considerable amounts of coisolated gluten and other prolamin components.

Gluten (vital wheat gluten, VWG) is a side product in that sector of the starch industry where wheat is used. Reversely, in the United States and Canada, starch is considered a side product of the wheat gluten industry which appreciates gluten much for many purposes. The processing properties of wheat are largely determined by gluten proteins. Presently, in Europe, 9 MMT wheat is processed in the starch industry and produces over 630,000 MT VWG ($\sim$7%). An increase of the VWG market is anticipated over the next years. About half of the VWG produced in Europe is exported to the United States. The European wheat milling industry applies 32 MMT wheat containing 10%–11% protein of which $\sim$7% is gluten: this equals to 2.25 MMT gluten (J. de Meester, Cargill, personal communication). Between 0.2% and 10% of gluten can be added to flour to improve the rheological and technical properties to the levels required by the baking industry for the production of steam buns, toasted breads, crusty breads, sweet breads, leavened and laminated sweet goods, laminated puff pastries, rolls and buns, crackers, cookies, sponge cakes, wafers, and snacks. These products can easily be produced and consumed and are highly compatible with the "Western lifestyle" [58]. "Seitan" is a traditional gluten-based (meat replacing) vegan product, originating from East Asia, prepared of wheat dough from which starch and bran have been removed by cold washing and that be cooked resulting in a meat-like structure that can be applied for further treatments like baking or grilling.

The intake of VWG has tripled since 1977 from 0.37 to 1.12 g per capita per day. However, even this 1.12 g VWG is a minor addition to the 13.7–15.1 g of total daily gluten consumption per capita from bread [76].

Gluten is also a preferred ingredient in feed, especially in aquaculture due to its water insolubility and binding capacity in feed pellets and providing "green" proteins. For example, in France about 12% of the annually produced wheat is used for feed. In nonfood applications, gluten is also applied for the preparation of biopolymers. Low-grade wheat grains (from bad harvests or in cases of excess of wheat grains unsuitable for the human market) will be used to feed livestock, especially poultry. Other uses include the production of alcohol, adhesives, soaps, rubbers, cosmetics, and varnishes [58].

6.9.2 Gluten-free products

The wide range of applications leads to increasing demands for transparency to customers and producers about the myriad products that contain ingredients derived from wheat ([58]

and references therein). This is today especially relevant to individuals who suffer from CD, wheat allergy, and non-celiac wheat sensitivity (NCWS). It was, for example, hypothesized that the possible increase of the prevalence of NCWS parallels the increased use of highly processed wheat ingredients [77]. The presence of "hidden" gluten and contaminating gluten in processed food products intended as gluten-free (GF) may be a health hazard to celiac and allergic sufferers [78].

Wheat is an important component of many supermarket items and was found in 29.5% of 10,235 labeled food items [79]. Wheat was obviously present in bread and baked goods, biscuits, pasta products, cereal breakfasts, and so on; wheat was also commonly present in highly processed foods, such as sweets, frozen meals, packed soups and chips, and in processed foods that are not necessarily staples including preprepared meals, snack bars, chocolates, and marinades, which is more or less comprehensible; however, it was also found often but inconsistently and unexpectedly in foods such as vinegars and dressings, gourmet products, vitamins, ice creams, popcorn, coffee, nuts, rice crackers, soy sauce, canned vegetables, frozen poultry, cheeses, chilled milk, seafoods, mostly as wheat starch, gluten, glucose, sometimes as caramel color, with none of these processed foods drawing attention to the wheat connection on their product ingredient label. Interestingly, wheat was also found in nonfood items like pet foods (for dogs and in general), hair care and hair color products, and in some skin care products [79]. Wheat starch-derived sorbitol may be problematic to CD patients who are highly sensitive to gluten (T. Aarsen, personal communication).

When wheat is chemically and physically disassembled to become an (food) industrial ingredient, its presence and identity may be exchangeable for similar components from other sources, and it becomes hidden and often invisible [79]. This issue (where is wheat used in) was also addressed in an overview search on the evolution of the gluten content in grain-based GF products in the period from 1998 to 2016 [80]: 3141 GF (labeled and not certified) foods were checked using R5 mAb tests, with the Prolamin Working Group gliadin standard solution [81] as reference and for calibration. Gluten contamination could be detected in 371 products (12%), but, in accordance with European legislations on food ingredient information including gluten content (allowing a gluten content of maximum 20 ppm [82]), the frequency clearly decreased over time from 13% (in 1998–2002) to 3% (in 2013–16), indicating that GF foods have become more safe but still require testing [80]. And further, some celiac patients are proven super sensitive even to smallest amounts of gluten (far less than 20 ppm). They feel good after consuming cow's milk from "grass cows," but they experience an uncomfortable feeling after eating beef and drinking milk from cows fed on wheat-containing feedstuff (winter feed, stable feed); they got similar ill-making responses from food derived from pigs and poultry (also eggs) only when these are fed on wheat as major component. For reason of improving shelf life, structure improvement, or better cutability, meat, fish, and chicken products may be injected with

protein, often wheat (gluten). Processed (aromatized) teas and coffees may give problems because of processing with maltodextrin which is legally not required to be labeled according to its origin and thus can be derived from wheat starch (contaminated with minor traces of gluten). These foods and drinks are complicating dramatically the life of highly gluten-sensitive individuals, especially because the real cause or origin of the contamination is difficult to trace back and its occurrence not always consistent (Tine Aarsen, personal communication).

6.10 Wheat/gluten consumption and (gastrointestinal) health

In 39 UK-adapted wheat cultivars dating from between 1790 and 2012, the contents of dietary fiber components (arabinoxylan and beta-glucan) and polar metabolites (sugars, amino acids, organic acids, choline, and betaine) were determined to investigate possible changes that might have occurred during more than two centuries of wheat breeding. Quantities of these components appeared strongly sensitive to environmental factors that differed between the study years. Arabinoxylan and betaine tended to increase, whereas some amino acids tended to decrease between the older and newer cultivars. Protein was not monitored. Overall, this study confirmed that intensive breeding did not significantly affect the content of health-beneficial compounds [83].

Similarly, the gastrointestinal health effects related to gut inflammation and gut barrier integrity were absent in mice fed with a modern wheat cultivar Gallagher (since 2012 widely grown in the United States) and a blend of the two heirloom cultivars Turkey and Kharkof under normal versus Western diet conditions. Major components of both wheat types were identical except for the wet gluten content which was lower in the modern cultivar Gallagher. The doses and composition of the mice feed resembled those of human food in clinical intervention studies [84].

Regarding NCWS, it was hypothesized that processing and consumption factors may be involved. For example, the increased use of additional VWG; modern ways of grain fractionation and refining of grains (which remove the bran that contains most of the fibers and the germ with many micronutrients); the additions of salt, sugars, and fat; and intensive kneading in ultraprocessed cereal-based foods may have rendered the gluten component less digestible and more interactive with the colon microflora, possibly resulting in increased inflammation. This hypothesis on the microflora-mediated inflammation response from intensively processed gluten requires further research in a cohort study of populations consuming ultraprocessed versus minimally processed wheat-containing foods [77].

In this regard, the dietary intervention of RNA interference (RNAi)—induced low-gliadin wheat bread in patients with NCWS showed no differences with a GF diet but provided

a better gut microbiota profile. The consumption of low-gliadin bread by NCWS subjects increased the level of butyrate-producing bacteria and favored a microbial profile that may have a key role in maintenance and improvement of gut permeability [85]. The scientific attention toward the gut microbiome is also increasing within the context of CD. Recent data highlight the possibility that CD results from the triggering of a bacterial pathogen-specific T-cell response that cross-reacts with gluten epitopes. Peptide mimics to DQ2.5-glia-alpha 1, DQ2.5-glia-omega 1, and DQ2.5-glia-alpha 2 with a negative charge at the canonical deamidation sites have been identified from several bacterial and some yeast species. Once initiated, a gluten-driven expansion of the cross-reactive T-cell receptor repertoire and epitope spreading may occur, making CD prominent [86]. Gluten itself are also considered to play a direct role toward the microbiome: the microbial balance (homeostasis) seems to be sensitive to the presence or absence of peptides from digested gluten. The balance may become disturbed (dysbiosis) and the mucosal and immune responses may become modulated. However, to what extent these changes in homeostasis (and the occurrence of dysbiosis) is a cause or a consequence of the disease needs further explanation [87].

6.11 Perspectives

Cereal consumption including wheat is much older than the onset of agriculture. Grain processing (heating, milling) was already applied by Neanderthals. Mixed cultivations of the first domesticated wheat species einkorn and emmer on fields surrounded by wild relatives resulted in new hybridizations, allopolyploid speciation, and introgression-based diversification. Wheat is promiscuous, flexible, adaptive, and productive. “The” wheat genome occurs in many variants over many related *Triticeae* species. Wheat fits to many human needs. The consumption of whole grain foods significantly reduces the incidence of chronic diseases. That is why many governments worldwide strongly promote the consumption of such foods.

The versatility of wheat leads to many food, feed, and nonfood applications. Big food industries developed during the last half century. Ten companies control at least half of the total global food market (https://www.independent.co.uk/life-style/companies-control-everything-you-buy-kelloggs-nestle-unilever-a7666731.html). These industries developed large-scale production facilities that require uniform raw material for their processing lines to be able to make products that should be uniform all over the world. Therefore batches of the original primary products (with wheat in a prominent position) originating from various locations globally are pooled to ensure constant quality, before being refined, fragmented, and reconstituted into (flour) mixes that meet the specific technological processing requirements for, for example, cookies and pastries, for white or whole grain bread, and for all other (wheat-based food) products.

The big food industries should be praised in producing so much cheap and available food to increasingly feed the growing world population: hunger is gradually in decrease. They should be blamed for the production of highly (ultra) processed and uniform food products of poor nutritional quality (too much salt, too much fat, too much sugar, too few fibers) without offering a healthy and accessible alternative.

While the quality of the primary products did not change significantly during the last half century, the big food industries have transformed the daily consumption pattern with many new processed food products and matching advertisements. "Tasty" is the spell; but the consequence is that "You don't eat what you believe to eat." An unhealthy consumption pattern, combined with a low-exercise lifestyle, has considerably increased the consumer's prevalence of several serious chronic diseases with connected high medical and health-care costs [88]. This raised awareness. There is a growing consumer's trend to avoid processed foods and to focus on "fresh, local, sustainable, traditional, and fair" food products. The individual choice of food can be considered an expression of the consumer's identity in an increasingly globalizing world [89]. Trends often lack a scientific basis. This is partly due to the fact that the health effect of one food product, or of one component therein, is difficult to determine scientifically.

One trend is to avoid bread and other wheat-containing products. This is mainly based on self-diagnosis of symptoms suggesting a form of non-celiac wheat (or gluten) sensitivity (NCWS) [90]. However, no biomarkers for this condition are known. If there really are biological causal factors (food compounds), they are still unclear. Wheat gluten can be excluded as the causal agent on the basis of sound scientific research [91]. The involvement of fermentable oligo-, di-, monosaccharides and polyols (FODMAPs) seems less likely. These are indeed present in wheat but also in many other foods, and they may cause some unpleasant bowel effects due to their interaction with the intestinal microbiome but generally are considered to support gut health [92]. Wheat ATIs may play a role as they possibly act as an immune-related inflammatory substance [93]. In general, sensitivity to wheat may be part of a much broader phenomenon, and likely related to irritable bowel syndrome, with limited causality to wheat alone. A different factor is psychological and can be found in popular books as well as in social media, which fanatically blame wheat and bread, and preach a belief in the detrimental health effects of wheat consumption [78].

In 2016, to reveal the true factors behind the present wheat/gluten avoidance, an international intervention study (the Well-on-Wheat project, www.wellonwheat.org; [94]) was started. It aims to compare the effects on intestine (physiological) and well-being (psychological) of yeast- and sourdough-fermented whole grain bread prepared from bread wheat, spelt, and emmer, and it will also pay attention to the possible occurrence of placebo and nocebo effects. The results will be helpful to correctly advise consumers on wheat consumption.

Some diseases caused by wheat have been studied thoroughly [95]. Of these, CD is the most common. It is a serious health and quality of life-impairing condition that affects

1%–2% of the human population. It presently receives attention from the cereal agronomic, breeding, and food sector. Celiac-safe wheat species do not exist, although some einkorn lines may have lower quantities of specific epitopes. Such lines may at best be qualified as "low in gluten" [64]. Due to the large number of gluten genes in each wheat plant and the complexity of the wheat genome, wheat that is celiac-safe but retains baking quality cannot be produced by conventional breeding alone [64]. RNAi has been used to downregulate the gene expression of gliadin families. This has led to wheat lines with highly reduced amounts of gliadins while maintaining reasonably good baking properties [96]. Recently, targeted gene editing using CRISPR/Cas9 has been applied to gliadins as well [67,73]. This produces wheat plants with silenced, deleted, and/or edited gliadins. Ultimately, including this in regular breeding programs, combined with advanced screening techniques [41], will make it possible to produce wheat that is safe for people with CD. However, although the technology is here, the application of gene editing for the production of celiac-safe wheat will depend on national and international regulatory frameworks on genetically modification technology [64]. These frameworks were put in place 20 years ago to protect against potential risks of new genomic technology, but currently they effectively block the application of technology to remediate a concrete disease affecting at least 4.5 million people in the EU.

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Conflict of interest

The authors declare no conflict of interest.

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