/REVIEW

Biotechnology of Breadmaking: Unraveling and Manipulating the Multi-Protein Gluten Complex

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Breadmaking is one of humankind's oldest technologies, being established some 4,000 years ago. The ability to make leavened bread depends largely on the visco-elastic properties conferred to wheat doughs by the gluten proteins. These allow the entrapment of carbon dioxide released by the yeast, giving rise to a light porous structure. One group of gluten proteins, the high molecular weight (HMW) subunits, are largely responsible for gluten elasticity, and variation in their amount and composition is associated with differences in elasticity (and hence quality) between various types of wheat. These proteins form elastomeric polymers stabilized by inter-chain disulphide bonds, and detailed studies of their structures have led to models for the mechanism of elasticity. This work has also provided a basis for direct improvement of wheat quality by transformation with additional HMW subunit genes.

It is now 250 years since the isolation of wheat gluten was first described, by Jacopo Beccari Professor of Chemistry at the University of Bologna[1]. He showed that wheat flour could be divided into two fractions, one of which was water-soluble with characteristics similar to sugars, and the other water-insoluble and similar to substances of animal origin[2]. These fractions were called amylo and glutinis, and correspond to the present day starch and gluten, respectively. Thus gluten was one of the earliest proteins to be prepared. However, even by this time its importance was well established, if not recognized, due to its contribution to the functional properties of wheat doughs. There is no doubt that the use of wheat doughs to produce leavened breads is one of humankind's oldest forms of biotechnology, being well established in ancient Egypt before 2000 BCE[3]. The early leavened breads were almost certainly based on a sour dough system in which a starter containing microorganisms was mixed with fresh dough and allowed to ferment. A similar system is still used in the United States (being particularly popular in the San Francisco area) with a characteristic flavor introduced by the action of *Lactobacilli*[4]. This system was replaced by the use of bakers yeast in the 19th Century, resulting in a more consistent and lighter product.

In addition to leavened bread, a wide range of other wheat products have developed in different cultures. These include various types of flat breads (e.g. Indian sub-continent) and pocket breads (middle east), noodles (China and S.E. Asia) and pasta (reputedly introduced into Italy by Marco Polo in the 14th Century). In fact, wheat products are highly culturally determined, and have assumed religious significance as well as economic and nutritional importance.

Wheat gluten is largely responsible for the functional properties of dough that are exploited in all these end uses. Consequently it has been the subject of a vast volume of research, carried out over more than a century. As with other types of proteins, our knowledge has benefitted enormously from the application of molecular genetics and advances in biophysical methods over the past decade of so. Although still far from complete, it provides an excellent basis for the application of wheat transformation to explore and improve the quality of wheat for both traditional and novel end uses.

What is Gluten?

The gluten proteins comprise the major grain storage proteins, which are deposited in the developing starchy endosperm. Thus their only biological role is to provide a store of amino acids for germination. The gluten proteins are synthesised on the rough endoplasmic reticulum (ER), and are directed into the lumen via a standard signal peptide-mediated mechanism. They are then deposited in protein bodies which may be derived from direct accumulation within the ER lumen or transport via the Golgi apparatus to the vacuole: the precise pathway and mechanism of protein body formation is still unclear, and indeed may vary between individual proteins and stages of grain development[5,6]. The individual protein bodies range in diameter up to about 20 μm (Fig. 1A). However, as the endosperm cells fill with starch the protein bodies become disrupted and finally coalesce to form a matrix of storage proteins surrounding the starch granules in the mature dry tissue (Figs. 1B, 1C). When the endosperm is milled and the flour mixed with water and kneaded, the storage proteins are brought together to form a continuous proteinaceous network called gluten. The structure and properties of gluten are, therefore, at least partly determined by molecular interactions established in the developing grain and it is important that these should be understood if the functional properties of gluten are to be manipulated.

Wheat gluten proteins, and the major storage proteins of most other cereals, are characterized by their insolubility in water or aqueous solutions of salts, but solubility in mixtures of alcohols and water (typically 60–70% (v/v) ethanol or 50% (v/v) propan-1-ol)[6]. This property allows the gluten fraction to be readily isolated, by gently washing a dough to remove starch and soluble components. This was the method used by Beccari in the 18th Century and is still used today by cereal chemists and in grain quality laboratories. The resulting fraction accounts for about 10% of the weight of the dough, and consists mainly of proteins (≈ 70–80% on a dry weight basis) together with some starch and lipids. It also exhibits unusual physical properties which are usually described as a combination of elasticity with extensibility or viscous flow (Fig. 1D). These properties are crucial for breadmaking as they allow the entrapment of carbon dioxide released by yeast during proving. This expands the gluten network to give a light porous structure which is fixed by baking (Fig. 1E, 1F). It is also important that the gluten should have a precise balance of these properties: excessive elasticity would limit the expansion, while insufficient elasticity would fail to retain the carbon dioxide.

The elasticity of gluten is often referred to as dough

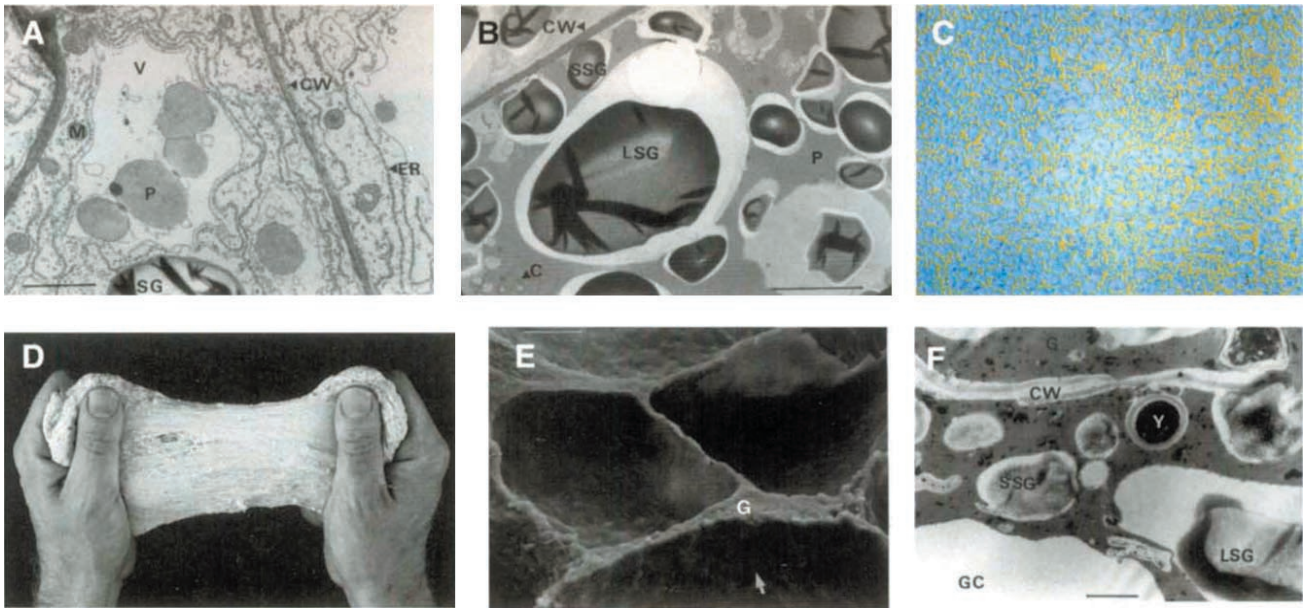

FIGURE 1. The Origin of Gluten

A. Transmission electron microscopy of an inner endosperm cell of a young (13 days after anthesis) caryopsis of wheat (cv. Maris Freeman) shows deposits of gluten protein (P) within a central vacuole. A starch granule (SG), cell walls (CW), rough ER (ER) and mitochondria (M) are also clearly seen. The scale bar is 2 μm.

B. Transmission electron microscopy of a similar cell from the inner starchy endosperm of wheat (cv. Maris Freeman) at a later stage of development (46 days after anthesis) shows a proteinaceous matrix (P), resulting from disruption and coalescence of the protein bodies, surrounding large starch granules (LSG) and small starch granules (SSG). The cell wall (CW) and remnants of cytoplasm (C) are also seen. The scale bar is 5 μm.

C. Light microscopy of the endosperm of a mature grain of cv. Hereward shows that the endosperm cells are full of starch granules surrounded by a proteinaceous (gluten) matrix. The latter is stained with toluidine blue and displayed, after computer image enhancement, in yellow.

D. Wheat gluten can be isolated from dough by washing, forming a cohesive visco-elastic mass.

E. Scanning electron microscopy of a cut fragment of white bread shows the cut surface of the gluten matrix (G) and the concave walls of gas cells (white arrow). The scale bar is 50 μm.

F. Transmission electron microscopy of a section of white bread roll shows a gluten matrix (G) surrounding partially gelatinised small starch granules (SSG) and large starch granules (LSG), yeast cells (Y) and fragments of cell wall (CW). A thin layer of gluten is always present between starch granules and gas cells (GC). The scale bar is 2 μm.

Parts A and B are previously unpublished results of Dr. M. Parker, from the study reported in reference 32. Part C is previously unpublished results of Ms. Michelle Amor and Dr. Charles Brennan. Parts D and E are from reference 33.

strength, and strong doughs are required for breadmaking and for other food uses such as making pasta and noodles. In contrast, less elastic (weaker) glutens are required for making cakes and biscuits (cookies). The poor quality of many wheats for making breads, pasta and noodles is usually related to low gluten elasticity and it is not surprising that this property has been the main focus of research.

Elasticity is an unusual property of proteins, and we know of no other examples of elastic proteins derived from plants. However, there are several examples from the animal kingdom, the best documented of which is the connective tissue elastin[7]. Elastin is found in vertebrates and is widely distributed in composites such as skin, arteries, veins and cartilage. Other examples are resilin (found in the elastic ligaments of insect flight muscles and in the jumping mechanism of fleas) and abductin (found in the inner hinge of bivalve mollusc shells, where it acts as an elastic pivot)[8]. In these systems the proteins act as an energy store. Abductins have high contents of glycine, whereas resilins have high contents of glycine, alanine and asparagine and elastins high contents of proline, valine, glycine and alanine. Apart from elastin these proteins are poorly characterised, but amino acid analysis data suggest the presence of repeated sequence motifs. In elastins, sequences of tetra, penta and hexapeptide repeats are found and it has been proposed that the pentapeptide repeats form elastic β-spiral-type structures, based on repetitive β-reverse turns. Synthetic polypentapeptide repeats have been demonstrated to be elastomeric and it has been proposed that these structures contribute to the elasticity of elastin through entropic mechanisms[7].

It is notable that in all these proteins the cross-links are derived from amino acids other than cysteine: in elastin the cross-links are formed by oxidation and condensation of lysine residues to form desmosine and isodesmosine and in resilin the cross-links are formed by tyrosine residues, di- and tertyrosine. The nature of the abduction cross-links is not known. Unlike disulphide bonds, these cross-links are not able to exchange and rearrange under stress. An intriguing question is whether the elasticity of gluten proteins has any biological significance in wheat. It is not possible to answer this definitively, but the fact that no other cereal protein fractions exhibit significant elasticity makes it unlikely.

Wheat Gluten Proteins

Gluten is not a single protein, but a complex mixture of components[9]. The precise number has not been determined, but over 50 individual proteins can be separated by two-dimensional electrophoresis under reducing and denaturing conditions. Each of these is the product of at least one gene, with no evidence for post-translational modification except

disulphide bond formation. Although all the individual gluten proteins have some degree of sequence similarly, the extent of this varies and it is possible to classify them into several groups and sub-groups[10]. However, the most durable classification was proposed before we had any detailed knowledge of the individual proteins. This classification is into two groups called gliadins and glutenins. The gliadins were defined as readily soluble in aq. alcohols, and we now know that this relates to their presence as monomers which either lack cysteine or have only intra-chain disulphide bonds. In contrast, the glutenins are insoluble in aq. alcohols, and consist of high M_r (probably up to 10×10^6) polymers stabilized by inter-chain disulphide bonds. These fractions also have functional significance as the glutenins are largely responsible for gluten elasticity and the gliadins for viscosity. However, treatment of the glutenin fraction with a reducing agent converts the polymers to alcohol-soluble monomers, resulting in a loss of elasticity.

There is little doubt that all the individual proteins contribute in some way to the functional properties of whole gluten. However, it appears that one group of proteins are of particular importance in determining elasticity. This group is called the high molecular weight (HMW) subunits of glutenin, and consists of three, four or five individual proteins in different genotypes. The evidence for the importance of this group is of two types. First, it has long been known that the elasticity of gluten is positively correlated with the proportion of proteins present in high M_r (above about 1×10^6) polymers. It is also known that these polymers are enriched in the HMW subunits, implying that the HMW subunits are responsible for elasticity[11]. The second line of evidence is genetic rather than biochemical. This is the observation that differences in breadmaking quality are strongly correlated with allelic variation in the number and/or structure and properties (observed as differences in mobility on SDS-PAGE) of the HMW subunits (Fig. 2)[12]. A series of detailed genetic, molecular and biochemical studies, carried out in a number of laboratories since about 1980, have allowed us to propose explanations for these effects[13,14].

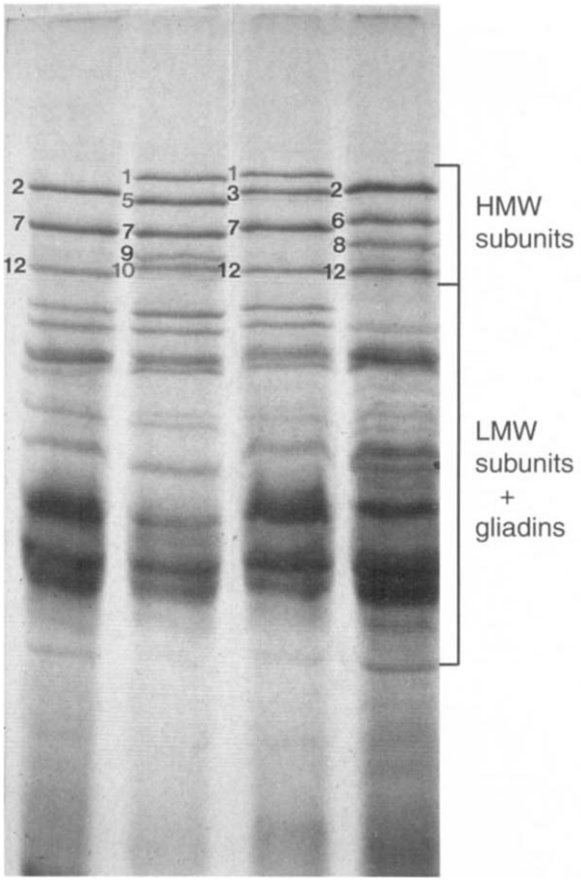

FIGURE 2. Polymorphism of HMW subunits. SDS-PAGE of reduced gluten proteins from four cultivars shows allelic variation in the number (3, 4 or 5) and composition of the HMW subunits. The presence of subunit 1 is associated with good breadmaking quality when compared with the null (i.e. silent allele). Similarly the presence of subunit 5+10 (which are inherited together) are associated with good quality when compared with the allelic pairs of subunits 2+12 or 3+12.

Quantitative and Qualitative Effects of HMW Subunits on Quality

Although different cultivars of bread wheat contain three, four or five HMW subunits, all have six HMW subunit genes[12]. Two genes are present on the short arms of each of the group 1 chromosomes (1A, 1B, 1D) of hexaploid wheat, which encode a high M_r x-type subunit and a low M_r y-type subunit. The variation in HMW subunit number results from specific gene silencing, with the 1Ay gene being silent in all bread wheats and the 1By and/or 1Ax genes expressed in only some cultivars[12]. This variation in gene expression has an impact on the total amount of HMW subunit protein. For example, the presence of a 1Ax subunit is associated with higher quality when compared with lines in which this gene is not expressed. We have shown that this is associated with an increase in the amount of HMW subunit protein, from about 8% of the total extractable protein in lines in which four HMW subunits are expressed to about 10% in lines containing five subunits[15]. Similar results were reported by Seilmeier et al. (1991)[16]. This implies that the association of a 1Ax subunit with quality is quantitative, with more protein resulting in a higher amount of elastic polymer and hence improved quality. The demonstration of an effect of gene dosage on the amount of HMW subunit protein also suggests that it should be possible to engineer plants for improved quality by adding additional copies of expressed HMW subunit genes. This is the simplest strategy for quality improvement and such work is in progress (see below). However, variation in quality is also associated with allelic differences in HMW subunit composition rather than amount. This is presumably due to qualitative differences in the properties and structures of the individual HMW subunits. To manipulate this aspect of quality, and indeed to carry out any fine tuning of gluten functionality (as opposed to coarse adjustment by varying HMW subunit amount), it is necessary to understand the structure of HMW subunits and how this may relate to their biophysical (and hence functional) properties.

HMW Subunit Structure

The amino acid sequences of nine individual HMW subunits are currently available, derived from the analysis of the corresponding genes[13,14,17]. This shows that the mature proteins (after signal peptide cleavage) have M_rs between 67,495 and 88,137. All have a similar structure, with short N- and C-terminal domains flanking an extensive central domain consisting of repeated sequences based on short peptide motifs. Although so far it has not been possible to determine detailed 3-D structures by X-ray crystallography or nmr spectroscopy a detailed picture has nevertheless been built up using a combination of approaches, as summarized in Figure 3.

Several aspects of HMW subunit structure are relevant to their role in forming elastic polymers. The first is the number and distribution of cysteine residues, as these are potential sites for intermolecular cross-linking. The degree of cross-linking determines the elastic properties of materials, a high density of covalent cross-links results in rubber-like elasticity whereas a lower density of cross-links results in more exten-

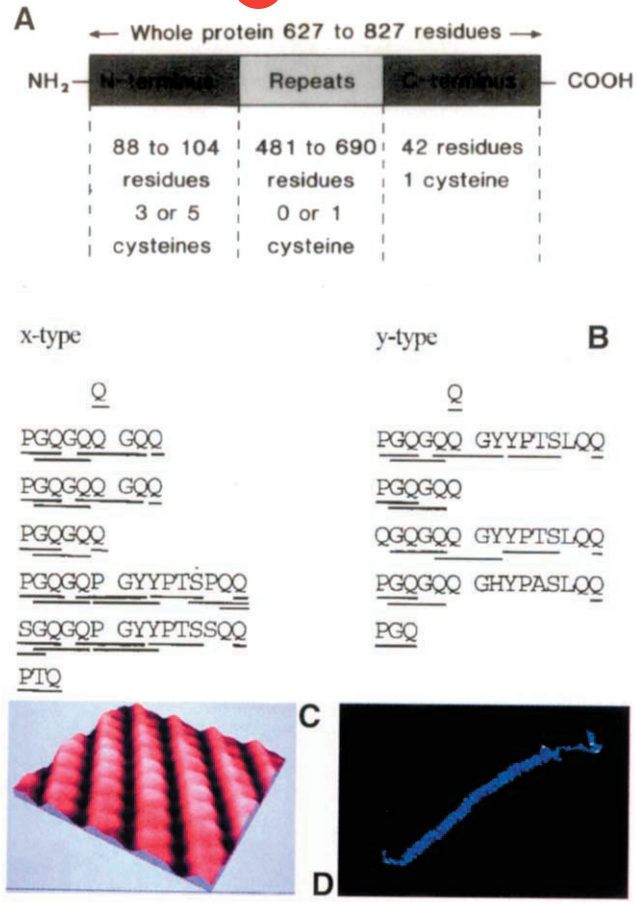

FIGURE 3. Structural Analysis of the HMW Subunits of Glutenin

A. Schematic summary of the amino acid sequences of HMW subunits, based on published data for nine subunits[13,14,17]. A repetitive domain of 481 to 690 residues is flanked by shorter non-repetitive N-terminal and C-terminal domains. Four to seven cysteine residues are present, concentrated in the non-repetitive domains.

B. The repetitive domains consist of tandem and interspersed repeats based on two or three short peptide motifs. These are hexapeptides and nonapeptides in the y-type subunits, with additional tripeptides in the x-type subunits. Secondary structure prediction indicates the formation of regular β-reverse turns, both within individual motifs and spanning the junction between adjacent motifs. The figure shows sequences derived from the repetitive domains of typical x-type and y-type HMW subunits, aligned to demonstrate the repeat structure. Predicted β-reverse turns, which involve four amino acid residues, are underlined. Standard single letter codes for amino acid residues are used. The non-repetitive N-terminal and C-terminal domains are predicted to be rich in α-helix (not shown).

C. Scanning Tunnelling Microscopy (STM) of a purified HMW subunit deposited on a graphite substrate in the hydrated solid state shows aligned rods of diameter about 1.9nm with diagonal striations of pitch about 1.5nm. The diameter agrees well with hydrodynamic studies with showed an extended rod-like conformation of dimensions about 50nm x 2nm. The pitch is consistent with the repetitive sequences forming a loose spiral based on β-reverse turns. The N-terminal and C-terminal domains are not resolved by STM.

D. Computer modelling using Quanta and CHARM modelling software packages (Molecular Simulations Inc.) shows a similar conformation with the repetitive sequences forming a loose spiral structure while the non-repetitive N- and C-terminal domains are rich in α-helix. The subunit modelled (called 1Dx5) has four cysteine residues which are shown in yellow. Taken in part from references 9,34–36.

sible materials. The cysteine residues in the HMW subunits are mainly located in the N- and C-terminal domains; the central repetitive domain can therefore undergo deformation/reformation under stress/relaxation. Secondly, the central repetitive domain forms a spiral structure based on repetitive β-reverse turns in the repeat motifs. Deforming this domain results in disruption of a stable state, the reformation of which contributes to elasticity. The repeat motifs are rich in glutamine residues, which can form hydrogen bonds both within and between subunits. Spectroscopic studies indicate a high degree of hydrogen bonding between subunits[18], which contribute to the overall stability of the structures[19]. However, the precise nature of the elastic mechanism of the HMW subunits and their contribution to gluten elasticity remains unclear.

Manipulation of Wheat Gluten Structure and Functionality

Although wheat transformation has only been achieved over the past 2–3 years, refinement in the procedures mean that it is now carried out in several laboratories, with frequencies exceeding those for other crops, such as maize, with longer standing transformation systems[20–22]. The current system in use in our laboratory, and the advantages and limitations, are summarized below. Grain composition and quality is one of the most attractive initial targets for the application of transformation technology due to our sound biochemical background and the availability of genes.

The simple and most obvious strategy to improve gluten quality is to increase the number of expressed HMW subunit genes. This should result to an increased proportion of HMW subunit protein, leading in turn to more high M_r polymers and greater gluten elasticity. This strategy is being followed in several laboratories using currently available genes controlled by their own endosperm-specific promoters. The first generation of transgenic plants has given promising results, with levels of transgenic expression varying but in some cases exceeding those of the corresponding endogenous genes—the reason for this is not known. It is now necessary to multiply the grain in order to quantify the impact on the amount and composition of gluten and on the functional properties of doughs.

These initial series of experiments may well generate new lines of wheat with genuinely useful changes in properties which can be incorporated into breeding programs. However, it is at best a very coarse approach and the long term aim should be to fine-tune the properties of gluten by making more subtle changes based on our knowledge of HMW subunit structure. This will also allow the various contributions to the elastic mechanism to be dissected and quantified.

The easiest structural feature to explore is the role of cross-linking, by making mutations to alter the number and distribution of cysteine residues. This should in turn affect the size and cross-linking patterns of the glutenin polymers, with effects on function. Similarly, the overall contribution of hydrogen bonding could be evaluated by increasing or decreasing the lengths of the repetitive domains: this should increase or decrease the stability, respectively[18].

It is also important to explore the properties and contribution of the β-spiral conformation, particularly since differences in the stability of this structure have been proposed to account for allelic differences between good and poor quality subunits[23]. This will be difficult to achieve by modifying the currently available HMW subunit genes as it would be necessary to make changes to each of the highly reiterated peptide units. A more promising approach is to design and express novel HMW subunit-related proteins, with subtle alterations in the repeat motifs which should affect the stability and properties of the β-spiral structure. This approach has been suc-

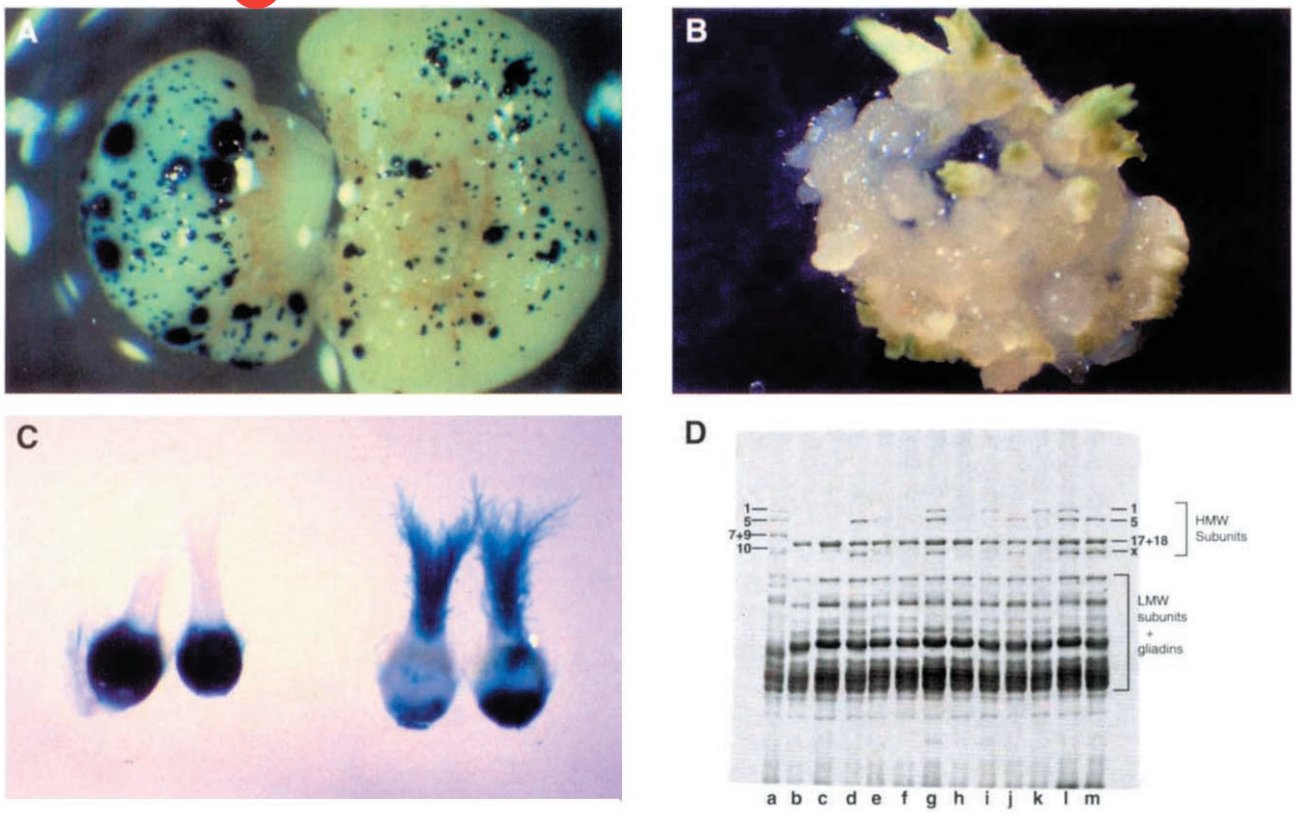

FIGURE 4. Transformation of Wheat and Tritordeum

A. Scutellum explants after particle bombardment showing transient expression of the β-glucuonidase (GUS) gene under control of a maize ubiquitin promoter.

B. Shoots developing from somatic embryos produced on a scutellum culture of wheat (cv. Florida). Herbicide or antibiotic selection, to isolate transformants, is usually applied shortly before this stage of culture development.

C. Differing patterns of GUS gene expression in developing carpels of two independent transgenic tritordeum lines.

D. SDS-PAGE of reduced gluten proteins from single seeds of wheat transformed with genes encoding HMW subunits of glutenin. Lane a shows proteins from the cv. Sicco which has five HMW subunits numbered 1, 5, 7 + 9 (migrating as one band) and 10. Lanes b-m show proteins from a wheat line which contains only two subunits called 17 + 18 (migrating as one band)[31]. Tracks b and c are from single seeds of control plants that had been regenerated but not transformed. Lanes d-m are from single seeds of plants transformed with separate genomic fragments encoding HMW subunits 1 and 5. Some seeds are clearly expressing both subunits, although the levels vary, while others are only expressing subunit 1 (lane k) or subunit 5 (lanes d, j, m). A novel band, labelled x, is also present in all seeds expressing subunit 5: the origin of this is not known. Further details are provided in the text.

cessfully exploited by Professor Dan Urry of the University of Alabama, who has developed a range of bioelastic polymers for biomedical applications based broadly on the repeated peptide sequences present in elastin[24]. It is possible to design similar modifications to the repeat motifs of the HMW subunits which would be expected to stiffen or weaken the β-spiral structure. For example, by altering the regularity of the β-reverse turns, either to produce protein molecules with perfectly conserved repeat motifs or proteins with a reduced propensity to form β-reverse turns. Such studies should indicate the contributions of the intrinsic elasticity of the proteins to the overall elasticity of gluten.

The functional properties of the HMW subunits are expressed in wheat doughs as part of the gluten fraction, a complex mixture of over 50 proteins. Thus it is ultimately necessary to evaluate the functional properties of the modified subunits in transgenic wheat in order to ensure that they are correctly incorporated. However, it is also possible to carry out preliminary studies in heterologous systems, using a protein engineering approach. Thus Bekes and co-workers have incorporated HMW subunits expressed in *E. coli* into doughs, using partial reduction and re-oxidation[25]. Although the incorporated subunits had the expected effects on dough elasticity, it seems unlikely that this procedure would lead to the same molecular interactions as those established when the protein is expressed in developing grain. We are therefore using a different approach, by constructing synthetic polymers based on HMW subunit repeats expressed in *E. coli*, and determining their biophysical properties[26]. This approach is providing information on the basic mechanism of glutenin elasticity which will be applied to the production of a new generation of transgenic plants in which gluten structure is finely adjusted to end use requirements.

Wheat Transformation: Current Status

Since the first report of transformed wheat in 1992 (ref. 27) transgenic wheat plants have been recovered in a number of public and commercial laboratories[21,28,29]. In all the published reports, gene transfer has been achieved via particle bombardment of tissue cultures derived from immature scutella, although immature inflorescence tissue may also be used as target tissue[22]. While transformation efficiencies up to 4% (based on the number of independent transformants recovered per bombarded explant) have been reported[29], in most experiments the wheat cultivars used were pre-selected for good response to *in vitro* culture, rather than varieties of agronomic importance. A priority is therefore to establish robust transformation procedures applicable to a range of germplasm.

In our laboratory we are addressing this in current UK rec-

ommended varieties. Although transformation efficiencies are at present lower than in "model" responsive genotypes, transformants of the breadmaking cultivars Baldus and Canon and of the feed cultivar Brigadier have been recovered, indicating that wider application of procedures is feasible (authors' unpublished results). We have also used similar methods to transform several genotypes of tritordeum, a novel cereal combining the genomes of tetraploid pasta wheat and *Hordeum chilense* (a wild barley species)[22].

A more important limitation to early exploitation of wheat transformation technology may, however, be the paucity of characterized tissue-specific or developmentally-regulated promoters suitable for use in cereals. Although a wide range of such promoter sequences have been identified from dicot species, these generally do not function well in cereals. An exception to this general situation is the availability of endosperm-specific promoters where a number of cereal sequences have been isolated and characterized[30].

Our wheat transformation protocol (Fig. 4) is based on a culture system in which somatic embryogenesis and shoot formation are induced as quickly as possible after culture initiation in order to minimise the frequency of variant regenerants. Cultures are bombarded one day after initiation and antibiotic (geneticin) or herbicide (PPT) selection is applied at about three weeks, when defined somatic embryos are present. Selection pressure is maintained until plantlets have rooted *in vitro*, when leaf samples are taken for reporter gene assays and PCR analysis to confirm transgene integration. With this procedure, transformants can be transferred to the glasshouse four to five months after culture initiation and few abnormal or sterile plants are recovered.

In our first experiments on the genetic manipulation of gluten quality a series of near-isogenic wheat lines which show the expression of between zero and five HMW subunit genes[32] are being transformed with the genes encoding subunits 1 and 5, singly and in combination, under the control of their own promoters. Transformants analyzed to date have shown the expected endosperm-specific expression of the transgenes and lines with high levels of subunit expression have been identified (Fig. 4D).

These preliminary results, and similar studies in progress in other laboratories, demonstrate that the manipulation of gluten structure and functionality by transformation with genes for HMW subunits and other gluten proteins is technically feasible. We have no doubt that genetic engineering will have a major impact on the end use properties and utilization of wheat within the next decade.

Acknowledgments

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