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Shewry P., Tatham A., Barcelo P., Lazzeri P.

*in*

Di Fonzo N. (ed.), Kaan F. (ed.), Nachit M. (ed.).  
Durum wheat quality in the Mediterranean region

Zaragoza : CIHEAM

Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 22

1995

pages 227-240

Article available on line / Article disponible en ligne à l'adresse :

<http://om.ciheam.org/article.php?IDPDF=95605375>

To cite this article / Pour citer cet article

Shewry P., Tatham A., Barcelo P., Lazzeri P. **Molecular and cellular techniques in wheat improvement**. In : Di Fonzo N. (ed.), Kaan F. (ed.), Nachit M. (ed.). *Durum wheat quality in the Mediterranean region*. Zaragoza : CIHEAM, 1995. p. 227-240 (Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 22)



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## Molecular and cellular techniques in wheat improvement

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**SUMMARY** - The application of genetic engineering to the improvement of wheat has two prerequisites. The first is the availability of methods to transfer genes and ensure that they are expressed at the required level and show the correct pattern of tissue specificity and development regulation. The second is an understanding of the traits of interest at the molecular level and the availability of suitable genes to manipulate these traits. The present article summarises the current status of research on the genetic engineering of bread and durum wheats focusing on opportunities for manipulating resistance to biotic and abiotic stresses, and grain quality for breadmaking and pasta making.

**Key words:** Wheat, transformation, proteins, resistances.

**RESUME** - "Techniques moléculaires et cellulaires pour l'amélioration du blé". L'application du génie génétique à l'amélioration du blé présente deux pré-requis. Le premier est la disponibilité de méthodes de transfert de gènes en s'assurant qu'ils s'expriment au niveau désiré et suivent la démarche correcte de spécificité du tissu et de régulation du développement. Le second est la connaissance des caractères d'intérêt au niveau moléculaire et la disponibilité des gènes qui se prêtent à la manipulation de ces caractères. Le présent article résume la situation actuelle de la recherche en matière de génie génétique des blés durs et tendres en insistant sur les possibilités de manipuler la résistance aux conditions adverses biotiques et abiotiques, ainsi que sur la qualité du grain pour la fabrication du blé et des pâtes.

*Mots-clés :* Blé, transformation, protéines, résistances.

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### Introduction

Wheat is the most important and widely grown crop in the world, the total yield in 1991 exceeding 550 million tonnes. This is reflected in the amount of effort which has, and still is, being applied to crop improvement, with a total of about 17,000 varieties being produced by the 1970's (Feldman, 1978). A number of species have been cultivated, but only two are grown to any extent today: hexaploid bread wheat (genetic constitution AABBDD) and tetraploid pasta wheat (AABB). The latter is well suited to drier regions such as the Mediterranean basin and similar areas in Asia and America.

The vast majority of currently grown cultivars of bread and pasta wheats have been produced by conventional plant breeding, employing the wide range of genetic variation within these species. However, it has also proved possible over the past 20 years to transfer useful genes from wild species of *Triticum* (notably tetraploid wild emmer) or related genera of the Triticeae (e.g., rye, *Aegilops*

*ventricosa*) by chromosome engineering (see Feldman, 1988), thus increasing the range of variation available to breeders. The development of genetic engineering technology should take this process one stage further, by allowing single genes or combinations of genes from any species of plant, animal or microbe (or even *ab initio* designed genes) to be inserted into wheat to confer desired traits.

In the present paper we will briefly review the application of genetic engineering technology to wheats, and discuss its potential applications in the improvement of grain quality. However, because the vast majority of the research has been carried out on bread wheat rather than pasta wheat, it is inevitable that much of the discussion will relate to this species, although we will refer to pasta wheat where possible.

## Applications of cellular and molecular techniques to wheat

### Cellular techniques in plant breeding

Although cell biological techniques are often viewed as an adjunct to genetic engineering, they have also had a major impact on classical cereal breeding, making the process more efficient and facilitating the transfer of genes from wild relatives which cannot readily be crossed. Three aspects of this are particularly relevant to wheat improvement. Haploidy is of value as it allows the production of homozygous genotypes from heterozygotes in a single stage, thus eliminating the need for repeated selfing. Two systems can be used to produce haploids in wheat: wide crossing with *H. bulbosum* or maize followed by spontaneous elimination of the foreign chromosomes, or regeneration from haploid gametophytic tissues such as anthers (microspores) or ovaries. In both cases tissue culture techniques are required to rescue the haploid embryos or regenerate from haploid tissues, and chromosome doubling may be spontaneous or induced by colchicine. Such haploid populations have been used to facilitate RFLP-mapping studies as well as in plant breeding. Embryo rescue and tissue culture are also used in wide crossing to transfer genes from wild relatives, as the hybrid embryos rarely develop naturally on the plant. Cell and tissue culture techniques are widely used for some crops to multiply germplasm and eliminate virus infections. This is not the case for cereals which have limited capacity for vegetative multiplication and are efficiently propagated by seed. There have been some attempts to exploit the somaclonal variation which may arise during the tissue culture procedure as a source of useful variation. However, despite initial enthusiasm (Larkin and Scowcroft, 1981), this has not so far proved to be the case, most of the variation being deleterious or unstable. In addition, *in vitro* selection in tissue culture can be used to produce useful biochemical mutants, for example with increased levels of free amino acids.

### Genetic engineering technology

There are two requirements for genetic engineering of plants: the DNA must be delivered into the plant cells, which must then be regenerated to give a plant in which all the cells are transformed. Cereals have not proved amenable to *Agrobacterium* - mediated transformation and work has, therefore, focused on direct DNA delivery systems and on the development of regenerable protoplast systems or embryogenic tissue culture systems as recipients.

We have discussed the early work in this area in a recent review article (Lazzeri and Shewry, 1994), and will not discuss this further. It is sufficient to say that work reported over the past year has firmly established one approach at the forefront of wheat transformation technology. This is particle bombardment of embryogenic callus cultures or inflorescences. The former has resulted in efficient and reproducible transformation of hexaploid bread wheat (Vasil *et al.*, 1992; Weeks *et al.*, 1993), while bombardment of inflorescences has given transformed plants of *Tritordeum* (an amphidiploid between tetraploid wheat and the wild barley *Hordeum chilense*) (Barcelo *et al.*, 1993) (Fig. 1). A further approach which has considerable potential is to deliver DNA into similar tissues by electroporation rather than bombardment, but results achieved using this technique in wheat have yet to be published.

While most work on wheat transformation has focused on bread wheat, cell culture systems allowing regeneration from a range of explants and tissues have been developed for cultivars of *T. durum*.

These include immature embryos (Bennici *et al.*, 1988; Barcelo *et al.*, 1989), long term callus cultures (Borrelli *et al.*, 1991), and suspension and protoplast cultures (Yang *et al.*, 1993). The DNA transfer methods which have been developed for bread wheat can almost certainly be applied to these culture systems, so genetic engineering of durum wheat is now feasible.

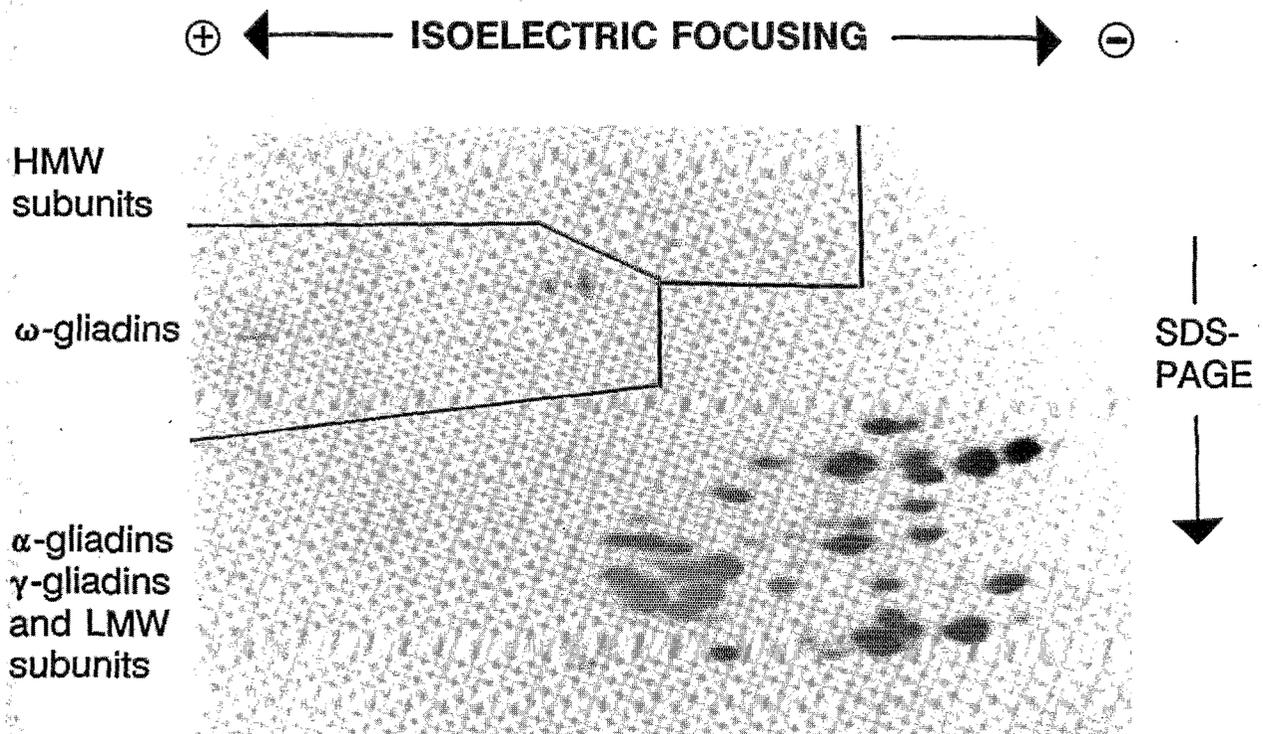


Fig. 1. 2-D IEF/SDS-PAGE of total prolamins (gluten proteins) from bread wheat cv. Chinese Spring.

## Targets for genetic engineering

### Yield and agronomic performance

Grain yield is, and is certain to remain, a major target for all cereal breeders. However, at present it is one of the least amenable of all characters to the techniques of genetic engineering. This is because it is a highly complex character resulting from the interactions of many genes. Although genetic engineering cannot yet be applied to increasing yield, it could be used to explore some of the contributing factors, for example the roles of source/sink relations (see Sonnewald *et al.*, 1991) and endosperm cell number (Singh and Jenner, 1982).

### Manipulation of resistance to abiotic and biotic stresses

Resistance to stresses is of key importance, particularly for a crop such as durum wheat which is grown in dry conditions. A number of strategies have been developed for conferring resistance to microbial pathogens (see recent review by Lamb *et al.*, 1992) and to insect pests. Several molecular approaches have been used to confer resistance to viruses in other species of plant, notably tobacco. These include the incorporation of viral coat protein genes (Beachy, 1988) or satellite DNA (Gerlach *et al.*, 1987; Harrison *et al.*, 1987) into the plant genome. In the case of fungal pathogens it is possible to confer resistance by exploiting natural disease resistance mechanisms. A number of plant proteins have been shown to be toxic to fungal pathogens, either alone or in combination. These may be produced as a result of infection or damage, or be constitutively expressed in storage tissues such as seeds or tubers. They include  $\beta$ -glucanases, endochitinases, ribosome-inactivating proteins, lectins,

thionins and membrane-destabilising proteins. Because these proteins are the products of single genes, they may be readily transferred between species, and some have already been shown to confer resistance in transgenic plants. For example, a barley ribosome-inactivating protein confers resistance to soil borne fungal pathogens in transgenic tobacco plants (Logemann *et al.*, 1992). Other plant anti-fungal compounds are secondary products such as flavonoid and terpenoid phytoalexins. Being synthesised by multienzyme pathways, these pose more problems for genetic engineers. However, it has recently been shown that the transfer of two stilbene synthase genes from grapevine into tobacco results in the production of the phytoalexin stilbene and resistance to infection by *Botrytis cinerea* (Hain *et al.*, 1993). These strategies are based on natural wide spectrum disease resistance mechanisms, and therefore confer resistances to a range of fungal pathogens. The naturally occurring genes responsible for the gene-for-gene resistances to specific species and races of pathogens have not yet been characterized but should ultimately allow the specificity of engineered resistances to be fine tuned.

There has also been considerable work on identifying and exploiting sources of resistance to insect pests. The antimicrobial proteins discussed above may also deter feeding by insect pests, or be accompanied by other proteins which deter feeding. The most well known of these is the cowpea trypsin inhibitor which confers resistance to the Lepidopteran pest *Heliothis virescens* in transgenic tobacco (Hilder *et al.*, 1987), but they also include inhibitors of cysteine proteinases (which may confer resistance to Coleopteran larvae), lectins and inhibitors of insect  $\alpha$ -amylases. In addition, genes encoding the insecticidal  $\delta$ -endotoxins of *Bacillus thuringiensis* have been used to confer insect resistance in transgenic plants including maize (Koziel *et al.*, 1993).

Abiotic stresses can include extremes of temperature (heat or cold stress), water availability (drought or waterlogging) or high levels of minerals such as salt, aluminium, manganese, boron and heavy metals (notably copper) (see Manyowa and Miller, 1991). So far the most successful approach to these problems has been to exploit natural variation present either in the crop itself or in wild relatives (for example, salt tolerance in *Laphopyrum elongatum*, aluminium tolerance in *Aegilops uniaristata*). However, studies in progress to determine the mechanisms of such tolerances should open the way to genetic engineering approaches. In fact, some success has already been achieved with salt tolerance, Tarczynski *et al.* (1993) showing that the introduction of a bacterial gene encoding mannitol-1-phosphate dehydrogenase into tobacco results in the production of mannitol and increased tolerance of salinity.

Heat stress is also of considerable interest in relation to Mediterranean agriculture. The basic biochemical and molecular events which occur when wheat is heat stressed are undoubtedly similar to those which have been characterized in more detail in other plants (Key *et al.*, 1985) and other organisms (Burden, 1986). However, specific effects on the developing grain may also lead to effects on grain quality. Blumenthal *et al.* (1990) showed that decreases in dough strength in grain grown for short periods above 35°C were associated with specific effects on gluten protein gene expression, while Bradl and Ho (1991) showed that selective destabilization of mRNAs for secretory proteins (i.e. those passing into and through the ER lumen) occurs in barley aleurones. The effects of high temperature on the development and functional properties of durum wheats are clearly worth investigating.

## Manipulating grain quality

The quality of cereal grain is, of course, determined by the end use. In the case of wheat there are two major end uses: as feed for livestock, and for processing into food (notably baked goods and pasta) for human nutrition. Although wheat has low nutritional quality for monogastric livestock (e.g., poultry and pigs) due to its low contents of lysine and threonine, there has been little interest in this aspect of quality. The overwhelming interest is in food quality: breadmaking quality for bread wheat and pasta-making quality for durum wheat. In the case of breadmaking, the quality is dependent on the visco-elastic properties conferred on doughs by the gluten proteins: good quality cultivars form doughs which are highly elastic, and poor quality cultivars doughs which are more extensible. Dough elasticity is also important in durum wheats, where it is again determined by the gluten proteins. However, in addition, it is also necessary to have the correct surface properties, with an absence of clumping or stickiness when cooked. The basis for this property is less well understood, but recent studies indicate that proteins may also be involved.

We will therefore discuss these aspects of bread and durum wheat quality, starting with a brief introduction to the structure and properties of the gluten proteins.

## Wheat gluten proteins

The gluten proteins correspond to the major grain storage proteins, and account for over half of the total grain protein at maturity. They are deposited in the developing starchy endosperm in discrete membrane-bound deposits called protein bodies. As the endosperm cells become distended with starch these bodies become disorganised, and the proteins form a matrix around and between the starch granules. When the endosperm is milled, wetted and worked to form dough the proteins form a network, but they can be isolated as a cohesive proteinaceous mass (called gluten) by washing the dough to remove most of the starch and the water-soluble components.

Gluten has a unique combination of two physical properties: elasticity and viscous flow (extensibility). It is these properties which appear to determine functionality in food systems, and a precise balance is required for different applications. For example, whereas strong (i.e. elastic) gluten is required for bread and pasta making, highly extensible gluten is preferred for cakes and biscuits.

The gluten proteins of wheat resemble the major storage proteins of barley (hordeins) and rye (secalins) in containing high contents of the amino acids proline and glutamine and low levels of charged acidic and basic amino acids (including lysine, resulting in the low nutritional quality of the whole grain). The proteins are also insoluble in water but soluble in alcohol/water mixtures, and are together classified as prolamins (Shewry *et al.*, 1986). However, despite the presence of related proteins, it is not possible to prepare gluten from either barley or rye, and neither can be baked to give leavened bread.

Wheat gluten consists of a complex mixture of proteins (Fig. 2) which are classically divided into two groups (Fig. 3). The gliadins are readily soluble in alcohol/water mixtures in the native state, and consist of single monomeric proteins which contain no disulphide bonds or only inter-chain disulphide bonds. In contrast, the glutenins consist of subunits which form high  $M_r$  polymers stabilized by inter-chain disulphide bonds, although intra-chain bonds may also be present. These polymers may be insoluble in alcohol/water mixtures, although the individual reduced subunits are soluble. These two groups of gluten proteins also have functional significance; the gliadins being associated with gluten viscosity (extensibility) and the glutenins with elasticity. These two fractions can also be further sub-divided, the gliadins into  $\omega$ -gliadins,  $\gamma$ -gliadins and  $\alpha/\beta$ -gliadins, and the glutenins into high molecular weight (HMW) and low molecular weight (LMW) subunits.

Because of their functional importance, the gluten proteins have been studied in detail using genetical, molecular, biochemical and biophysical approaches. This has revealed details of the numbers and locations of their controlling genes and loci, their amino acid sequences, their conformations and their relationship to various functional properties (see Payne, 1987; Shewry *et al.*, 1989; Tatham *et al.*, 1990). It has also shown that it is more valid to classify them into three groups, which do not correspond to the more widely used gliadin/glutenin classification (Shewry *et al.*, 1986). Two of these groups correspond to the  $\omega$ -gliadins (the S-poor prolamins) and the HMW subunits of glutenin (the HMW prolamins) respectively, while the third group (the S-rich prolamins) comprises gliadin ( $\gamma$  and  $\alpha/\beta$ -) and glutenin (LMW subunit) components. The amino acid sequences of typical members of these groups are summarised in Fig. 4.

All wheat prolamins are characterized by the presence of repeated sequences. These are rich in proline and glutamine and appear to be based on similar motifs in the S-rich and S-poor groups. However, whereas these repeats are present only in the N-terminal parts of the S-rich prolamins, they account for almost the whole protein in the S-poor group. The repetitive sequences present in the HMW subunits are located in the centre of the proteins, and are based on several motifs which are not related to those present in the S-rich and S-poor prolamins.

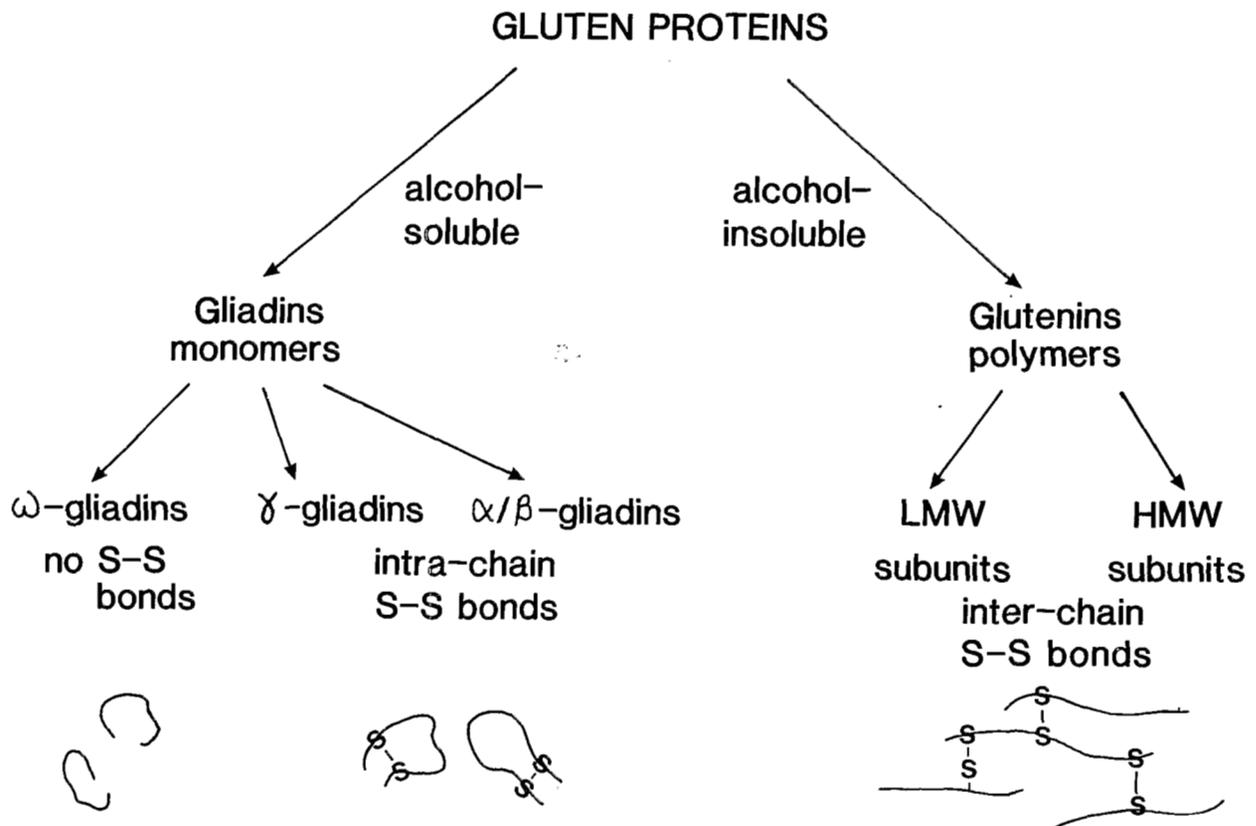


Fig. 2. Summary of the major groups of wheat gluten proteins. The  $\omega$ -gliadins and HMW subunits are defined as S-poor prolamins and HMW prolamins respectively. The  $\alpha/\beta$ -gliadins,  $\gamma$ -gliadins and LMW subunits are S-rich prolamins.

## Gluten proteins and breadmaking quality

The gluten proteins are clearly of great importance in determining the functional properties of wheat doughs. It is therefore important to understand the molecular basis for these properties if we wish to use genetic engineering to improve quality. Although most of the work has been carried out on bread wheat, much of the information gained is undoubtedly also relevant to the quality of durum wheat. Three aspects of gluten protein structure are of interest and will be discussed here: the structures of the individual proteins, their interactions with each other and their interactions with other dough components (starch, lipids).

The repeated sequences present in all wheat prolamins are largely responsible for the unusual amino acid compositions (being rich in proline and glutamine) and solubility properties of the whole proteins (see Shewry, 1993). Biophysical studies show that these repetitive domains are not globular (unlike the non-repetitive domains of the S-rich and HMW prolamins), but form unusual spiral supersecondary structures based on repeated  $\beta$ -turns and (in the S-rich and S-poor types only) poly-L-proline II structure (Field *et al.*, 1987; Tatham *et al.*, 1990; Miles *et al.*, 1991; l'Anson *et al.*, 1992). This results in the repetitive domains having extended structures. There is no doubt that the repetitive domains and the structures that they adopt are of great importance in determining the properties of gluten. In particular, the viscosity (extensibility) of gluten almost certainly results primarily from strong hydrogen bonding and hydrophobic interactions between the repetitive domains of the gluten proteins, notably the monomeric gliadins. In genetic engineering terms, viscosity could be increased by increasing the gliadin: glutenin ratio, for example by increasing the proportions of the S-poor  $\omega$ -gliadins. However, similar results may be more readily achieved by decreasing the proportion of glutenin subunits, and Payne and Seekings (1993) have recently reported the development of a novel cultivar of bread wheat (Galahad 7) which has high gluten extensibility and contains only a single HMW glutenin subunit compared with the three, four or five subunits present in other cultivars (see below). However,

this cultivar has been produced by classical plant breeding, by combining naturally occurring mutant null alleles of the genes encoding the various HMW subunits.

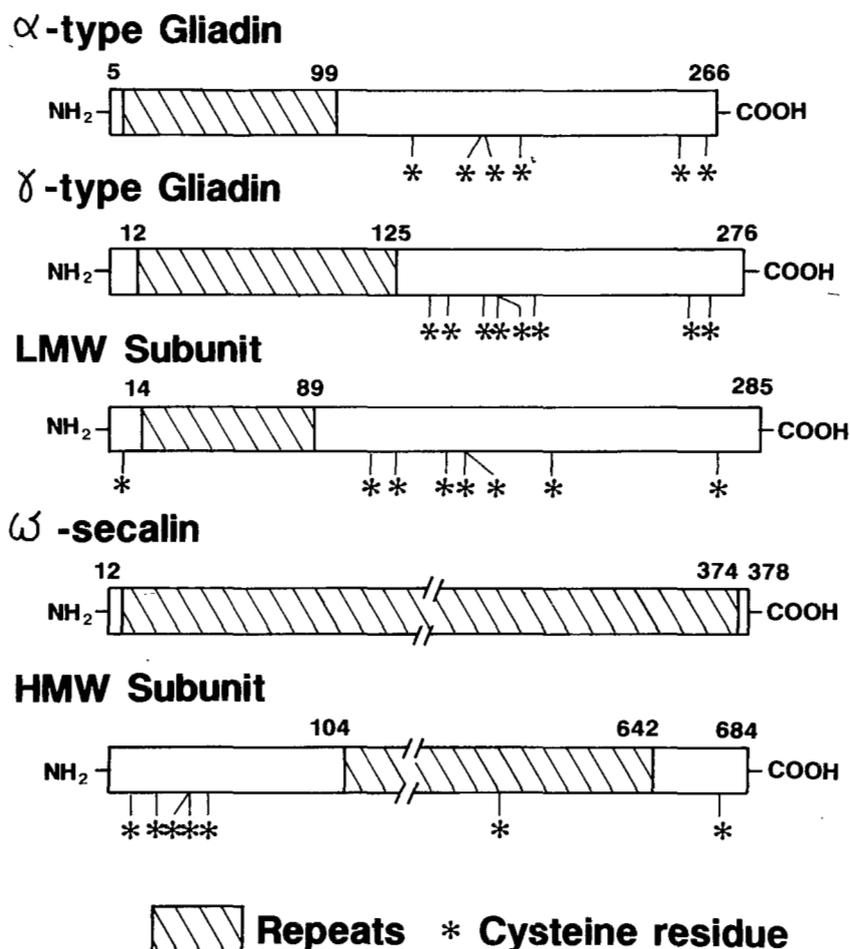


Fig. 3. Schematic sequences of a typical  $\alpha$ -gliadin,  $\gamma$ -gliadin, LMW subunit and HMW subunit of wheat, and a  $\omega$ -secalin (a homologue of  $\omega$ -gliadin) of rye. The repeated sequences present in the gliadins, LMW subunit and  $\omega$ -secalin are based on related motifs and are rich in proline and glutamine. The repeated sequences in the HMW subunits are not related to those present in the other proteins. See Shewry *et al.* (1989), Shewry and Tatham (1990) and Hull *et al.* (1991) for details of original references.

Whereas gluten viscosity is mainly determined by non-covalent interactions, particularly between the gliadins, elasticity is associated with the covalently linked glutenin polymers and, in particular, with the HMW subunits. Although the HMW subunits only account for about 20% of the total glutenin fraction (and 6-10% of whole gluten, see Halford *et al.*, 1992), two lines of evidence indicate that they are associated with high elasticity and good breadmaking quality. Firstly, they are only present in high  $M_r$  (above about  $1 \times 10^6$ ) polymers, the amounts of which are associated with good breadmaking quality (see, for example, Field *et al.*, 1983). Secondly, allelic variation in their number and composition is strongly correlated with variation in breadmaking quality (see Payne, 1987). Thus, all cultivars contain two subunits encoded by chromosome 1D and one encoded by chromosome 1B, while additional 1B-encoded and/or chromosome 1A-encoded subunits are present in some cultivars only. Variation in breadmaking quality is correlated with the absence or presence of a subunit encoded by chromosome 1A, and with allelic variation in the two subunits encoded by chromosome 1D (Payne, 1987).



subunits encoded by chromosome 1A (Payne, 1987). However, variation in quality is also associated with allelic variation in expressed HMW subunits. In this case the effect on quality could result from differences in HMW subunit structure, either in their ability to form cross-links (for example, one quality-associated subunit encoded by chromosome 1D contains an additional cysteine residue compared with an allelic subunit associated with poor quality) or in the intrinsic elasticity of the repetitive domain (see Shewry *et al.*, 1992).

In the case of bread wheat, increases in gluten elasticity could be obtained by inserting additional genes for HMW subunits to increase the total amount of HMW subunit protein. In addition, these genes could possibly be mutated to give more subtle differences, due to effects on the cross-linking or other properties of the glutenin polymers.

It must be remembered, however, that wheat gluten is present as a network in dough, where it is intimately associated with other dough components. These interactions are still incompletely understood, but reported correlations of breadmaking quality with polar lipid content (Chung, 1985) and the demonstration by nuclear magnetic resonance spectroscopy that lipids act to plasticise gluten proteins (Tatham *et al.*, 1990) indicate that they are potentially of great importance in modulating the functionality of the gluten proteins.

## Gluten proteins and pasta quality

We have discussed breadmaking in some detail because it is the most fully understood aspect of wheat quality, and the information gained can possibly be applied to the manipulation of durum wheat quality. However, studies of durum wheats indicate that the high gluten strength of good quality cultivars relates to their contents of S-rich prolamins rather than to the HMW subunits. Durum wheat cultivars can be divided into two groups characterized by the absence or presence of  $\gamma$ -gliadins called bands 42 and 45 respectively (Damidaux *et al.*, 1978, 1980). Each of these bands is, in fact, a marker for the complex *Gli-B1* locus, which encodes a mixture of  $\gamma$ -gliadins,  $\omega$ -gliadins and LMW subunits of glutenin, and it is probable that specific blocks of LMW subunits associated with gliadins 42 and 45 are responsible for the associated weak and strong dough respectively (Payne *et al.*, 1984). In contrast, there appears to be little effect of the HMW subunit composition on quality (Autran and Feillet, 1987).

The absence of any major effect of HMW subunit composition could indicate that these proteins play little role in determining pasta making quality. On the other hand it could result from limited genetic variation in the composition of these proteins in durum wheat cultivars. Durum wheats lack the two chromosome 1D-encoded HMW subunits that are present in all bread wheats, and most cultivars contain only one or two HMW subunits which are both encoded by chromosome 1B (more than 80% being null at the *Glu-A1* locus) (Autran and Feillet, 1987; Branlard *et al.*, 1989).

The LMW subunits are clearly a key target for attempts to improve durum wheat quality by genetic engineering. However, attempts to manipulate the amounts and properties of these proteins would currently be limited by our poor knowledge of their structures and functional properties, and by the complexity of the LMW subunit gene family (see Sabelli and Shewry, 1991). A simpler approach would be to attempt to explore and manipulate pasta quality by insertion of additional genes for HMW subunits. Insertion of one or more genes could have a major impact on the total amount of HMW subunit protein (as only one or two genes are normally expressed), leading to increased dough elasticity.

## Pasta surface quality in durum wheats

The quality of durum wheats for pasta making is determined not only by dough visco-elasticity but also by the surface quality on cooking. The latter appears to depend not on the prolamins storage proteins that form the major part of gluten, but on minor protein components which may also be present in extracted gluten. Early studies indicated that cooking quality was associated with a high ratio of reactive to total disulphide bonds (Fabriani *et al.*, 1970), and Kobrehel and co-workers subsequently reported a positive correlation between cooking quality and the total contents of sulphhydryl groups and

disulphide bonds in two low  $M_r$  gluten proteins called DSG (for durum wheat sulphur-rich glutenins) -1 and -2 (Kobrehel and Alary, 1989a,b). These proteins have  $M_r$ s by SDS-PAGE of about 14,100 and 17,100 respectively, and were subsequently shown to have similar N-terminal amino acid sequences to two proteins previously characterized from bread wheat and called CM (for chloroform/methanol-soluble) 16 and CM3 (Shewry *et al.*, 1984; Barber *et al.*, 1986; Kobrehel and Alary, 1989b). This relationship has since been confirmed by comparison of cDNA sequences of CM16 from pasta and bread wheats (Lullien *et al.*, 1991).

The CM proteins are not prolamins, although they do have some sequence similarity to the non-repetitive domains of the S-rich and HMW prolamins, indicating a common evolutionary origin (Kreis and Shewry, 1989; Shewry, 1993). Their *in vivo* function is unknown, although they may be active *in vitro* as inhibitors of serine proteases (trypsin) or insect  $\alpha$ -amylases, either as single subunits or as components of dimeric or tetrameric complexes (see García-Olmedo *et al.*, 1987). Their precise functional role in determining pasta cooking quality is also not known, although Kobrehel *et al.* (1991) have proposed that they associate with gliadins and glutenins by strong non-covalent interactions.

A number of cDNA and genomic clones for CM proteins have been isolated, from barley as well as wheat (see García-Olmedo *et al.*, 1987). In addition, a related  $\alpha$ -amylase inhibitor from bread wheat has been successfully expressed in *E. coli* to explore the basis for its activity against insect  $\alpha$ -amylase (García-Maroto *et al.*, 1991). It should therefore be possible to use protein engineering to explore the interactions between CM3, CM16 and other CM proteins, other gluten proteins and other dough components, and to use genetic engineering to insert wild type or mutated genes in order to manipulate quality.

## Conclusion

Recent developments in transformation technology for small grain cereals should soon be applied to durum wheat, allowing specific manipulations to be made to improve aspects of agronomic performance or grain quality. The latter will be facilitated by the detailed understanding of quality-related proteins which has been obtained over the past few years, allowing the composition to be manipulated to optimise quality for traditional and novel end uses.

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