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Comparative transcriptional and proteomic profiling of bread wheat cv. Bobwhite and its derived transgenic line over-expressing a *lmw-gs* gene

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ABSTRACT

Since wheat is indisputably one of the major food crops in the world and the single most important source of plant protein in the human diet, it has always been a target of choice for classical and biotechnological-oriented breeding programs.

The quality of the wheat end-use products is largely determined by the gluten proteins, a complex mixture of polypeptides accounting for up to 80% of the total seed proteins. Gluten proteins are the determinants of the visco-elastic properties of the wheat doughs, and their quantity in the seeds is directly correlated with the quality of end-use products.

Recent efforts to increase the quantity of gluten proteins in the seeds focused on the introduction of additional gene copies by means of genetic engineering technology. We have thus produced and extensively characterized an independent transgenic bread wheat line overexpressing a LMW-GS (Low Molecular Weight-Glutenin Subunit), whose relative quantity in the seed is expected to positively affect the technological properties of the flour.

In order to define the consequences of transgene(s) insertion/expression and the effects of genetic transformation on the global endosperm gene expression, we carried out a comparative proteomic and transcriptional profiling between the seeds of the transgenic line with its non-transformed counterpart.

Microarray analysis showed that, during the seed development, 542 unigenes were significantly differentially expressed. Those genes for which a reliable annotation was available have been classified, according to their putative functional category, to provide an overview of the genome responses to genetic transformation and trasgene(s) expression. By expression pattern matching, we identified a number of genes strongly correlated to the expression of the transgenes during the process of seed maturation. The implications of the *lmw-gs* over-expression on the partitioning and accumulation of storage reserves in developing wheat grains are also discussed.

Evidence for differential expression of several seed storage-related genes was confirmed at the protein level, both in developing and mature seeds, with quantitative proteomic analyses of the corresponding encoded subunits.

Our data define in part the downstream responses in developing seeds to genetic transformation and over-expression of a *lmw-gs* transgene in bread wheat.

1. INTRODUCTION

1.1 Wheat

Wheat is the world's most popular crop. It is grown over a large area and under a wide range of conditions. Over 600 million tons (Mt) are produced annually on 215 million hectares (Mha), an average of 2.79 t ha^{-1} (Tab. 1.1). At present, wheat is the staple food in more than 40 countries for over 35% of the world's population and provides over 20% of the calories and proteins in human nutrition.

Its key role is due to several reasons. It can grow over a wide range of climatic conditions, elevations, and soil fertility. It is easily transported and safely stored over long periods of time, and perhaps, most important, it can be consumed by humans with minimal processing: its seeds can be ground into flour or semolina which form the basic ingredients to prepare leavened and unleavened bread, noodles, cakes, biscuits and pasta. Moreover, wheat-derived products are widely accepted by people of all religions and races. Basically, in one form or another, wherever we are in the world, we consume wheat at every meal.

In particular, wheat endosperm, as a major component of the human diet, is not only a source of carbohydrates, but also of proteins, minerals and vitamins and it can be a sufficient protein supplier when balanced with other foods rich in lysine and methionine. The protein content of wheat grains can vary from 10 to 18% of the dry weight and the majority of the proteins accumulate in the endosperm as storage proteins that represent a source of carbon and nitrogen during the germination process.

	2004 Wheat production (Mt)	2004 Wheat area harvest (Ha)
World	627,130,584	215,765,044
Africa	21,682,410	9,675,588
North & Central America	87,110,539	30,663,190
South America	24,017,168	9,715,534
Asia	255,118,303	96,419,605
Europe	218,539,154	57,260,121
Oceania	20,663,010	12,031,006

Tab. 1.1: Wheat production and area harvest in different continents and in the world (FAOSTAT data, 2005).

Storage proteins form a complex called gluten which shows, upon mixing with water, unique rheological properties such as elasticity and extensibility. By modifying the rheological properties of gluten, wheat flour can be used to produce a wide variety of food products.
Owing to the importance of wheat as a food crop, it has been, and it is still is, extensively studied. The wheat storage proteins, in particular, have been widely characterized to understand their contribution to the physical and chemical properties of the wheat flour/dough (Osborne, 1924; Shewry and Tatham, 1990; Shewry et al., 1999 for general review) with the final goal to improve the wheat end-product quality, although many aspects still remain unclear due to the peculiar structure and organisation of the endosperm proteins into gluten.

1.2 Origin of wheat

It is widely accepted today that wheat was first grown as a food crop since 10,000-8,000 B.C.. The origin of the genus *Triticum* is found in Asia and part of Africa, in the area that extends from Syria to Kashmir, and southwards to Ethiopia. This is where cultivated wheats gradually evolved from wild plants. Emmer is generally regarded as one of the ancestors of the wheats commonly grown today, because it closely resembles the wild species of wheat found in the mountainous regions of Syria and the area formerly part of Palestine. Moreover, crude wheat plants, such as einkorn and emmer, and many wild species of grass were common to the same area.

According to the modern taxonomy, wheat belongs to the genus *Triticum* of the family *Gramineae*; it includes several species forming a polyploid series, with a basic chromosome number (x) equal to 7, comprising diploid (2n=2x=14), tetraploid (2n=4x=28) and hexaploid (2n=6x=42) wheats.
Today's commercial wheat, tetraploid and hexaploid wheats, contain two and three homeologous genomes, respectively. These genomes are named A, B, D and G, according to the donor species, each of which consists of seven pairs of chromosomes numbered 1 to 7. They are products of natural hybridisation of ancestral types, none of which nowadays is still of any commercial importance. In the hybridisation process, spontaneous crosses between wild species with different chromosomes have been followed by spontaneous doubling of chromosomes to originate a fertile allopolyploid (Fig.1.1).

Diploid wheat (einkorn) comprises three species: *T. boeoticum*, *T. urartu*, and *T. monococcum*, the first two of which are wild and the last is cultivated. All contain the A genome that is shared with polyploid wheats. *Triticum monococcum* was probably domesticated from its wild progenitors in the Karacadag Mountains of south-eastern Turkey about 10,000 years ago.

Tetraploid *T. turgidum* contains the A and B genomes. There has been an ongoing controversy regarding the diploid source of the B genome, but the current consensus of opinion is that it was *Aegilops speltoides* Tausch or a close relative within the Sitopsis section of the *Aegilops* genus.

The cultivated form var. *durum* (often called *T. durum*) is widely grown in regions with a Mediterranean climate as durum or pasta wheat.
The cultivated tetraploid wheats have evolved from two wild type groups, *T. dicoccoides* (AABB) and *T. araraticum* (AAGG) (Feldman, 2001), derived from diploid progenitors.
A second tetraploid species, *T. timopheevii*, as its wild progenitor *T. araraticum* , contains the G genome, which is closely related to the genome of *Aegilops speltoides* (S genome). *T. timopheevii* is still grown to a limited extent in Armenia and Transcaucasia.
Of all cultivated tetraploid wheats, today *T. durum* types are by far the most important, even though they are only grown on about 10% of the wheat total cultivated area, the remaining 90% being dedicated to the hexaploid wheats.
Nowadays, durum wheat, that is particularly suited to the production of pasta products, but also of some types of bread in Southern of Italy and couscous in North of Africa, is getting more and more importance with the increase of pasta products demand.

The hexaploid wheats originated some 6,000-7,000 years ago by natural hybridisation of tetraploid wheat, most likely *T. dicoccum* (AABB) with the diploid wild grass *Aegilops squarrosa* (DD), also known as *T. tauschii* (Miller, 1987). The resulting hexaploid species *T. aestivum* (AABBDD), the common “bread wheat”, is the dominant species in world agriculture. Most bread wheat is the free threshing var. *aestivum*, but other free threshing types (vars. *compactum* and *sphaerococcum*) are grown in restricted areas.
The hexaploid wheat species *T. zhukovskyi,* whose genomic composition is AAAAGG, could have been originated recently by interspecific hybridization of cultivated tetraploid *T. timopheevvii* (AAGG) with the cultivated diploid *T. monococcum* (AA).

The evolution of the polyploidy complex of the genus *Triticum* and a possible classification of cultivated wheats along with its wild progenitors are shown in Fig. 1.1 and Table 1.2, respectively.

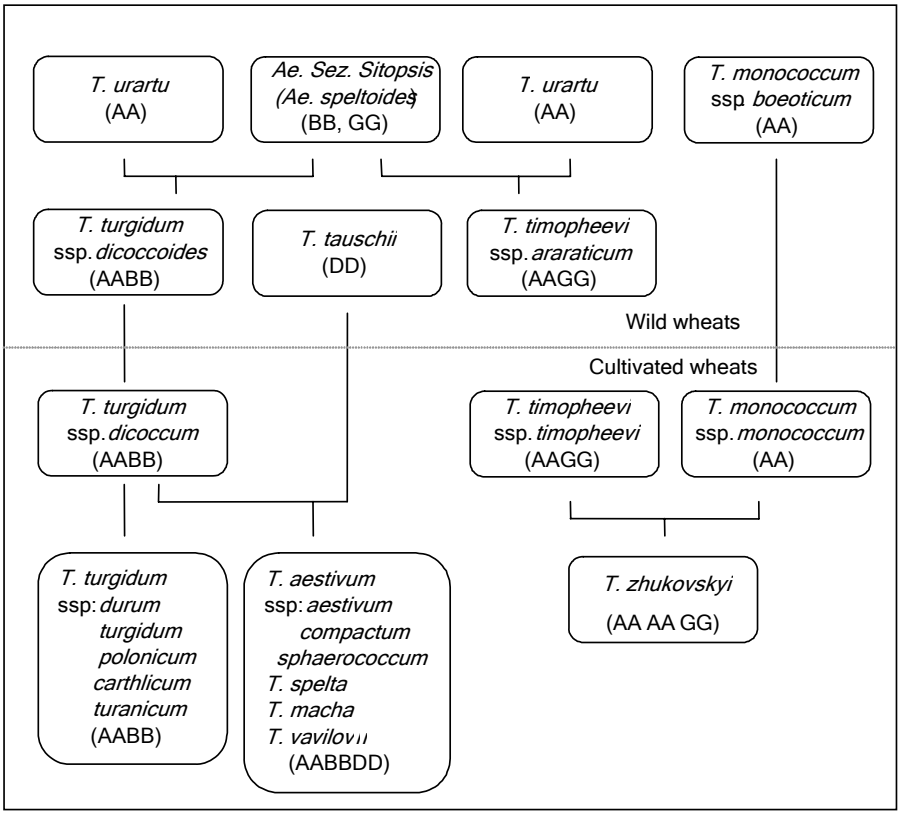


Fig. 1.1: A schematic representation of the evolution of the polyploid wheats. Letters in brackets indicate the genomic composition of the species.

Species	Genome	Wild (hulled)	Cultivated (Hulled)	Cultivated (Free-threshing)
DIPLOID (2n=14)				
Aegilops speltoides	S (G)	All		
Aegilops squarrosa	D	All		
Triticum urartu	A	All		
Triticum monococcum	A	var. boeoticum (wild einkorn)	var. monococcum (cultivated einkorn)	var. monococcum (cultivated einkorn)
TETRAPLOID (2n=28)				
Triticum timopheevii	AG	var. araraticum	var.timopheevii	var. militinae
Triticum turgidum	AB	var.dicoccoides (wild emmer)	var. dicoccum (cultivated emmer)	var. durum var. turgidum var. polonicum
HEXAPLOID (2n=42)				
Triticum aestivum	ABD		var. spelta var. macha var. vavilovii	var. aestivum var. compactum var. sphaerococcum

Tab. 1.2: Classification of cultivated wheats and related wild species
Redrawn from Feldman et al. (1995), modified.

It should be noted that the high chromosome number of hexaploid wheats definitely contributed to their wider adaptability than other species; this explains the great dispersion from South-Western Asia in various directions.

Moreover, the unique milling and baking properties of common bread wheat are not found among the diploid and tetraploid wheats. Since only the hexaploid group possesses the D set of chromosomes, the desirable quality characteristics of bread wheats have been attributed preponderantly to the presence of this third genomic component.

By crossing and selecting, man has produced numerous cultivars of hexaploid wheat; nowadays it is still the most important species grown in Europe, North America and Australia.

1.3 Wheat grain anatomy and composition

The wheat grain is botanically a single-seeded fruit, called a ‘‘caryopsis’’ or ‘‘kernel’’ (Fig. 1.2 and Fig. 1.3). It develops within floral envelopes (the ‘‘lemma’’ and ‘‘palea’’), which are actually modified leaves. At maturity, the wheat kernel averages ~2.5-3.0 mm thick (or high as it stands on its base), ~3.0-3.5 mm wide, ~6.0-7.0 mm in length, with an average weight of ~30-40 mg.

Wheat kernels are rounded on the dorsal side, with a longitudinal ‘‘crease’’ (a deep groove) running the full length of the ventral side. The shape of the groove is a characteristic feature of some species and varieties.
The wheat grain is constituted by three distinct parts: the bran, the starchy endosperm and the embryo (called “germ” by millers). These different components account for 13-17%, 80-85% and 2-3% of the dry weight, respectively.
The main inner volume of the grain is thus taken up by the starchy endosperm, which becomes the white flour that is released and crushed to fine particles by the flour miller.

1.3.1 The bran

The bran of the wheat grain is composed by a series of different cell layers. The ‘‘pericarp’’ (fruit coat) surrounds the entire seed and consists of two portions, the outer pericarp and the inner pericarp. The outer pericarp is composed by the epidermis (epicarp), the hypodermis, and by the innermost layer, called the remnants of thin-walled cells. The inner pericarp, adjacent to the remnants, is composed of intermediate cells (cross cells and tube cells). The cross cells are long and cylindrical (~125x20 μm). They are tightly packed, with little or no intercellular space. The tube cells are similar in size and shape to the cross cells, but they are not packed tightly and do not form a continuous layer; thus have many intercellular spaces. A further inner layer of cells is the seedcoat (also called “testa”) which is firmly joined to the tube cells. Grain color, usually red or white, is related to pigment in the seedcoat. Tightly bound to the internal surface of the “testa” is the nucellar epidermis (‘‘hyaline layer,’’ or ‘‘perisperm’’).
The total pericarp has been reported to comprise ~5% of the kernel volume.

1.3.2 The endosperm and the embryo

The mature wheat grain consists of two genetically different organs, the endosperm and embryo, that arise from separate fertilization events. The pollen tube delivers two sperm cells into the embryo sac, one of which fuses with the egg cell to give the diploid zygote. This gives rise in the mature grain to the embryonic axis and to a single cotyledon, called the scutellum. The second sperm cell fuses with two polar nuclei to form a triploid endosperm cell that expands, cellularizes, divides, and differentiates to give two tissues: the starchy endosperm, which comprises about 80% of the dry matter and 72% of the protein in the mature grain (Pomeranz, 1988), and the aleurone, which completely surrounds the kernel and is generally one cell thick. It is the intermediate cell layer between the nucellar epidermis and the peripheral cells of the starchy endosperm. The majority of the mineral matter located in bran is found in the aleurone layer, which also has high content of thiamine. The aleurone cells are heavy-walled, essentially cube-shaped, and free of starch. They can vary in thickness from 30-70 μm within a single kernel and have thick (6-8 μm), double-layered cellulosic walls.

	Whole Grain	Starchy Endosperm	Bran	Embryo (Germ)
Proteins	16	13	16	22
Fats	2	1.5	5	7
Carbohydrates	68	82	16	40
Dietary fibers	11	1.5	53	25
Minerals (ash)	1.8	0.5	7.2	4.5
Other components	1.2	1.5	2.8	1.5
Total	100	100	100	100

Tab. 1.3: Chemical composition of the whole wheat grain with its various parts. Numbers are average percentages converted on a dry matter basis. Redrawn from Belderok (2000).

Essentially, mature starchy endosperm comprises cells in which starch granules are embedded in a matrix of storage proteins. These two components, together with cell walls and minor constituents, accumulate as a source of nutrients for the accompanying embryo when it begins to germinate. The nutrients are made available as a result of hydrolysis by enzymes produced in the embryo itself or in the aleurone, or both. Most of the starchy endosperm consists of food reserves: 82% carbohydrates (mainly starch), 13% proteins (Wieser et al., 1998) and 1.5% fats. The content of mineral and of dietary fibres is low, 0.5% and 1.5% respectively (Pomeranz, 1988).

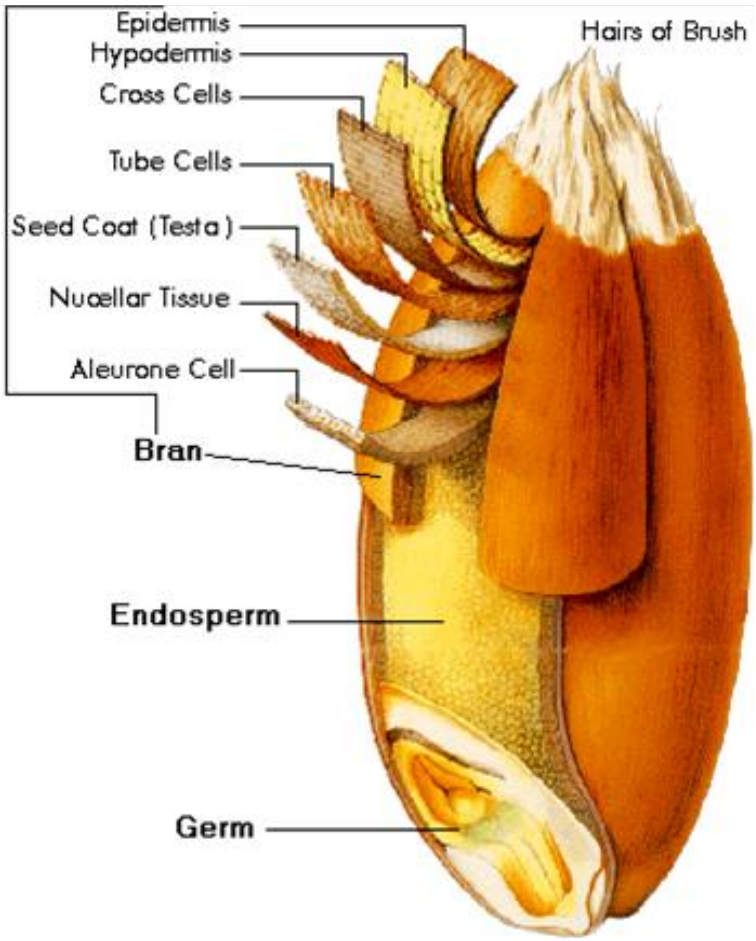


Fig. 1.2: Structure of the wheat grain.

The wheat embryo is composed of two major parts, the embryonic axis (rudimentary root and shoot) and the scutellum, which functions as a storage organ. The scutellum is adjacent to the endosperm and contains some storage compounds (notably oil and globulin storage proteins), but it does not contain gluten proteins. The germ is quite rich in vitamin E and in B-vitamins. It contains many enzymes. The germ is a rich source of protein (25%), sugar (18%), oil (16% of the embryonic axis, and 32% of the scutellum are oil). The sugars are mainly sucrose and raffinose. On incineration, the germ gives a high level of ash (5%). Recovery of the germ during the milling process is an important step because of its value in the food and pharmaceutical industries.

Fig. 1.3: Cross-section of the wheat kernel.

1.3.3. Endosperm development

The development of the wheat plant is often described using the Feekes, Zadoks or Heun scales that assign numbers to visual landmarks that are useful for crop management purposes (Chang et al., 1974; Large, 1954). These subjective scales divide grain development into early, medium and late milk stages, and early, soft and hard dough stages. The temporal pattern of grain development also can be described in terms of transition points in the accumulation of total dry matter, starch, protein, and water, in order to pinpoint times in grain development when changes in gene expression and protein accumulation are likely to occur (Altenbach et al., 2003). Endosperm development begins with fertilization of a diploid cell, followed by repeated division of the triploid nuclei, gradual formation of cell walls, and partitioning of the original vacuolated cell into a characteristic cellular pattern (Olsen, 2001; Olsen et al., 1999; Lohe and Chaudhury, 2002). Next is a period of cell expansion in which water content increases and starch and protein reserves accumulate. Endoreduplication of DNA within the nuclei continues during this phase and has been reported to influence final grain size in maize (Engelen-Eiges et al., 2000). The maximum amounts of starch and protein that accumulate in each grain depend on the number of endosperm cells, determined early in grain fill, and the final size of the cells, which is influenced by water uptake, cell-wall extensibility and rate and duration of grain fill (Egli, 1998). Cell-wall-loosening enzymes may play a role in determining the extent of cell enlargement (Chanda and Singh, 1998). In maize, endosperm expansion was also reported to be influenced by cell division in the peripheral layers (Vilhar et al., 2002).

Cell expansion and water accumulation stop before the cessation of dry matter accumulation, starch and protein replace cell water, and the kernel begins to desiccate (Berger, 1999; Lopes and Larkins, 1993; Rogers and Quatrano, 1983). Late in development, the formation of a waxy layer at the chalaza impedes input of sugars and amino acids into the grain (Sofield et al., 1977a; Cochrane et al.,1983), protein and starch deposition cease and the grain reaches maximum dry weight or physiological maturity. At approximately this time the endosperm tissue undergoes a form of apoptosis, or programmed cell death, similar to that observed in other plant and animal tissues (Beltrano et al., 1994; Young and Gallie, 1999; Young and Gallie, 2000). Cell death, visualized by viability staining, progresses gradually throughout the endosperm tissue and is accompanied by internucleosomal fragmentation of genomic DNA. Only the aleurone cells remain viable. Finally, kernels desiccate rapidly, losing all but 10–15% of their water content, at which time they are ready for harvest.

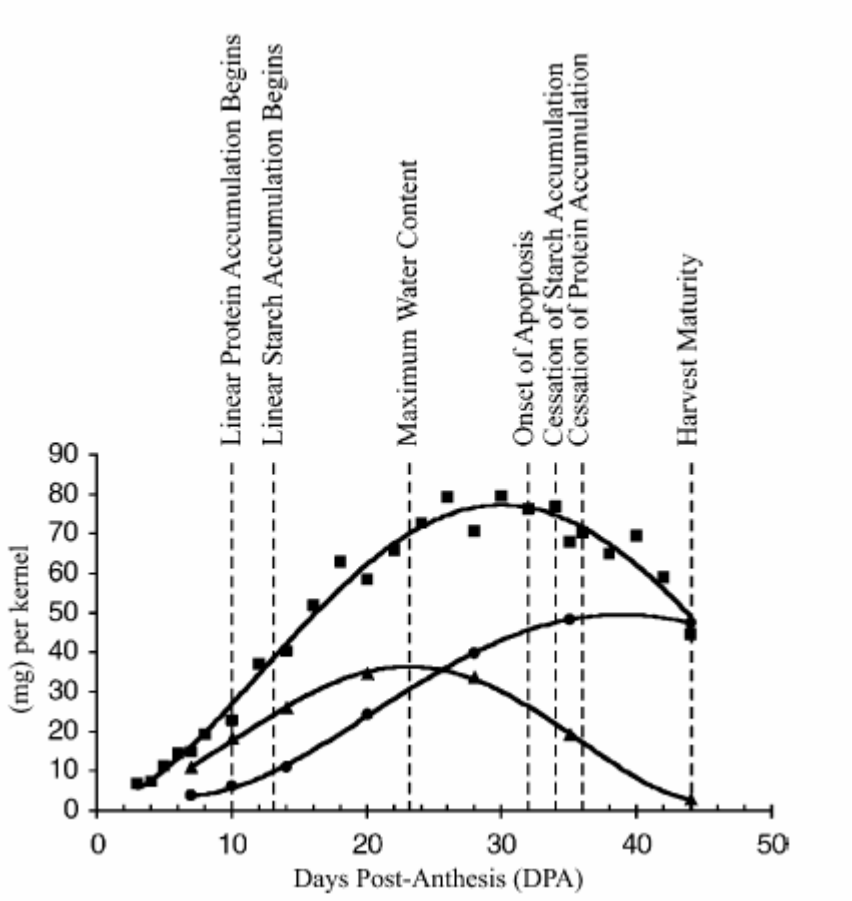


Fig. 1.4: Temporal patterns in the accumulation of starch and protein described by the water content (►), fresh weight (■) and dry weight (●) during the grain development. Proteins were measured by a nitrogen combustion analysis, starch was measured by a specific assay kit, and the onset of apoptosis was estimated by the analysis of fragmented genomic DNA. Plants were grown under a 24°C/17°C day/night regimen. Taken from DuPont (2003), based on data from Altenbach (2003).

1.3.4 Starch

Starch is the major component of the endosperm of the cereal grains. It is the stored form of energy that is released on germination when amylase enzymes (both synthesized and deposited during the period of grain filling or *de novo* synthesized upon germination) break the starch down to glucose units for the developing embryo, roots, and shoots. For feed and food, starch also provides the major source of energy, providing it in a ''slow-release'' form that is well suited to our digestive systems.

Wheat starch is composed only of glucose units; the glucose units are linked α-1,4 to form linear chains and branches are formed through the connection of α-1,4 linked chains via α-1,6 linkages.

Starch is generally described as containing two broad classes of molecules, amylose and amylopectin, that differ in degree of polymerisation and branch frequency. Amylopectin is a very large molecule with a degree of polymerisation from 10^5 to 10^7 and contains frequent branch points, on average approximately one branch for every 15–20 glucose units.

Amylose has a lower degree of polymerisation (10^3 to 10^4) and contains from zero to a few branch points. These differences in amylose and amylopectin are functionally important and are reflected in the variety of applications these polymers find in the food and chemical industries. In hexaploid and durum wheats, amylose content ranges from about 18 to 35%, although waxy wheats containing effectively zero amylose have now been produced.

Starch is deposited in granules in the wheat endosperm in amyloplasts, specialised starch biosynthetic organelles derived from the same proplastids as chloroplasts, but containing no photosynthetic apparatus. The precise molecular events that occur at the initiation of the starch granule remain obscure. In wheat, granule initiation occurs in two phases, in the period 3–7 days after anthesis, during which time the large (25-40 μm) “A” granules are initiated (Briarty et al., 1979). Granule initiation then appears to cease until mid endosperm development, when a second, much more prolific, period of granule initiation occurs leading to the development of the small (5-10 μm) “B” granule populations. A third burst of “C” granule initiation has also been observed in wheat. A-granules develop a bulbous protuberance that develops into an apposition plate that progressively extends around the granule, eventually encircling the granule. The deposition on the faces of the equatorial plate appears to proceed in a diurnal manner, producing the characteristic alternating layers of starch.

The B-granules were seen in lateral evaginations of the amyloplast membrane, and multiple small B-granules were seen in a single evagination. B granules remain spherical and do not proceed through the equatorial plate formation pathway typical for the A-granule. There are marked differences in the patterns of starch deposition in different cereals that result in different starch granule size distributions and morphologies.

In addition to starch, the starch granule contains two other important components. Firstly, the interior of the starch granule contains a range of starch biosynthetic enzymes that account for about 0,5% of the mass of the granule (Denyer et al., 1995; Rahman et al., 1995). Secondly, the granule contains lipids, complexed within the amylose fraction (Morrison and Gadan, 1987).

These lipids are thought to exert important effects on the interactions of the granule with water during gelatinisation and swelling (Morrison et al., 1993). The properties and functionality of wheat starch are controlled not only by the nature and composition of the starch granule, but are also strongly influenced by the nature of the endosperm material in which the granule is embedded in the desiccated grain. The hardness of the grain controls the manner in which the endosperm and starch granule is fractured during the milling process, leading to important effects on processing performance.
In summary the key features of the deposition of starch in the wheat endosperm that control functionality are starch content, grain hardness, granule size distribution and shape, the presence of endogenous lipids in the granule, amylopectin structure, and the ratio of amylose to amylopectin. These differences in starch deposition define the ways in which starch responds to heat and water during the utilisation of starch in the complex foods prepared from cereal flours. Each of these features may be amenable to modification by molecular/genetic changes in genomic DNA.

1.3.5 Seed storage proteins

Wheat grains contain relatively little protein compared to legume seeds, with an average of about 10–12% dry weight.
Nevertheless, they provide over 200 mt of protein for the nutrition of humans and livestock, which is about three times the amount derived from the more protein-rich (20–40%) legume seeds. In addition to their nutritional importance, cereal seed proteins also influence the utilization of the grain in food processing. In particular, the unique property of wheat flour to form an extensible and elastic dough reside primarily in the storage proteins of its endosperm.

The scientific study of cereal grain proteins extends back to 1745, with the pioneering work of Giacomo Beccari, professor of Chemistry at the University of Bologna. He was the first one to describe and call “gluten” that cohesive protein mass obtained after washing wheat flour with water (Beccari, 1745). Since then more systematic studies have been carried out, notably by Osborne (1859–1929). Osborne developed a classification of wheat seed proteins based on their solubility in a series of solvents, for example, albumins in water, globulins in dilute saline, prolamins, soluble in alcohol-water mixtures and glutelins, soluble in dilute acid or basic solutions.

1.3.5.1 Storage albumins and globulins

In classifications of storage proteins based on solubility, those that are soluble in water and in salt solutions are called albumins and globulins, respectively.
Many water or salt-soluble wheat proteins are located in the embryo and aleurone layers; others are distributed throughout the endosperm. They may amount to about 20% of the total proteins in the caryopsis. Albumins are usually more prevalent than globulins.

Payne and Rhodes (1982) noted that soluble proteins are complex mixtures containing: 1) metabolic enzymes that survived dehydration, 2) hydrolytic enzymes necessary for germination, 3) enzyme inhibitors.
Also present are proteins related to legumins (the seed storage proteins of legumes), called "triticins", which were extensively studied by Singh and Shepherd (1985). They account for about 5 % of the total seed proteins and are located in the protein bodies of the starchy endosperm. When considering their solubility properties, they behave as globulins, but no important link with pasta or breadmaking quality has been established.

The storage globulins of maize and wheat have been characterized in some detail (Kriz, 1999 and Singh et al., 2001). They are readily soluble in dilute salt solution and have sedimentation coefficients of about 7. They have limited sequence similarity with, and may be homologous to, the 7S vicilins of legumes and other dicotyledonous plants; they also have similar structures and properties (Kriz, 1999).
The 7S globulins are stored in protein bodies and appear to function solely as storage proteins. However, they do not appear to be absolutely required for normal seed function, at least in maize, where a null mutant behaves normally in terms of development and germination (Kriz and Wallace, 1991). Furthermore, although the aleurone and embryo are rich in proteins compared with the starchy endosperm, the globulins in these tissues have limited impact on the end use properties of the grain. In wheat, the aleurone and embryo account only for about 10% of the grain dry weight and are usually removed by milling before human consumption.

Genes for the major albumins and globulins of wheat have been assigned to chromosome groups 3, 4, 5, 6, and 7 (Garcia Olmedo et al., 1982). A major component of the albumins of low molecular weight (14,000–16,000 Da) is encoded by the short arm of chromosome 3D, as suggested by its absence in the water extracts of aneuploid strains nullisomic-3D, tetrasomic-3B, and ditelosomic-3DL of Chinese Spring (Pogna et al., 1991). In amino acid composition, albumins and globulins differ from the gluten proteins in having lower amounts of glutamic acid and more lysine. In fact, due to the lysine, this family of proteins has an amino acid composition that fits the dietary requirements of humans and monogastric animals. Unfortunately, because they are present in the wheat endosperm in minor proportions, their presence it is not enough to overcome the lack of lysine in wheat flour.

1.3.5.2 Gluten proteins

Gluten proteins may amount to about 80% of the total proteins in the caryopsis.
Gluten is classically divided into alcohol-soluble (gliadin) and insoluble (glutenin) fractions, which are further separated by electrophoresis.
The gliadins consist of monomeric proteins, which are separated into α, β, γ, and ω groups by polyacrylamide electrophoresis at low pH (Woychik et al., 1961). The glutenins consist of polymeric proteins stabilized by inter- and intrachain disulfide bonds.

These bonds need to be reduced before the component subunits can be separated into two groups, high molecular weight (HMW) and low molecular weight (LMW) subunits, with the latter being further divided into B-, C-, and D-type subunits according to size, isoelectric points, and composition (D'Ovidio and Masci, 2004).

It was long considered that the gliadin and glutenin fractions comprised different types of proteins that corresponded to the prolamins and glutelins, respectively, as defined by Osborne. However, a range of biochemical and molecular studies carried out over the past two decades have demonstrated that this is not the case and we now know that all gluten proteins are structurally and evolutionarily related and they can all be defined as "prolamins" (for the high content of the amino acids proline and glutamine) in that they are soluble in alcohol–water mixtures either as protein monomers (gliadins) or as reduced subunits (glutenins). Furthermore, it is possible to define three groups of prolamins, which contain gliadin and/or glutenin proteins. These are: 1) the high molecular weight prolamins, which comprise only the HMW subunits of glutenin polymers; 2) the sulfur-poor (S-poor) prolamins, which comprise ω-gliadins and D-type LMW subunits of glutenin polymers, and 3) the S-rich prolamins, which comprise α and β-type gliadins, γ-type gliadins, and the B- and C-type LMW subunits of glutenin polymers. Furthermore, the C and D groups of LMW subunits are highly similar in sequence to individual α-type/γ-type and ω-gliadins, respectively, and are considered to be derived from these components by mutations resulting in the presence of additional cysteine residues, which are able to form interchain disulfide bonds. In contrast, the B-type LMW subunits form a discrete group and no closely related gliadin components have been identified (D'Ovidio and Masci, 2004).

The main distinction between the gliadin and glutenin proteins is that the former are monomeric and the latter polymeric. Nevertheless, this classification has been retained by cereal chemists mainly because it has functional significance, with the glutenins being primarily responsible for the elasticity (strength) of the gluten and the gliadins for viscosity.

1.3.5.2.1 Gliadins

Gliadins are heterogeneous mixtures of single-chained polypeptides which are, in their native state, soluble in 70% aqueous alcohol. In accordance with their mobility in A-PAGE (acid-PAGE), they are divided into four groups: α- (fastest mobility), β-, γ-, and ω-gliadins (slowest mobility) (Woychik et al., 1961).

However, according to the amino acid analyses and N-terminal sequences, Kasarda et al. (1983) suggested that the gliadins can be arranged into three major groups of α/β-, γ-, and ω-gliadins.

The molecular weight range is ≈30,000 to 75,000 Da. Using one-dimensional electrophoresis, gliadins of a single wheat grain can be separated into 20–25 components (Bushuk and Zillman 1978; Autran et al 1979; Wrigley et al 1982; Metakovsky et al 1984). Two-dimensional electrophoresis allows better separation with a resolution of up to 50 components (Wrigley 1970; Payne et al 1982; Lafiandra and Kasarda 1985; Pogna et al 1990). Due to extensive

polymorphism, these proteins have been widely used for cultivar identification in hexaploid and tetraploid wheats.

The γ-gliadins differ from α- and β-gliadins in the amount of aspartic acid, proline, methionine, tyrosine, phenilalanine, and tryptophan (Bietz et al 1977). The ω-gliadins differ in amino acid composition from other gliadins and do not have cysteine. The ω-gliadins are characterized by high levels of glutamine (+glutamate) (40–50 mol%), proline (20–30 mol%), and phenylalanine (7–9 mol%), which represent >80% of the total amino acid residues (Tatham and Shewry 1995). All gliadins are low in the ionic amino acids (histidine, arginine, lysine, and free carboxylic groups of aspartic acid and glutamic acid). Glutamic and aspartic acids exist almost entirely as amides. Also, gliadins can be classified according to their N-terminal amino acid sequence.

Genetics and polymorphism

The first reports on the chromosomal location of gluten protein genes date back to the late 1960s when Boyd and Lee (1967) and Shepherd (1968), using starch gel electrophoresis, analyzed the compensating nullisomic–tetrasomic and ditelosomic series developed by Sears (1954, 1966) in the bread wheat cv. Chinese Spring. These authors reported that gliadin proteins are controlled by genes present on the short arm of the homoeologous group 1 and 6 chromosomes. The poor resolution of the one-dimensional separations and the overlapping of many gliadin components meant that it was possible to assign only a few of them to specific chromosomes.

The development of more refined two-dimensional electrophoretic techniques, in which starch was replaced by polyacrylamide gels, resulted in increases in the resolution and number of components detected, but essentially confirmed the chromosomal assignments of gliadin coding-genes in Chinese Spring as well as in different bread wheat cultivars (Brown et al., 1981b; Payne et al., 1982; Lafiandra et al., 1984). Similar studies of durum wheat (Joppa et al., 1983; Lafiandra et al., 1987) and wild relatives of wheat have confirmed these results (Lafiandra et al., 1993a).

Extensive studies of the inheritance of gliadins in the progeny of specific crosses have indicated that the major gliadin genes occur in tightly linked clusters, termed blocks, with intrablock recombination being rare (Sozinov and Poperelya, 1980). It is now generally accepted that the ω- and γ-gliadins are controlled by clusters of tightly linked genes present at the *Gli-1* loci (*Gli-A1, Gli-B1,* and *Gli-D1*) on the short arms of the homoeologous group 1 chromosomes, whereas the α- and β-gliadins are controlled by the *Gli-2* loci (*Gli-A2, Gli-B2*, and *Gli-D2*) present on the short arms of the group 6 chromosomes (Payne, 1987). This spatial separation of gliadin genes on the group 1 and 6 chromosomes has been attributed to an ancient interchromosomal translocation, with the *Gli-2* locus originating from the translocation of a γ-type gene from chromosome 1 to chromosome 6, followed by divergence of the coding sequence to give rise to the α-type sequence (Shewry et al., 1984b). The *Gli-1* loci have been shown to be present on the distal parts of the group 1 chromosomes, showing independent or loose linkage with their

respective centromeres (Shepherd, 1988). The *Gli-2* loci have been studied in less detail, but telocentric mapping showed 35% recombination between the *Gli-A2* locus and the centromere (Payne, 1987). The individual *Gli-1* and *Gli-2* loci exhibit extensive polymorphism, as detected by electrophoretic techniques, with allelic blocks differing in the numbers, proportions, and mobilities of different components. This results in a great diversity of gliadin patterns, providing the basis for distinguishing different wheat cultivars (Metakovsky, 1991).

Amino acid sequences and structure of ω-gliadins

ω-gliadins have a very high level of glutamine and proline with a low level of sulfur amino acids. Their methionine level may be <0.1% and they are totally lacking in cysteine and are not able to produce -S-S- type bonding. Also, they have few basic amino acids and a higher level of phenylalanine in comparison with the other gliadin groups (Kasarda et al 1983; Tatham and Shewry 1995). Nevertheless, their surface hydrophobicity is lower than that of the α- and γ-type gliadins. They are the first peptides to elute from the RP-HPLC column (Popineau and Pineau 1987). They are also the most hydrophilic of the gluten proteins in terms of total amino acid composition with only a few residues with charged side chains (DuPont et al. 2000).
On the basis of the N-terminal sequences, three different types of ω-gliadins have been observed in wheat and in related proteins such as C-hordeins and ω-secalins. These sequences are named ARQ-, KEL-, and SRL-types on the basis of the first three amino acids of their N-terminal sequences (Kasarda et al 1983; Tatham and Shewry 1995). According to previous reports by Kasarda et al (1983) and Tatham and Shewry (1995), the ARQ-type is thought to be the ancestral sequence type. The KEL-type differs from the ARQ-type in its lack of the first eight residues and also with respect to a highly conserved sequence in the first 10 positions. The third type of ω- gliadin N-terminal sequence is the SRL-type, which is characteristic of ω-gliadins encoded by chromosome 1B (Tatham and Shewry 1995; DuPont et al 2000). The primary sequences of C-hordeins (Tatham et al 1989) and ω-gliadins from the 1D chromosome (Hsia and Anderson 2001) are based on an octapeptide repeat motif made almost entirely from glutamine and proline residues in the sequence PQQPFPQQ. It appears likely that this repeat motif is related, from an evolutionary point of view, to the repeats present in the S-rich prolamins. However, ω-gliadins encoded at chromosome 1B are characterized by different internal peptides rich in glutamine such as QQXP, QQQXP, and QQQQXP, where X is F, I, or L in order of predominance (DuPont et al. 2000).

The structural studies of C-hordeins (Tatham et al 1985b; Field et al 1986), which are related to the gliadins, served as a model for understanding the structure of the S-poor prolamins (Tatham and Shewry 1995). The ω-gliadins are homologous to rye ω-secalins and barley C-hordeins (Tatham and Shewry 1995). The DNA sequences of two full-length wheat ω-gliadin genes (ωF20b and ωG3) containing significant 5' and 3' flanking DNA sequence have been completed (Hsia and Anderson, 2001). The general protein structure is consistent in 19 amino acid residues corresponding to a signal peptide followed by 10–11 amino acid residues from the non-

repetitive N-terminal region, then a repetitive region encompassing 90–96% of the protein and a C-terminal region with 10–11 amino acid residues (Hsia and Anderson, 2001).
The ω-gliadins do not have a compact structure, and circular dichroism spectra indicate β-turns and only low levels of α-helices and β- sheets (Tatham and Shewry 1985).

Amino acid sequences and structure of α/β- and γ-gliadins

α/β- and γ-gliadins are also characterized by high levels of glutamine and proline, where ≈90% of the glutamic and aspartic acid residues are amidated (Bietz et al 1977; Ewart 1983; Kasarda et al 1983). They are relatively high in leucine and low in basic amino acid.
The α/β- and γ-gliadins are rich in sulfur with six and eight cysteine residues, respectively. As a result, three and four intramolecular disulfide bonds are formed (Kasarda et al 1984; Köhler et al 1993; Müller and Wieser 1995, 1997).
On the basis of N-terminal sequences, the α/β-gliadins are represented by a very small sequence of five amino acid residues (VRVPV) (Bietz et al 1977). Peptide motifs based on the pentapeptides PQQQP and PQQPY are always present in a repetitive region that follows the N-terminal region of the α/β-gliadin proteins (Shewry et al 1986). The N-terminal region of the γ-gliadins is formed by 12 amino acid residues (NMQVDPSGQVQW) that precedes a series of repeats based on the consensus motif PQQPFPQ (Autran et al 1979; Kasarda et al 1983; Shewry and Tatham 1990).

The complete amino acid sequence from a number of α/β-gliadins (Kasarda et al. 1984) and γ-gliadins (Sugiyama et al 1986; Rafalski 1986) comes from the analysis of cDNA and genomic DNA sequences. The sequences show that the primary structure of these proteins is divided into several domains of variable size, a trait common to all prolamins. The N-terminal is a short domain with 5–14 amino acid residues. The central repetitive domain has up to 100 residues organized as repeat sequences of one or two motifs composed of glutamine, proline, and hydrophobic amino acids (phenylalanine or tyrosine). Finally, in the C-terminus non-repetitive domain, there is a succession of segments of polyglutamine and unique sequences particularly rich in lysine and arginine that contain all the sulfur amino acids (Thompson et al 1994; Müller and Wieser 1997). In their structure, α-gliadins have six cysteine residues, while γ-gliadins have eight.
They form three and four disulfide bonds, respectively. There are no free cysteines, and all S-S linkages are intramolecular, preventing gliadins from participating in the polymeric structure of glutenin. In γ-gliadins, the repetitive domain is rich in β-reverse turns and may form an extended structure. The non-repetitive domain is rich in α-helices (Tatham et al 1990b). This feature is also present in α-gliadins, but the β-turns have an irregular distribution when compared with γ-type gliadins (Tatham et al 1987, 1990c).

Relationship of gliadins to wheat quality

Sozinov and Poperelya (1980, 1982) analyzed progeny from many different crosses to demonstrate that allelic variation at the *Gli-1* and *Gli-2* loci was associated with variation in breadmaking properties, and also established a ranking order of the different blocks of gliadin components. The contributions of different gliadin components to variation in breadmaking performance has also been reported by others, including Pogna et al. (1982), Wrigley et al. (1982), Branlard and Dardevet (1985a), and Metakovsky et al. (1997b,c), with allelic variation present at the *Gli-1* loci being more effective in influencing breadmaking properties compared with the *Gli-2* loci. In most of these studies correlations between gluten components and breadmaking properties of flour have been assessed by the SDS sedimentation test, an indirect test in which good breadmaking performance is associated with the formation of a large sediment when the flour is suspended in water containing SDS and lactic acid.

Studies by Moonen et al. (1982) and Payne et al. (1987b) have demonstrated that large sedimentation volumes result from the formation of an extensive gel, comprised exclusively of the larger and more insoluble glutenin polymers, leading to the conclusion, supported by genetic studies (Pogna et al., 1988, 1990), that the superior quality associated with gliadins present at the *Gli-1* loci is the result of linkage with genes encoding LMW subunits at the *Glu-3* loci. Similarly, reports that differences in dough strength were correlated with the presence of certain *Gli-2* alleles (Metakovsky et al., 1997b; Branlard et al., 2001) could be related to the association with C-type LMW glutenin subunits, which have been reported to be closely linked to gliadin components encoded by genes on the group 6 chromosomes (Masci et al., 2002).

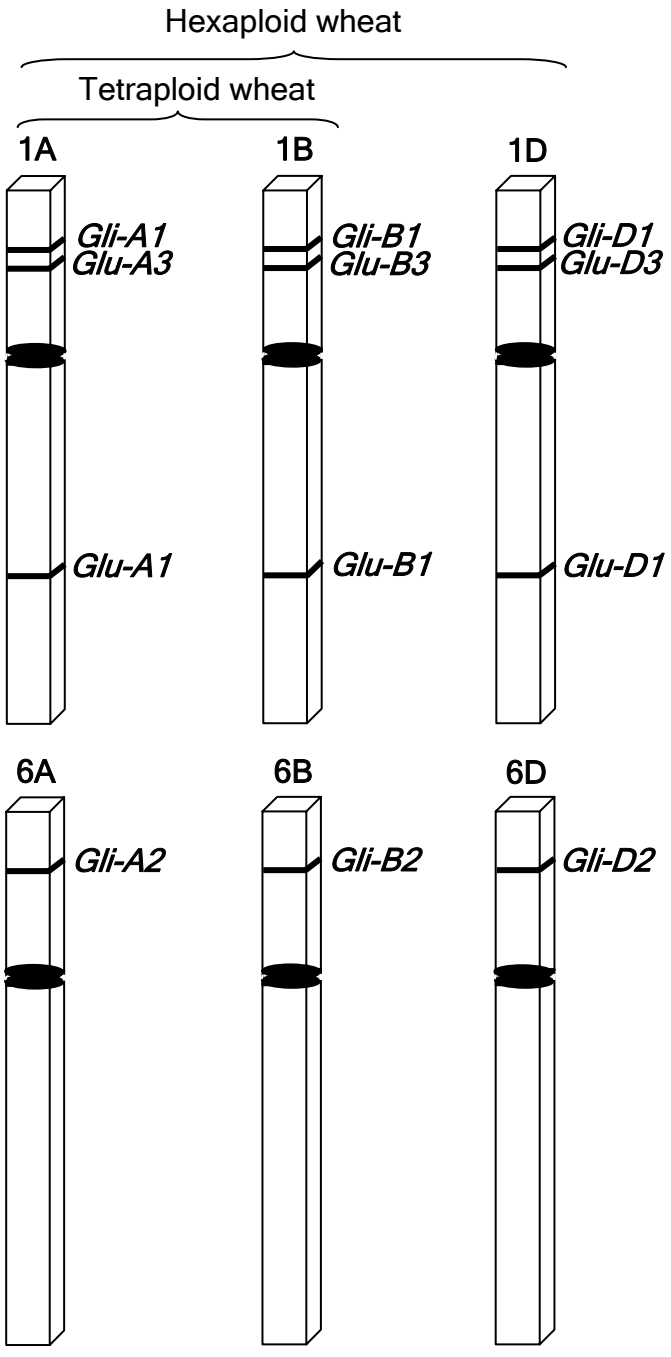
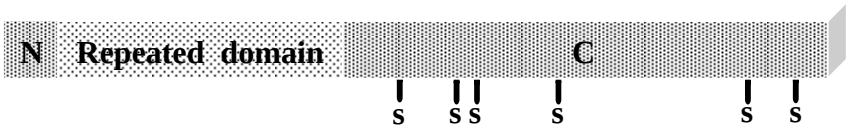
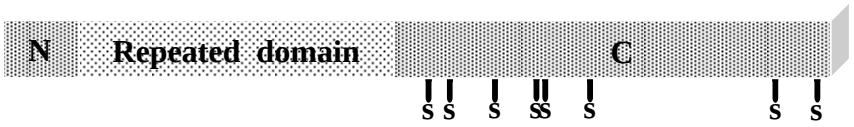


Fig. 1.5: Schematic representation of the chromosomal locations for the genes encoding the gluten proteins of tetraploid and hexaploid wheats.

α/β-gliadin



γ-gliadin



ω-gliadin

Fig. 1.6: Schematic illustration of α/β-, γ-, and ω-gliadins. Black bars with letter s indicate the approximate position of cysteine residues, that are all involved in intramolecular disulphide bonds.

1.3.5.2.2 Glutenins (glutenin polymers)

Based on gel filtration (Huebner and Wall 1976; Bietz and Simpson 1992) and flow field-flow fractionation (FFF) studies (Wahlund et al 1996; Stevenson and Preston 1996), the molecular weight of glutenin polymers reach over twenty millions daltons. They are the largest protein molecules in nature (Wrigley 1996).

These proteins are heterogeneous mixtures of polymers formed by disulfide-bonded linkages of polypeptides that can be classified in four groups according to their electrophoretic mobility in SDS-PAGE after reduction of disulfide bonds (the A-, B-, C- and D-regions of electrophoretic mobility). The A-group (with an apparent molecular weight range of 80,000–120,000 Da) corresponds to the HMW-GS (Payne and Corfield, 1979). The B-group (42,000–51,000 Da) and C group (30,000–40,000 Da) are LMW-GS distantly related to γ- and α-gliadins (Payne and Corfield 1979; Payne et al 1985; Thompson et al 1994). Finally, the D-group, also belonging to the LMW-GS group, is highly acidic and related to ω-gliadins (Jackson et al 1983; Masci et al 1993).

1.3.5.2.2.1 HMW-GS (High Molecular Weight – Glutenin Subunits)

The HMW-GS are minor components in terms of quantity, but they are key factors in the process of breadmaking because they are major determinants of gluten elasticity (Tatham et al 1985a) to the extent that they appear to promote the formation of larger glutenin polymers.
The apparent molecular weights of HMW-GS estimated by SDS-PAGE are ≈80,000–130,000 Da. However, true estimates calculated from derived amino acid sequences indicate lower molecular weights (60,000–90,000 Da) (Anderson et al 1988, 1989; Anderson and Green 1989).

Genetics and polymorphism

Orth and Bushuk (1974) and Bietz et al. (1975), using the Chinese Spring aneuploids line, located the HMW subunit genes on the long arms of the homoeologous group 1 chromosome. Subsequently, the study of different sets of intervarietal chromosome substitution lines, using discontinuous SDS-PAGE systems, allowed the results obtained in Chinese Spring to be extended to other bread wheat cultivars (Lawrence and Shepherd, 1980; Payne et al., 1980; Galili and Feldman, 1985). These results provided the evidence that in bread wheat the HMW subunits are encoded by genes at complex loci, designated *Glu-1*, present on the long arm of the homoeologous group 1 chromosomes *(Glu-A1, Glu-B1, Glu-D1*). Each locus contains two tightly linked genes (Harberd et al., 1986) encoding subunits designated as x- and y-type based on their molecular weights and biochemical characteristics (Payne et al., 1981a). The x-type subunits generally have a slower electrophoretic mobility in SDS-PAGE and higher molecular weight than the y-type subunits.
However, because of the silencing of some genes, only three to five HMW subunit genes are expressed in different bread wheat cultivars. In particular, two subunits are always expressed by the *Glu-D1* locus, two or one by the *Glu-B1* locus and one or none by the *Glu-A1* locus. When only one subunit is expressed by the *Glu-B1* or *Glu-A1* loci, this is always the x-type subunit. A similar situation occurs at the *Glu-A1* and *Glu-B1* loci in durum wheat. However, the y-type gene present at the *Glu-A1* locus may be expressed in cultivated and wild diploid wheats (*T. monococcum* subsp. *monococcum*, subsp. *boeoticum*, and *T. urartu*), in the wild tetraploid wheat *T. turgidum* subsp. *dicoccoides* (Waines and Payne, 1987; Levy et al., 1988), and also in cultivated and wild forms of tetraploid wheats with the genomic formula AAGG *(T. timopheevii* subsp. *timopheevii* and subsp. *araraticum*) (Margiotta et al., 1998).
Allelic variation has also been reported in the subunits encoded by each *Glu-1* locus in bread wheat cultivars (Lawrence and Shepherd, 1980).
Payne and Lawrence (1983) developed a numbering system to identify different HMW-GS which also provides a chromosomal location of the genes and is the system in current use. Originally, the assignment of ascending numbers was related to the mobility in SDS-PAGE, lower numbers equating to lower mobility. As new subunits have been identified, there has been difficulty in following this logical order. Thus, there are some subunits, such as 20, with lower mobility and

higher number than the original subunits. When identifying subunits numerically, it is customary to include both the genome from which the subunit is derived and the indication of whether it is an x-type or y-type subunit (e.g., Dx5, By9).

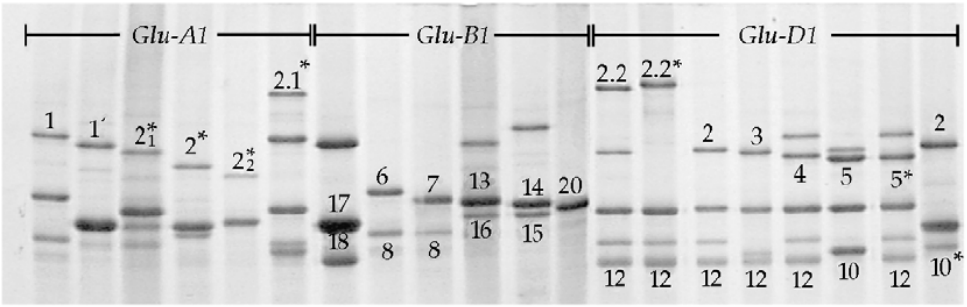


Fig. 1.7: SDS-PAGE of allelic HMW-GS present at the three *Glu-1* loci. Taken from Shewry et al. (2003).

Analyses of large collections of bread wheat landraces have identified rare alleles at the different *Glu-1* loci. Payne et al. (1983) and Lafiandra et al. (1997) have described the identification of subunits with high molecular weights encoded by the *Glu-D1* and *Glu-A1* loci. Null forms lacking x- and/or y-type subunits have also been detected (Bietz et al., 1975; Payne et al. 1984a; Lafiandra et al., 1988). Combination of the different null *Glu-1* alleles has resulted in the production of genotypes with unusual HMW subunit

Compositions. For example, Lawrence et al. (1988) have developed a set of bread wheat lines in which the number of subunits increased progressively from zero to five while Lafiandra et al. (2000b) have produced wheat lines with single x- or y-type subunits. This material has proved useful in determining the relative effects of individual HMW subunits on flour breadmaking properties and provided the possibility of developing wheats suitable for different end uses (Lawrence et al., 1988; Payne and Seekings, 1996; Lafiandra et al., 2000a).

Although the genes encoding x- and y-type subunits are tightly linked, rare cases of recombination have been reported to occur (Payne, 1987; Singh and Shepherd, 1988b). The *Glu-1* loci have been mapped relative to the centromeres by telocentric and translocation mapping, with the latter giving larger map distances (28–34 cM) than the former (7.6–10.1 cM). The identification of two chromosome mutants lacking about half of the long arms of chromosome 1B and 1D and also lacking the corresponding *Glu-B1*- and *Glu-D1*-encoded HMW subunits led Payne (1987) to conclude that the *Glu-1* loci are physically located on the distal halves of the chromosome arms. This was subsequently confirmed by Curtis and Lukaszewski (1991), who used C-banding patterns to study the distribution of recombination along chromosome 1B.

Amino acid sequences and structures of the HMW-GS

The availability of cloned cDNA and genomic sequences coding for HMW-GS (Forde J. et al 1985; Halford et al 1987, 1992; Anderson et al 1988, 1989, 1991; Anderson and Green 1989; D'Ovidio et al 1994, 1995b, 1996, 1997a) has allowed the complete amino acid sequences to be

deduced, providing a basis for gene structural analysis, biochemical modelling and biophysical studies. Such molecular and biophysical studies give us today a detailed picture of the HMW-GS structure (Shewry et al 1989, 1992, 1997).

HMW-GS have high content of glutamic acid (mostly as the amidated form glutamine), proline, glycine and low contents of lysine. Structural features include a central repetitive domain (composed of short amino acid motifs that constitute ≤85% of the protein sequence), and two non-repetitive terminal domains that contain the majority of the cysteine residues. These domains presumably form the molecular basis of the role of the HMW-GS in gluten functionality.

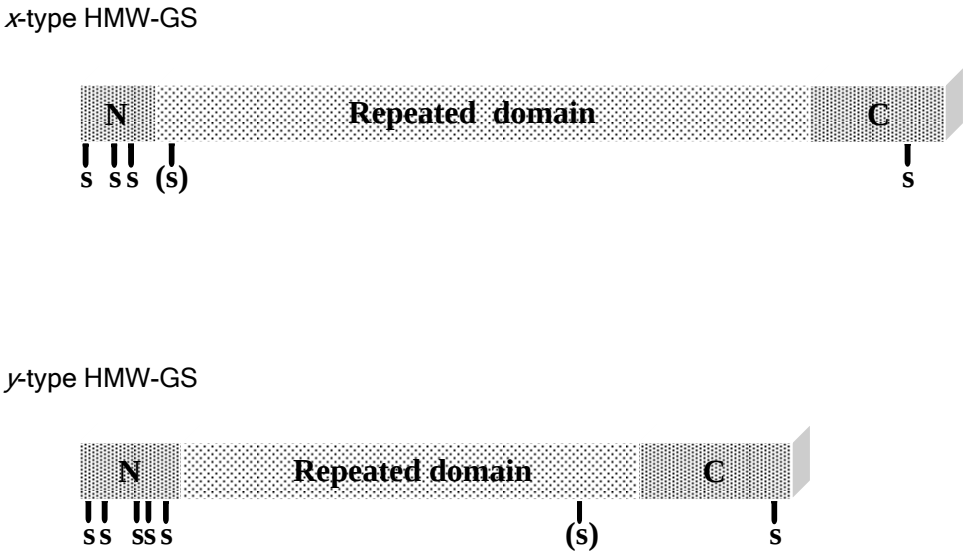


Fig. 1.8: Structure of the *x*- and *y*-type HMW-GS. Black bars with letter s represent cysteine residues which may be involved in either an inter- or intramolecular disulphide bond. (s) indicates the cysteine residues not found in some *x*- and *y*-type HMW-GS.

The N-terminal region has a non-repetitive, conservative sequence of 81-140 residues with three to five cysteine residues. Cysteine residues provide intermolecular disulfide bonds between HMW-GS and LMW-GS to form protein polymers with a range of different sizes that could reach up to tens of millions of daltons (Shewry et al 1992; MacRitchie 1992; Wrigley 1996). In the first 16 amino acids residues, it is possible to distinguish only small differences such as the sixth residue that could be E (glutamic acid) in Dx-type glutenin subunits or R (arginine) in Dy-type subunits (EGEAS-QLQCERELQE). At position 10 in all HMW-GS, there is a C residue (cysteine). Some differences have been observed for the By7 subunit at positions 12 and 14 (Shewry et al 1984; Anderson et al 1991). The C-terminus is a non repetitive domain consisting of 42 residues that include one residue of cysteine.

The amino acid composition of HMW-GS has indicated the hydrophilic nature of the central repetitive domain and the hydrophobic characteristics of the N- and C-terminal domains (Shewry et al 1989). The proportion of the different amino acids is mainly defined by sequences of repeated polypeptide motifs. The sequences PGQGQQ, GYYPTSPQQ form >90% of the

repetitive domain (Anderson and Greene 1989; Shewry et al 1992, 1997). The repetitive domains of x-type also have a tri-peptide motif (GQQ). The central domains of y-type HMW-GS often have the second proline in the GYYPTSPQQ repeat motif replaced by a leucine. Both x- and y-type repetitive domains are predicted to adopt a β-turn conformation (Tatham et al 1990a). Miles et al. (1991) suggested that the β-turns may be organized in a β-spiral structure, ranging from 490 to 700 residues, rich in glutamine, proline, glycine and poor in sulphur (0 or 1 cysteine). Miles et al. (1991) and Shewry et al. (1992, 1997) also proposed an α-helical arrangement of the amino acids for both N- and C-terminal regions. Belton et al. (1994, 1999) noted that the high level of glutamine residues in the central repetitive domain has a very high capacity to form both intra- and intermolecular hydrogen bonds and this feature could therefore be involved in elasticity through formation of intermolecular hydrogen bonds. In the dough, some of these bonds break on stretching, giving rise to unbonded mobile regions (loops) and bonded regions (trains). Thus, the loops can be stretched and then reform when the stress is removed, which accounts for the elastic restoring force of the dough, as in rubber elasticity.

It has been proposed that the length variation observed for the HMW-GS encoded at the *Glu-1* locus is mainly due to variations in the length of the central repetitive domain (Halford et al 1987; Anderson et al 1988, 1989; Shewry et al 1989, 1992; D'Ovidio et al 1994, 1995b). This hypothesis has been confirmed by PCR analyses using primers specific for the N-terminal, C-terminal, and repetitive regions of HMW-GS genes at the *Glu-D1* locus in hexaploid wheats (D'Ovidio et al 1995b). Other authors (Payne et al 1983; Shewry et al 1989; D'Ovidio et al 1996) point out that the most likely mechanism giving rise to variation in size of glutenin subunit is an unequal crossing-over event. This is a relatively common process among genes belonging to multigene families in eukaryotes (Baltimore, 1981). Unequal crossing-over could also produce very long genes (as a result of insertion of several blocks of repetitive motifs) according to D'Ovidio et al. (1996) or very short ones (as a result of deletion of several blocks) such as subunit 12.4^{t} present in *T. tauschii* (Gianibelli et al 1996a).

Relationship of HMW-GS to wheat quality

The establishment of allelic variation in HMW glutenin subunits as a major contributor to genetic variation in breadmaking quality was pioneered by Payne and co-workers with a series of studies starting at the end of the 1970s (Payne et al., 1979).

Based on the analyses of a large numbers of cultivars, Payne et al. (1987a) also developed a scoring system for the HMW-GS in which individual subunits are graded with numbers based on quality evaluations. A given cultivar can then be assigned a *Glu-1* score, which is the sum of the contributions of each of the three HMW-GS loci. The HMW-GS score has been shown to have more influence in some sets of wheats than in others (MacRitchie et al 1990). This is likely to be due to the complex interaction of factors that define wheat quality. These factors, in which HMW-GS have a major role, also include LMW-GS, gliadins, and abiotic stresses. One aspect that is sometimes overlooked when using the scoring system is that subunits with the same electrophoretic mobility in SDS-PAGE differ in some other features like small differences in

protein sequences and surface hydrophobicity. For example, after the *Glu-1* score was established, Sutton (1991) found differences in retention time for subunit 8 in some cultivars when subjected to RP-HPLC. He concluded that two different subunit 8 were involved (8 and 8*). Also, different electrophoretic mobilities were reported for subunit 7 (7 and 7*). Thus, four different alleles, instead of just one, are expected for this pair (7+8; 7*+8; 7+8*; 7*+8*) (Marchylo et al 1992b). Interestingly, there are contrasting effects on quality within these pairs and, hence, the score originally given to the pair 7+8 is sometimes misleading. Nevertheless, reference to HMW-GS composition has proved valuable in the segregation of lines in the process of breeding for specific quality targets (Cornish 1995; Cornish et al 1999).

Locus			
Glu-A1	*Glu-B1*	*Glu-D1*	Score
-	-	5 + 10	4
1	17 + 18	-	3
2*	7 + 8	-	3
-	7 + 9	2 + 12	2
-	-	3 + 12	2
null	7	4 + 12	1
-	6 + 8	-	1

Tab. 1.4: Quality scores assigned to individual HMW glutenin subunits or subunits pairs (from Payne et al., 1987a).

Several HMW-GS alleles have been closely associated with breadmaking quality. The studies of Payne et al. (1981b) mainly involved the analysis of the random progeny from various crosses between lines with different HMW subunits composition and contrasting breadmaking properties. He showed that allelic variation in subunits affected breadmaking properties as measured by the SDS sedimentation test. For example, the allelic pairs of HMW subunits 1Dx5 + 1Dy10 and 1Dx2 + 1Dy12 encoded by the Glu-D1 locus were correlated with good and poor breadmaking properties, respectively. We now know that the observed differences in breadmaking properties (i.e., dough strength) were due to differences in molecular size of glutenin polymers (Gupta and MacRitchie, 1994). When comparing the allelic pair of HMW subunits 1Dx5 + 1Dy10 Vs. 1Dx2 + 1Dy12, Anderson and Green (1989) and Kasarda (1999) both consider the presence of an extra cysteine residue in Dx5 as the determinant of the allelic differences observed. Similarly, Payne et al. (1981b) also showed differential effects of allelic subunits encoded by the *Glu-B1* and *Glu-A1* loci.

Analyses of varieties grown in different countries have essentially confirmed the role of allelic difference at the three *Glu-1* loci in determining breadmaking properties (MacRitchie et al., 1990). Subsequently, Payne et al. (1987c) used a set of near-isogenic lines developed in the bread wheat cultivar Sicco [HMW subunit composition 1Ax1 (*Glu-A1*), 1Bx7 + 1By9 (*Glu-B1*), 1Dx5 + 1Dy10 (*Glu-D1*)] to show effects associated with the replacement of subunits 1Dx5 + 1Dy10 with the allelic pair 1Dx2 + 1Dy12. SDS sedimentation, farinograph and baking tests clearly showed the superior effects on breadmaking of the former pair compared with the latter. Furthermore, near-isogenic lines of Sicco, in which HMW subunits had been eliminated, demonstrated that the removal of subunits 1Dx5 + 1Dy10 had severe effects on the SDS sedimentation volume and loaf volume. These results were similar to those obtained by Lawrence et al. (1988), who crossed a mutant line of the cultivar Olympic, which is null at the *Glu-B1* locus, with a line of the cultivar Gabo, which is null at the *Glu-A1* and *Glu-D1* loci, to generate a series of lines containing from zero to five subunits.

By the middle of the 1980s, the results of extensive genetic and biochemical studies had clearly demonstrated that differences in number and type of HMW glutenin subunits strongly affected the breadmaking properties through effects on the amount and size distribution of glutenin polymers.

Halford et al. (1992) carried out quantitative analyses of total protein extracts from 22 bread wheat cultivars and demonstrated that the presence of the allelic subunits 1Ax1 or 1Ax2*, when compared with the null allele, resulted in an increase in the proportion of total HMW subunits from about 8 to 10%. This led them to suggest that the increase in breadmaking quality associated with the presence of subunit 1Ax1 or 1Ax2* may result from an increase in the total proportion of HMW glutenin subunits, which can in turn result in a higher amount of large glutenin polymers. Popineau et al. (1994) used the near-isogenic lines developed in the bread wheat cultivar Sicco to show that elimination of HMW subunits associated with the *Glu-A1* and *Glu-D1* loci strongly reduced the amount of the large size glutenin polymers, as measured by size-exclusion chromatography (SE-HPLC), with a marked decrease in gluten viscoelasticity. The substitution of the pair 1Dx5 + 1Dy10, normally present in Sicco, with the pair 1Dx2 + 1Dy12 also negatively affected the size distribution of glutenin polymers and gluten viscoelasticity. These results were paralleled by studies performed by Gupta and MacRitchie (1994), who used recombinant inbred lines and biotypes differing in allelic composition at the *Glu-B1* (1Bx17 + 1By18 vs 1Bx20 + 1By20) or the *Glu-D1* locus (1Dx5 + 1Dy10 vs 1Dx2 + 1Dy12) to show that the allelic pairs 1Bx17 + 1By18 and 1Dx5 + 1Dy10 were associated with the production of larger amounts of large-sized glutenin polymers. No quantitative differences were found between the pairs of allelic combinations tested. However, subunit 1Dx5 contains an additional cysteine residue compared with subunit 1Bx2 while subunit 1Bx20 contains two cysteine residues compared with four in subunit 1Bx17. It has been suggested that these differences may be critical in determining the size distribution of glutenin polymers (Lafiandra et al., 1993b, 1999; Gupta and MacRitchie, 1994).

More recently, doubled-haploid populations have been used to examine in detail the contributions of HMW subunits in influencing breadmaking properties (Kammholz et al., 1998;

Killermann and Zimmermann, 2000; Lukow, 2000; Cornish et al., 2001). Killermann and Zimmermann (2000) used four doubled haploid populations differing in glutenin subunits associated at the *Glu-1* and *Glu-3* loci and demonstrated that the effects of the different alleles were strongly dependent on the genetic background of the material. In fact, comparison of the allelic pairs 1Dx5 + 1Dy10 versus 1Dx2 + 1Dy12, and of 1Bx7 + 1By9 versus 1Bx6 + 1By8, consistently showed superior effects of 1Dx5 + 1Dy10 and 1Bx7 + 1By9, whereas comparison of the null allele (the silenced *Glu-A1* locus) with subunit 1 at the *Glu-A1* locus showed significant positive effects only in one population.

The effect of individual proteins (HMW-GS, LMW-GS, hordeins, gliadins) on dough properties can also be evaluated by studying the mixing behaviour of a base flour, modified either by incorporation or addition of the specific proteins (Bekes et al 1994a,b). Recent advances in microscale mixing and protein engineering systems have proved to be valuable in elucidating structure and functional relationship in gluten proteins (Bekes et al 1998). Chain-extender proteins such as HMW-GS and LMW-GS increased dough strength and stability estimated by mixograph parameters (Sissons et al 1998; Lee et al 1999a). Likewise, chain-extender proteins with longer repetitive domains increased the stability and strength more than proteins with shorter domains (Bekes et al 1998). Polypeptides containing a single cysteine can act as chain terminators during the formation of the glutenin polymers, producing decreases in dough strength and stability (Kasarda, 1989; Buonocore et al. 1998; Tamas et al. 1998; Greenfield et al. 1998; Masci et al. 1999).

1.3.5.2.2.2. LMW-GS (Low Molecular Weight – Glutenin Subunits)

The LMW-GS represent about one-third of the total seed protein and ≈60% of total glutenins (Bietz and Wall 1973). Despite their abundance, LMW-GS have received much less research attention than the HMW-GS. This has been mainly due to the difficulty in identifying them in one dimensional SDS-PAGE gels, since LMW-GS largely overlap with gliadins. However, improved resolution of capillary electrophoresis (Bean and Lookhart, 2000) and recent advances in two-dimensional gel-based mapping approaches (Skylas et al. 2000, Vensel et al. 2005), allowed us to get new insights about the genetic, structural and functional characteristics of these subunits.

LMW-GS can be classified, as proposed by Jackson et al. (1983), in B-, C-, and D-subunits on the basis of molecular weight and isoelectric point. Based on SDS-PAGE estimates, their molecular weight range is 20,000-45,000 Da (D'Ovidio and Masci, 2004). While most of the LMW-GS belong to the B group ("typical" LMW-GS) we now know that the D group is actually composed of modified ω-gliadin components that have acquired a cysteine residue (ω-gliadins lack this amino acid residue). This finding was the first evidence that gliadin-like subunits were present and incorporated in the glutenin polymers (Masci et al., 1993, 1999).

LMW-GS with α- and γ-type gliadin-like N-terminal sequences are the most abundant proteins in the so-called C group, with at least thirty components being detected by two-dimensional analyses (Masci et al., 2002). As for the D subunits, it is probable that they form part of the

glutenin fraction because the numbers of cysteine residues is different from that in the α- and γ-gliadins. This has been demonstrated not only by comparisons of gene and deduced protein sequences but disulphide bonds linking LMW-GS with γ-type sequences were found by Keck et al. (1995) and Kohler et al. (1993). γ-gliadin clones with nine cysteine codons instead of the typical eight have been identified in durum and bread wheats (D'Ovidio et al., 1995a; Scheets and Hedgcoth, 1988) while α-gliadin genomic clones with numbers of cysteine residues other than the typical six have been identified by Anderson et al. (1997a, b).

Within the B group of LMW-GS, on the basis of N-terminal amino acid sequences, three subgroups of typical LMW-GS can be recognized, called LMW-s, LMW-m, and LMW-i types, according to the first amino acid residue of the mature protein: serine, methionine, or isoleucine, respectively. LMW-s type subunits are the most abundant in all genotypes analysed and their average molecular mass (35,000–45,000) is higher than that of LMW-m type subunits (30,000–40,000) (Tao and Kasarda, 1989; Lew et al., 1992; Masci et al., 1995). The N-terminal amino acid sequence of LMW-s type subunits is SHIPGL-, whereas the N-terminal sequences of LMW-m type subunits are more variable and include METSHIGPL-, METSRIPGL-, and METSCIPGL- (Kasarda et al., 1988; Lew et al., 1992; Masci et al., 1995; Tao and Kasarda, 1989). However, both LMW-s and LMW-m type subunits contain eight cysteine residues, two of which are involved in intermolecular disulphide bonds.

Among typical LMW-GS, the LMW-i type (Cloutier et al., 2001), which was first identified by Pitts et al. (1988), can be considered as a variant form. These LMW-GS lack the N-terminal region, starting directly with the repetitive domain after the signal sequence, with ISQQQQ- being the deduced N-terminal sequence of all LMW-i type genes isolated so far. Although the N-terminal region is missing, the typical eight cysteine residues are all present in the C-terminal domain. Cloutier et al. (2001) and Ikeda et al. (2002) presented evidence that they are expressed in the endosperm and incorporated in the glutenin polymers.

On the basis of the structural characteristics of the B, C and D groups, Kasarda (1989) suggested the existence of two functional groups of LMW-GS. One group, which includes the majority of the B-type subunits, acts as chain extenders of the growing polymers because of their ability to form two inter-molecular disulphide bonds. The second group, which includes most of the C and D-type LMW subunits, act as chain terminators of the growing polymer, having only one cysteine available to form an intermolecular disulphide bond.

Clarke et al. (2000) and Anderson et al. (2001) have identified genes encoding new types of low molecular mass wheat endosperm proteins, with sequence similarity to gliadins, LMW-GS, and to 1-hordeins. The encoded proteins are characterised by distinctive N-terminal sequences, a smaller central repetitive domain than in typical LMW glutenin subunits, and the presence of more cysteine residues.

Genetics and polymorphism

The application of different two-dimensional electrophoresis techniques to analyze the Chinese Spring aneuploid lines allowed Jackson et al. (1983) to assign the LMW subunit genes to the short arms of the homoeologous group 1 chromosomes. Subsequent genetic analyses did not reveal any recombination between genes encoding LMW subunits and gliadins, so Payne et al. (1984b) concluded that the complex *Gli-1* loci contained three families of genes corresponding to the ω- and γ-gliadins and the LMW subunits. In contrast, Singh and Shepherd (1988a) reported evidence of recombination between genes for an ω-gliadin and B-type LMW subunit proteins. This has subsequently been confirmed by other research groups and consequently the symbol *Glu-3* was assigned to the loci encoding the B-type LMW subunits (Pogna et al., 1990; Gupta and Shepherd, 1993). Recombination between B-type LMW glutenin subunits and gliadins encoded by the *Gli-A1* and *Gli-B1* loci on chromosomes 1A and 1B has also been observed in *T. durum*. Map distances of 1.3 cM between *Glu-A3* and *Gli-A1* and of 2.0 cM between *Glu-B3* and *Gli-B1* were reported by Ruiz and Carrillo (1993), confirming data reported by Pogna et al. (1990). The latter group also reported that *Glu-B3* was located between the centromere and *Gli-B1*, on the short arm of chromosome 1B.
Sreeramulu and Singh (1997) found evidence of new loci encoding LMW-GS in addition to the *Glu-3*. They studied two novel LMW-GS present in accessions of Indian bread wheat and mapped their encoding genes to loci present on chromosomes 1D (*Glu-D4*) and 7D (*Glu-D5*). Although their amino acid compositions differ from those of typical LMW-GS and other gluten proteins, they have been defined as LMW-GS because of their polymeric behaviour, and because polyclonal antibodies raised against them cross-reacted strongly with typical LMW-GS, but not with HMW-GS or gliadins. Moreover, their N-terminal amino acid sequences resembled those of B- and C-type LMW-GS, being KETXXI- for the *Glu-D4* encoded subunit (similar to the METSHI- sequence), and VXVPV- for *Glu-D5* encoded subunit (similar to VRVPV- peculiar of α-type gliadins). The possibility that typical LMW-GS are encoded by loci in addition to the *Glu-3* locus, is also suggested by Vaccino et al. (2002) who found a high recombination frequency between the *Gli-B1* locus and two DNA fragments which hybridised with a probe specific for typical LMW-GS, and were associated with two LMW-GS bands.

Gupta and Shepherd (1990a) observed extensive polymorphism on the basis of screening a collection of 222 hexaploid wheats from 32 countries. They detected 20 different band patterns (LMW-GS blocks), six for the *Glu-A3* locus, nine for the *Glu-B3* locus, and five for the *Glu-D3* locus. They also noted that some cultivars did not exhibit any LMW-GS encoded by *Glu-A3* locus. On the other hand, they found considerable polymorphism for the LMW-GS encoded by chromosome 1B.
A wide variability in number and electrophoretic mobility of LMW-GS was also observed in *Triticum* species like *T. monococcum* and *T. urartu* (A genome) (Rodriguez-Quijano et al 1997; Lee et al 1999b), *T. dicoccoides* (AB genomes) (Ciaffi et al 1993), and *T. tauschii* (D genome). Since the number of the distinct observed electrophoretic patterns for the LMW-GS is much

lower than the total number of randomly possible combinations of single LMW-GS bands, it is generally assumed that the genes controlling the LMW-GS are closely linked. They form clusters that are inherited together, similar to those controlling gliadin blocks (Gupta and Shepherd 1990a,b; Lagudah et al 1991).

Until recently, most attention has been paid to the B-type LMW-GS, since they are the most abundant and have the deepest impact on the technological properties of the wheat flour. However, the development of methods to purify fractions enriched in C-type LMW subunits (i.e., the mutant forms of α- and γ-type gliadins) has allowed their encoding genes to be mapped to the group 1 and 6 chromosomes, either tightly linked to or within the *Gli-1* and *Gli-2* loci (Masci et al., 2002). This confirms earlier reports that the group 6 chromosomes contain genes encoding glutenin-type proteins (Gupta and Shepherd, 1993; Pogna et al., 1995; Felix et al., 1996).

Classification based on		
SDS-PAGE mobility	**LMW-GS group**	**Predominant sequence type**
	B	LMW-s; LMW-m
	C	α-type; γ-type
	D	ω-type
N-terminal amino acid sequence	**LMW-GS type**	**N-terminal AA sequence**
	LMW-s	SHIPGL-
	LMW-m	METSH(R/C)I-
	LMW-I	ISQQQQ-
	α-type	VRVPVP-
	γ-type	NMQVDP-
	ω-type	KELQSP-
		ARQLNP-

Tab. 1.5: A summary of the possible classifications for the LMW-GS. Taken from D'Ovidio and Masci (2004).

Regarding the D subunits, they are encoded by loci on the short arm of chromosomes 1 (Payne et al., 1985). However, until their structural characteristics were defined, there was confusion over their encoding loci. Jackson et al. (1983) suggested that the *Glu-B2* locus, located on the satellite of the short arm at 17 cM from *Glu-B1* and 22 cM from *Gli-B1*, encoded for a D-type subunit, although the same locus was later found to encode a B-type subunit (Liu, 1995). This observation might be due to the fact that *Glu-B2* is a complex locus, or result from the use of different electrophoretic systems to identify the subunit. Nieto-Taladriz et al. (1998) have shown that a D-type LMW-GS is encoded by the same *Glu- B3* locus as typical B-type LMW-GS, thus confirming that it is a complex locus.

Amino acid sequences and structures of the LMW-GS

The typical LMW-GS are encoded by gene families at each of the orthologous *Glu-3* loci, whose copy numbers are not known. However, estimates of the total gene copy number, based on Southern blot analyses, varied from 10–15 (Harberd et al., 1985) to 35–40 (Cassidy et al., 1998; Sabelli and Shewry, 1991) in hexaploid wheat. Recent sequence analyses of bacterial artificial chromosomes (BAC) clones revealed that two LMW-GS genes in *T. monococcum* may be separated from each other more than 150 kbp (Wicker et al., 2003). Information on the structure of genes encoding LMW-GS derives from the characterisation of more than 70 DNA clones reported in data banks. These are isolated from 15 different genotypes belonging mainly to *T. aestivum* and *T. durum*. Based on these data the general structure of a typical LMW-GS can be proposed. This shows four main structural regions including a signal peptide of 20 amino acids, a short N-terminal region (13 amino acids) that usually contains the first cysteine residue, a repetitive domain rich in glutamine codons and a C-terminal region. As suggested by Cassidy et al. (1998), the C-terminal region can be further subdivided into three distinctive regions: a cysteine-rich region containing five cysteine residues, a glutamine-rich region containing a cysteine residue and stretches of glutamine residues, and a C-terminal conserved sequence containing the last cysteine residue. Most of the full-length genes vary from 909 bp to 1167 bp, in size with the molecular masses of the encoded mature proteins ranging from about 32,000 to 42,800.

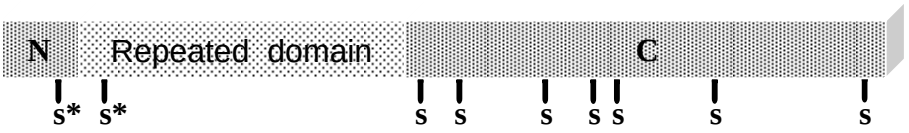


Fig. 1.9: Schematic illustration of a B-type LMW-GS. Black bars with letter s represent cysteine residues available for intermolecular or intramolecular disulphide bonds. s* indicates the first cysteine residue alternatively located in the N-terminal region or at the start of repetitive domain.

The number of repeats present in the repetitive domain is mainly responsible for this length variation, ranging between about 12 and 25.

Comparison of allelic genes suggests that this variation can result from deletion and/or insertion of repeat units (D'Ovidio et al., 1999), most probably caused by unequal crossing-over and/or slippage during replication as suggested for the evolution of other prolamins (Shewry et al., 1989). The length of each repeat unit can vary between 15 and 27 bp with the following consensus sequence: CCA_{1-2} TTT (T/C)C(A/G) CA(G/A) CAA_{1-4}. The repetitive domain is also mainly responsible for the general hydrophilic character of LMW-GS.

The locations of the cysteine residues in the sequence distinguishes between those LMW-GS that form intermolecular disulphide bonds (the first and the seventh cysteine residue) from those forming intra-molecular disulphide bonds. Based on the distribution of cysteine residues, the

LMW-GS proteins can be classified into three main different types: (i) those with one cysteine in the short N-terminal domain; (ii) those with a cysteine residue in the repetitive domain replacing that in the N-terminus; and (iii) those with eight cysteines in the C-terminal part of the protein. This different cysteine distribution could lead to functional differences.

Glutamine and proline account for almost 50% of the total amino acids, being about 30 and 15 mol %, respectively. A characteristic of LMW-GS genes is the preferential use of the CAA codon (about 70%) in place of the CAG triplet to encode glutamine residues, which is also typical of other endosperm proteins such as gliadins and HMW-GS.

The deduced sequences of the mature LMW-GS also show different N-terminal sequences, which have been used to classify the different gene sequences into groups (Tab. 1.5).

Comparison of some DNA-deduced N-terminal sequences with those effectively detected at the protein level showed that some correspond to proteins. However, the N-terminal sequences METSCIPGLERPS-, METSHIPGLERPS-, METSRIPGLE- and METSHIPGLEKPL-, found at the protein level by Lew et al. (1992), have not yet been detected at the gene level.

The secondary structures of LMW-GS, except for the D-subunits, have been proposed, and share an overall similarity with the structure of the S-rich gliadins (Tatham et al. 1987; Thompson et al. 1993, 1994). The N-terminal domains are rich in β-turns, possibly forming a regular spiral structure, while the short non-repetitive C-terminal domains are rich in α-helices and appear to be more compact (Thomson et al., 1992; Masci et al. 1998). Application of flexibility modelling to a specific LMW-GS (the so-called "42K LMW-GS") indicated that the repetitive domain was highly flexible, particularly where stretches of glutamines were present (Masci et al. 1998).

The 42K LMW-GS contains two cysteine residues that are likely to be involved in inter-molecular disulphide bonds, namely the first (Cys-43 according to Masci et al. (1998) or C^{b*} according to Köhler et al. (1993)) and the seventh (Cys-295 or C^{x}). These residues are predicted to be located or surrounded by regions of high flexibility, which might be a mechanism that facilitates polymerisation.

The remaining six cysteine residues in the 42 K LMW-GS are likely to be involved in intra-molecular disulphide bonds, as suggested by direct amino acid sequencing of cystine-containing peptides, similarity with the closely related γ-gliadins, and site-directed mutagenesis of cysteine residues (Keck et al., 1995; Köhler et al., 1993; Müller et al., 1998; Müller and Wieser, 1997; Orsi et al., 2001; Shewry and Tatham, 1997; Thompson et al., 1993, 1994). There is little doubt that the first (C^{b*}) and the seventh (C^{x}) cysteines are involved in inter-molecular disulphide bonds; this latter residue has in fact been found to be linked to several different polypeptides, namely HMW-GS and a modified γ-gliadin (probably a C-type LMW-GS) (Keck et al., 1995; Köhler et al., 1993).

The role of enzymes in the formation of disulphide bonds in prolamins is also unresolved. The use of protein disulphide isomerase (PDI) and cysteine-containing synthetic peptides based on LMW-GS indicated that it did have a strong oxidising effect, but this effect was inversely correlated to protein molecular weight, inferring that the enzyme does not play an important role in vivo (Bauer and Schieberle, 2000). However, in vivo studies indicate that PDI might be

involved in the assembly of wheat storage proteins within the ER, based mainly on its abundance in developing endosperm (DuPont et al., 1998; Grimwade et al., 1996; Shimoni et al., 1995). Moreover, the in vitro synthesis of a γ-gliadin in disulphide-deficient microsomes resulted in the formation of incorrect disulphide bonds, and this was reversed when microsomes were reconstituted with PDI (Bulleid and Freedman, 1988).
The dynamics of formation of both intra- and intermolecular disulphide bonds is also a matter of debate. LMW-GS are major components of the glutenin polymers, being about 5–6 times more abundant than HMW-GS (Clarke et al., 2000; Kasarda, 1989). However, it is not known whether they are randomly incorporated into the glutenin polymer, or their incorporation is subject to particular constraints due to their structure, timing of synthesis, or the involvement of specific enzymes. Lindsay and Skerritt (1998) proposed that the pattern of release of glutenin subunits from glutenin polymers by stepwise reduction indicates that this structure has a precise organisation. They found that LMW-GS were initially released as dimers, with monomers appearing after increasing the concentration of the reducing agent (DTT). B subunits were released at a low concentration of DTT, whereas C subunits were released over a wider concentration of reducing agent. This has been interpreted to indicate that B subunits are present in the biggest polymers, since inter-molecular bonds are broken first, whereas C subunits are involved in polymers with a wider range of molecular weight. On the basis of the results reported by these authors, B subunits may behave like a type of adhesive to stabilise the glutenin polymers, since once they are released, complete depolymerisation occurs.

Little is known about the structures of the D-subunits of the LMW glutenins. In terms of electrophoretic mobility and N-terminal sequences, the D-subunits are very similar to the S-poor ω-gliadins (Masci et al 1991a,b, 1993, 1999). Supposedly, the D-subunits are mutant forms of the monomeric ω-gliadins in which acquisition of a single cysteine residue allows cross-linking into the glutenin polymer. It has been proved that only one cysteine residue was involved in the structure of D-subunits, allowing them to act as chain terminators (Masci et al. 1999). Similarly, Gianibelli et al (1996b, 2002a) and Nieto- Taladriz et al (1998) have reported LMW-GS with a *Mr* of ≈70,000 Da and N-terminal sequences similar to that of the ω-gliadins, encoded at the *Gli-B1* locus. This subunit participated in the glutenin polymeric structure (Gianibelli et al., 2002a).

Relationship of LMW-GS to wheat wuality

LMW-GS are important for the end-use quality of durum wheat, in particular subunits encoded by loci present on chromosome 1B (Josephides et al., 1987). The best pasta-making characteristics are associated with the presence of a specific allelic form of typical LMW-GS, named LMW-2 (Payne et al., 1984b). This allele also seems to be important for determining breadmaking properties (Peňa et al., 1994).
LMW-2 comprises a group of polypeptides, encoded by the *Glu-B3* locus, which is genetically linked to the *Gli-B1* locus, which contains genes encoding γ- and ω-gliadins, designated 45 and 35, respectively. Most commonly grown durum wheat cultivars have either the LMW-2/γ-45 (plus

ω-gliadin 35) or the LMW-1/γ-42 (plus ω-gliadins 33, 35 and 38) allelic forms, the latter being associated with poor quality pasta-making properties. Because of the close association between γ-42 and γ-45 with LMW-1 and LMW-2, respectively, it was initially believed that quality characteristics were dependent on the presence of the specific gliadins rather than the associated LMW-GS.

However, many more recent studies have demonstrated the importance of LMW-GS, and it is now commonly accepted that γ-42 and γ-45 are only genetic markers for quality (Boggini and Pogna, 1989; Pogna et al., 1988). There are indications that the better quality associated with the presence of LMW-2 in durum wheat is mainly due to the fact that the subunits are more abundant than the LMW-1 subunits (Autran et al., 1987; D'Ovidio et al., 1992; Masci et al., 1995) and that structural differences may play only a minor role (D'Ovidio et al., 1999; Masci et al., 1998).

In support of this, D'Ovidio et al. (1999) showed that allelic genes encoding major components of the LMW-1 and LMW-2 groups differed only by 15 amino acid substitutions within the repetitive domain. Although it is not possible to exclude the possibility that other LMW-GS belonging to the LMW-1 and 2 groups are responsible for their different effect on end-use properties, this result supports the hypothesis that quantitative differences in LMW-GS are important. Further support comes from the poor processing properties of the durum wheat cultivar Demetra that possesses the LMW-2 group, but has a low total amount of LMW-GS, caused either by a lower number of subunits composing the LMW-2 and to a lower level of expression of those LMW-GS present (Masci et al., 2000b).

The main difference between the LMW-1 and LMW-2 protein groups is the presence of a slow moving *Glu-B3* coded LMW-GS in the LMW-2 pattern (D'Ovidio et al., 1999; Masci et al., 1995; Nieto-Taladriz et al., 1997; Ruiz and Carrillo, 1995, 1996). This slow-moving LMW-GS corresponds to the 42 K LMW-GS (Masci et al., 1998) in most genotypes and it is consistently the most abundant LMW-GS polypeptide (Masci et al., 1995). Allelic variants of the LMW-2 group have also been described and in all cases these are associated with good technological properties (Brites and Carrillo, 2001; Ruiz and Carrillo, 1995, 1996; Vàzquez et al., 1996).

The 42 K LMW-GS may also be present in good quality bread wheat (Masci et al., 2000a), but it is not associated with the LMW-2 group, which does not appear to occur in hexaploid wheat.

Although HMW-GS are the major group of gluten proteins that determine the bread-making characteristics of dough, LMW-GS also play an important role. In general, the LMW-GS are associated with dough resistance and extensibility (Metakovskii et al., 1990; Andrews et al., 1994; Cornish et al., 2001), and some allelic forms of LMW-GS show even greater effects on these properties than HMW-GS (Payne et al., 1987b; Gupta et al., 1989, 1994).

Similarly, null alleles of LMW-GS have detrimental effects on dough resistance and extensibility (Benedettelli et al., 1992).

Differences in the total amount of LMW-GS, associated with specific allelic forms, have also been reported to be an important cause of quality differences in bread wheat (Gupta and MacRitchie, 1994; Luo et al., 2001).

Since most of the qualitative evaluations of LMW-GS have been devoted to B-type subunits, the most abundant ones, little is known today about the role of C- and D-type subunits. Regarding the latter, the presence of a single cysteine residue may indicate a role as chain terminator, since the incorporation of the subunit would lead to the termination of the growth of the glutenin polymer (Masci et al., 1993; Tao and Kasarda, 1989; Egorov et al. 2000; Gianibelli et al., 2002a,b). Moreover, SDS-sedimentation tests on two biotypes of the bread wheat cultivar Newton differing at the *Gli-D1*/*Glu-D3* loci showed lower values for the biotype that possessed D-type subunits, providing further support for their negative contribution to visco-elastic properties of the dough (Masci et al., 1991b).

1.3.5.2.3 Polymer formation

As explained above, the polymeric protein of wheat endosperm is a mixture of polypeptides (subunits) held together by disulfide bonds, thus forming the protein matrix between the starch granules and gas bubbles in dough. Because these polymers have molecular sizes ranging up to tens of millions of daltons (Wrigley, 1996), it has been difficult to determine the precise structure and molecular weight distribution. In working toward the elucidation of structure, the proposal of models has been a useful tool for gaining a better understanding of the process of glutenin formation.

Ewart (1968, 1972, 1977, 1979), Graveland et al. (1985), and Kasarda (1989) have proposed ideas about polymer formation.

Ewart's is a linear model in which the subunits (no differentiation between HMW-GS or LMW-GS) are linked head-to-tail in a random fashion by disulfide bonds between polypeptides.

In Graveland's model, the backbone of the molecule comprises only HMW-GS with the x- and y-type subunits alternating in a head-to-tail arrangement and lateral attachments of LMW-GS; disulfide bonds maintain the structure.

The model suggested by Kasarda (1989) takes into account the proportion of HMW-GS and LMW-GS (10–20% and 20–80%, respectively). It proposes a cluster of LMW-GS structures connected through disulfide bonds located at the C-terminus with HMW-GS that act as connecting strings for further clusters of LMW-GS. It also incorporates the concept of chain-terminators as proteins (mainly LMW-GS) with only a single unreacted cysteine residue that would stop the polymerizing process. Recently, a more refined branched model of the glutenin macropolymer has been proposed (Lindsay and Skerritt 1998, 1999).

Although there is controversy about the manner in which glutenin subunits are incorporated into the glutenin polymer (Kasarda 1989; Ewart 1990), the results obtained so far favor a quasi-random process in which both HMW-GS and LMW-GS participate (MacRitchie and Lafiandra 1997). The polymers formed by LMW-GS were first observed by Bietz and Wall (1980) and more recently confirmed by Köhler et al (1993) and Vasil and Anderson (1997). Dimers of HMW-GS must also be considered in any model. They are common in partly reduced glutenin preparations (Lawrence and Payne 1983; Werner et al 1992; Shani et al 1994). Dimers of x- and y-type HMW-GS were widely observed and, to a lesser extent, dimers composed of only x-type

subunits were also detected. Dimers consisting of two y-type subunits were never observed (Shewry and Tatham 1997).
Although intermolecular disulfide bonding is the major factor defining polymer stability, NMR studies by Belton et al. (1995) and atomic force microscopy studies (Humphris et al 2000) indicated an important role of hydrogen bonding between adjacent HMW-GS and between HMW-GS and other proteins in stabilizing the structure of gluten. High proportions and regular spacing of glutamine residues would favor extensive hydrogen bonding.
Moreover, although the effect of the glutenin on wheat quality has largely been considered in relation to subunit composition, there is the need to also introduce the concepts of polymer as a whole, taking into consideration the interactions that occur with the wider range of components of dough. Polymer science indicates the importance of the size distribution for such molecules as a critical principle governing the physical properties of synthetic polymers (MacRitchie 1992; Weegels et al 1996a,b). For example, molecules below a certain size limit (threshold level) would not contribute to the strength properties of a mixed polymer. By analogy, size distribution of the gluten proteins is highly correlated to breadmaking quality (Southan and MacRitchie 1999).

1.3.5.2.4 Regulation of prolamin gene expression

Prolamin genes are subject to tissue-specific (starchy endosperm), developmental (they are expressed exclusively during mid- and late-development of the grain filling), and nutritional regulation, responding sensitively to the availability of nitrogen and sulphur in the grain (Duffus and Cochrane, 1992; Giese and Hopp, 1984).
This control of gene expression is exerted primarily at the transcriptional level (Bartels and Thompson, 1986; Sørensen et al., 1989).
Since they show similar patterns of expression, it is to be expected that prolamin genes would have regulatory sequences in common.
However, little is known about the mechanisms by which the expression of prolamin genes responds to sulphur. However, a motif involved in the response of S-poor and S-rich prolamin genes of wheat, barley and rye to nitrogen has been identified. This motif (called the “N motif” or “nitrogen element”) (Hammond-Kosack et al., 1993; Muller and Knudsen, 1993) is present within a highly conserved sequence called the “prolamin box”. The prolamin box was the first prolamin gene regulatory sequence to be reported and was identified through a comparison of the promoters of several gliadin and hordein genes (Forde B. G. et al., 1985). This revealed the presence of the conserved sequence, which is approximately 30 bp long, around 300 bp upstream of the transcription start site (it was first called the “-300 element”). The prolamin box contains two conserved motifs, TGTAAAGT and G(A/G)TGAGTCAT, with a more variable region in between. The former has been called the endosperm motif (E motif) (Hammond-Kosack et al., 1993) the latter the GCN4-like motif (GLM), nitrogen element, or N motif (Hammond-Kosack et al., 1993; Müller and Knudsen, 1993). The N motif is similar to the binding site of the GCN4 transcription factor, which is a component of the amino acid signaling pathway

in yeast. The E motif is present in the promoters of storage protein genes from a wide range of other species, including some zein genes (Coleman and Larkins, 1999).

The position of the prolamin box is highly conserved, with the first T of the endosperm motif close to position -250. The α-gliadin and LMW subunit gene promoters contain additional complete or partial boxes further upstream in the promoter, but these do not appear to be required for promoter activity and are not present in the γ- or ω-gliadin promoters. The N motif is inverted in S-poor genes from barley (C hordein) and rye (ω-secalin) (Shewry et al., 1999) but not in the prolamin box of the ω-gliadin gene.

Promoter regions containing the prolamin box have been shown to be functional by introducing promoter/ chloramphenicol acetyl transferase (CAT) reporter gene constructs into transgenic tobacco (Colot et al., 1987; Marris et al., 1988). Müller and Knudsen (1993) used an homologous, transient expression system, involving particle bombardment of cultured barley endosperms with C hordein promoter/β-glucuronidase (GUS) constructs. These experiments confirmed that the E and N motifs are separate elements and showed that the N motif exerts a negative effect on gene expression at low nitrogen levels and interacts with the E motif and other upstream elements to give high expression when nitrogen levels are adequate.

Hammond-Kosack et al. (1993) used in vivo footprinting and gel retardation assays to show that the E motif within the prolamin box of an LMW subunit gene bound a putative transcription factor, ESBF-1, during early grain development. The N motif bound a second putative transcription factor, ESBF-II, before maximum expression of the gene. This study was followed by a functional analysis of the prolamin box of this gene in transgenic tobacco, which showed that both motifs were required for seed-specific expression, and by the cloning of SPA, a bZIP transcriptional activator that recognized the N motif (Albani et al., 1997).

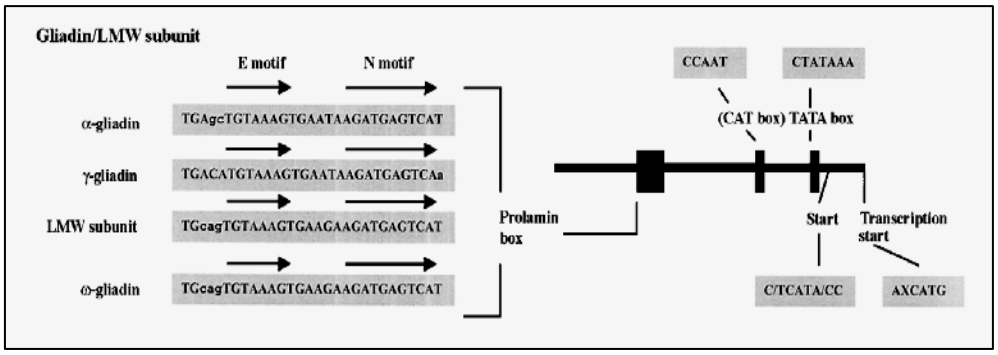


Fig. 1.10: A schematic diagram representing the regulatory sequences present in gliadin and LMW-GS genes. Taken from Shewry et al. (2003).

However, the prolamin box is not present in all prolamin genes. Zein gene promoters, for example, contain a highly conserved 15 bp element that has been suggested to act as a tissue-specific enhancer (Quayle and Feix, 1992). It contains the sequence TGTAAAG, which resembles the E motif, but the N motif is absent (Coleman and Larkins, 1999). The N motif is present in γ-zein promoters, but it is separated from the E motif and its function, if any, has not been investigated (Coleman and Larkins, 1999).

The prolamin box in its entirety is also not present in HMW prolamin gene promoters (Shewry et al., 1999).

Instead, HMW prolamin promoters contain a major regulatory element (identified by Thomas and Flavell, 1990) which is located in a 38 bp sequence with the consensus: 5'-GTTTTGCAAA GCTCCAATTG CTCCTTGCTT ATCCAGCT-3'.

The location of this sequence is highly conserved in all HMW prolamin promoters, beginning at position -185 to -189 (Shewry et al., 1999). The element contains the sequence TGCAAAG, which is similar to the E motif sequence TGTAAAG that is also present in zein genes, but it does not contain anything resembling the N motif. Sequences corresponding to parts of the N and E motifs are present in HMW prolamin promoters upstream from the major enhancer (Lamacchia et al., 2001). However, deletion of these sequences does not appear to affect activity of the promoter, at least when driving reporter gene expression in transgenic tobacco (Halford et al., 1989; Thomas and Flavell, 1990).

1.3.5.3. Seed storage proteins synthesis and deposition

Cereal seed storage proteins are produced by the secretory pathway and deposited in discrete protein bodies.

However, the mechanisms that determine the pathway of storage protein trafficking and deposition are still incompletely understood.

Protein bodies originate from the endomembrane system of the cell, which comprises the ER, the Golgi apparatus and various types of vesicle involved in the transport of components to and from specific cellular destinations.

Seed storage proteins are synthesized by mRNA associated with polyribosomes on the rough ER.

The protein translated from the mRNA differs from that deposited in protein bodies in the presence of a short N-terminal extension (the signal sequence which is usually ~20 amino acids) whose role is to lead the newly synthesized (nascent) polypeptide through the ER membrane into the lumen, thus entering the endomembrane system. This occurs by specific interaction of the signal sequence with a complex of proteins, the translocon.

Once the nascent polypeptide emerges into the ER lumen the signal sequence is removed by a specific enzyme (a signal peptidase) and the polypeptide chain commences to fold into its three-dimensional structure. These events, translocation, signal peptide cleavage and folding, occur when the protein is still undergoing synthesis and hence are termed cotranslational.

Many non-storage proteins play essential but non-specific roles in storage protein synthesis and accumulation.

For example nucleoside diphosphate kinase (NDK) is required for continued production of nucleoside triphosphates needed for DNA and RNA biosynthesis, which are prerequisites for storage protein biosynthesis (Morera et al. 1994). Other proteins may play very specific roles in post-translational processing of the storage proteins. The lumen of the ER in all eukaryotes contains chaperones and foldases such as binding protein (BiP) and PDI (Boston et al. 1996, Okita and Rogers 1996, Vitale et al. 1993). These proteins have been detected in wheat endosperm and in wheat and rice protein bodies (Levanony et al. 1992, Li et al. 1993, Roden et al. 1982, Shimoni et al. 1995). BiP is a member of the HSP70-related protein family and may be required for proper folding and oligomerization of newly synthesized proteins as they are translocated into the ER (Normington et al. 1989, Okita and Rogers 1996, Vitale et al. 1993). BiP may bind permanently to misfolded proteins (Vitale et al. 1993) and there is a correlation between BiP and storage protein misfolding in maize endosperm (Boston et al. 1991, Fontes et al. 1991). PDI catalyzes the formation of inter- and intramolecular disulfide bonds (Vitale et al. 1993), also appears to have a chaperone capability (Quan et al. 1995) and was demonstrated to catalyze intramolecular disulfide bond formation in a wheat gliadin (Bulleid and Freedman 1988). HSP70 is a cytoplasmic chaperone which also assists in folding newly synthesized polypeptides (Boston et al. 1996)

The significance of the chaperones in import of glutenin subunits into the ER, folding, assembly of the subunits into polymers, and formation of wheat storage protein bodies is subject to debate. Some authors raised the question of whether the patterns of accumulation of BiP and PDI are compatible with these chaperones playing an important role in storage protein accumulation in wheat (Grimwade et al. 1996, Shimoni et al. 1995). Because PDI transcripts peaked early in grain development, it was proposed that PDI might not be available to catalyze disulfide bond formation during the period of maximum storage protein synthesis (Grimwade et al. 1996). Also, it was reported that protein levels for BiP and PDI decreased at the time of maximum storage protein accumulation (Shimoni et al. 1995). However, in another study PDI activity was detected throughout the period of grain fill (Roden et al. 1982).

DuPont et al. (1998) showed that, unlike the gluten storage proteins, relative amounts of BiP, HSP70, NDK and PDI were high early in endosperm development. They then declined as a percentage of total protein while the storage proteins accumulated.

1.3.5.3.1 Deposition of storage albumins and globulins

The 2S albumin, 7S and 11S globulin storage proteins are synthesized on the rough endoplasmic reticulum (ER) membranes. They are then transported via the ER lumen, Golgi apparatus, and vesicles to a specific population of protein storage vacuoles where they form dense deposits. These vacuoles may subsequently divide to form protein bodies.

However, all three types of storage protein may also undergo modification, either in the vacuole or during their passage through the ER and Golgi apparatus.

The 2S albumins are synthesized as precursor proteins, which undergo folding and disulfide bond formation in the ER lumen. The precursors are then proteolytically processed in the vacuole to generate the mature structure.
The 7S and 11S globulin subunits are also folded in the ER lumen with the formation of a single intrachain disulfide bond. Both types of subunits are then assembled into trimeric structures stabilized by noncovalent forces.
Kermode and Bewley (1999) reviewed the precise details of this process.

1.3.5.3.2 Deposition of prolamines

The mechanisms of prolamin transport and deposition are less well understood than those of albumins and globulins. In maize and rice, the prolamins appear to accumulate directly within the lumen of the ER, leading to the formation of discrete protein bodies surrounded by a membrane of ER origin (Coleman and Larkins, 1999; Muench et al., 1999).
In wheat, barley and probably rye, there are now evidences that two routes operate: the direct accumulation of proteins into the ER (as in the maize and rice prolamines) and the transport to the vacuole (characteristic of the storage albumins and globulins). Evidences for both routes have been reviewed by Galili (1997) and Shewry (1999). In wheat, some prolamins, principally gliadins, are transported via the Golgi to the protein storage vacuole whereas others, principally glutenins, are retained within the ER. Shewry (1999) suggested that the polymeric prolamins (i.e. the high molecular weight glutenin polymers), forming insoluble aggregates into the ER lumen, would be preferentially retained in the ER, while monomeric forms (mainly gliadins), initially soluble in the luminal environment, would then be transported to vacuoles. However, it seems likely that this division is not so clear-cut.
Galili (1997) has proposed that the ER-protein bodies (mostly containing glutenins) subsequently become engulfed into vesicles and “internalized” into protein storage vacuoles, in a process similar to autophagy, leading to the fusion of the two protein-body populations.
The precise mechanism of protein body fusion in wheat remains to be resolved. However, the net result is the presence in mature, dry endosperm cells of a continuous matrix of proteins that surrounds starch granules and engulfs the remains of other cell structures. This matrix is the basis for the formation of the gluten network when wheat flour is mixed with water to form dough.
It is also probable that the relative amounts of prolamins deposited in the two types of protein body vary with the rate of protein synthesis and with the developmental state of the tissue. Thus, it can be envisaged that high levels of protein synthesis would lead to a high level of accumulation within the ER lumen and that this route would also be favored as the cells become distended with deposits of starch and proteins.
The protein bodies in developing wheat grains also contain dark-staining inclusions of the globulin storage protein triticin (Bechtel et al., 1991), which is presumably transported via the Golgi to the vacuolar protein bodies.

The mechanisms which determine whether prolamins are retained within the ER or transported via the Golgi to the storage vacuole are not known, and it is not possible to recognize either classical ER retention signals (i.e. the C-terminal tetrapeptides KDEL or HDEL) or vacuolar targeting sequences in these proteins. Expression of wild-type and mutant forms of γ-zein and γ-gliadin in heterologous systems has demonstrated that the proline-rich repetitive sequences are required for ER retention (Torrent et al., 1994; Geli et al., 1994; Altschuler et al., 1993; Altschuler and Galili, 1994), and it is possible that these regions form protein-protein interactions leading to the formation of insoluble aggregates which accumulate directly in the ER rather than being transported to the Golgi and vacuole (Coleman and Larkins, 1999; Shewry, 1999).
Okita and co-workers have proposed the existence of a specific mechanism leading to the retention of prolamins in the ER of rice. This involves an interaction with the molecular chaperone BiP (binding protein) which may bind the nascent polypeptide and retain it in the ER until assembly into a protein body (Li et al., 1993; Muench et al., 1999). This mechanism has not so far been reported for other cereals.

1.3.5.4 Allergenicity of the seed storage proteins in wheat flours

The prolamin superfamily comprises three major groups of plant food allergens, 2S albumins, nonspecific lipid transfer proteins (nsLTPs) and cereal alpha-amylase/trypsin inhibitors (Breiteneder and Radauer, 2004; Mills et al., 2004). All of these low-molecular proteins are cysteine rich, have similar three-dimensional folds that are rich in alpha-helices, and are stable to thermal processing and proteolysis. The 2S albumins are a major group of storage proteins present in many dicotyledonous plants. They include major allergens from tree nuts and seeds such as Brazil nut, walnut, sesame, and mustard. The nsLTPs play an important role in plant defence against fungi and bacteria (Blein et al., 2002). They have a wide distribution in fruits, nuts, seeds and vegetables and have attracted much interest for being highly important allergens for almost exclusively Mediterranean atopic populations. The family of cereal alpha-amylase and protease inhibitors mediates a certain degree of resistance to insect pests that feed on plant tissues.
This protein family comprises allergenic members that are produced in wheat, barley, rice, and corn. Just like the 2S albumins and the nsLTPs, the members of this protein family are capable to sensitise susceptible atopic individuals through ingestion or inhalation.
Basically, three factors confer allergenicity to a plant protein: its abundance in the plant tissues, the stability to digestion and its aggregation properties.
For example, the enzyme ribulose-1,5-bisphosphate carboxylase, which accounts for 30–40% of total leaf protein, has never been reported as an allergen. In contrast, nsLTPs are potent allergens but not very abundant. Recently, a single ω-gliadin component, the so-called fast ω_5-gliadin, has been identified as the major allergen in wheat-dependent, exercise-induced anaphylaxis (Morita et al., 2003). Thus, the amount of protein alone does not explain its allergenicity. While abundance is an important factor, it is probably secondary to protein stability. A compact three-dimensional structure, ligand-binding properties, presence of disulphide bonds

and glycosylation are all important facors determining protein stability (Breiteneder and Mills, 2005). All these factors are relevant to both the resistance of proteins to denaturation by food processing and to the harsh conditions of the gastro-intestinal tract. Ligand-binding can also have the overall effect of reducing mobility of the polypeptide backbone, increasing both thermal stability and resistance to proteolysis. Some proteins form a cavity while others possess a tunnel into which ligands fit. nsLTPs, which possess a lipid-binding pocket, show increased stability when the pocket is occupied by fatty acids or phospholipid molecules. One of the structural features clearly related to stability is the presence of disulphide bonds. Both inter- and intrachain disulphide bridges constrain the three-dimensional fold such that perturbation of the structure by heat or chemicals is limited and frequently reversible. Some of the members of the wheat prolamin superfamily (in particular nsLTPs, 2S albumins, cereal alpha-amylase/trypsin inhibitors, ω-gliadins) are all important plant food allergens. They are characterized by the presence of a high number of disulphide bonds, which can have a significant stabilizing effect on protein structure.

Many plant food allergens are also able to associate with cell membranes or other types of lipid structures found in food and show a propensity to aggregate as a result of food processing (Breiteneder and Mills, 2005). The allergenic 2S albumin was shown to interact with phospholipid vesicles. This led to the proposition that such interactions might affect the uptake and processing of the allergen in the gastro-intestinal tract indicating that the biologic activity of these proteins plays a role in attenuating their allergenic potential. Similarly, there is emerging evidence that nsLTPs are also able to interact with lipid structures. A propensity of certain proteins to aggregate might affect their ability to sensitise by generally enhancing their immunogenicity. Both 7S and 11S globulins are highly thermostable. Thus interaction with lipids and the formation of larger aggregates contribute to the allergenicity of plant food proteins in conjunction with the amount of protein ingested and the stability to processing and digestion.

1.4. Bread and pasta making

Wheat is the basic ingredient of many types of food, such as bread, pasta, biscuits and cakes. They all require flour with different characteristics. The general terms “baking quality” and “pasta quality” usually refer to the specific properties required for the production of leavened bread or pasta.

While criteria governing bread quality may vary in different countries, pasta quality is more univocal.

Pasta was introduced in Italy from China to Marco Polo (1254-1323). “Macaroni” and “lasagne” were well known in Italy in the 14th century, and in the 15th century consumption of pasta products greatly leaped forward in Italy, possibly because durum wheat, growing in the southern parts of country, proved to be optimal for the preparation of alimentary pasta. Furthermore the climate, especially around Naples, was very suitable for drying pasta in the open air, due to the presence of humid air from the sea during daytime and dry air from inland at night (Hoseney, 1985, 1986).

High quality pasta should have a yellow colour and maintain its shape when cooked in boiling water; moreover it should give a firm bite (known as 'al dente') and its surface should not be sticky after it is cooked. Furthermore water should be free of starch and the pasta should be resistant to over-cooking.
Qualitative characteristics of wheat dough such as strength, elasticity and extensibility are very important to have a good end products, and they are largely determined by protein content and composition. Proteins in fact, during dough development, form a continuous network throughout the dough; in particular gluten proteins are the major determinant responsible for the dough properties.
The right combination of the dough properties is critical to produce food products with the optimum quality. For instance, bread and pasta making require gluten with a good balance of elasticity and extensibility, while pasta making also requires a strong gluten to retain starch during cooking.

1.4.1 Rheology

Most food systems containing wheat flour start with a mixing of flour, water and various other ingredients to form a dough. The formation of a cohesive and elastic dough is unique to wheat flour.
Understanding dough's rheological properties is important to predict the behaviour of the dough during flour processing and the quality of end-products. Rheology is the study of deformation including elasticity and viscosity, and rheological properties of gluten is largely determined by glutenin polymers size (and thus by the quantity of glutenin subunits), composition and organization.. The molecular size distribution of polymeric proteins and the ratio of the HMW-GS to the LMW-GS are strongly correlated with the dough properties: larger polymeric proteins and an increased ratio of HMW-GS to LMW-GS are correlated with better quality doughs.
There are various instruments to study rheological properties of the doughs and several simple assays are available to predict the visco-elastic properties of the wheat flour.

1.4.1.1 Assay methods of the dough properties

The Zeleny sedimentation test

By measuring sedimentation, it is possible to combine information on protein quantity and quality in one value. The sedimentation test is based on the principle that high-quality wheat proteins swell in a lactic acid solution more than poor-quality proteins do. The Zeleny protocol requires flour of specific fineness, which is usually produced by a specific mills. In practice the test is executed as follows: 3.2 g of flour is suspended in 50 ml water in a glass cylinder with a scale from 0–100 ml. The cylinder is shaken a few times to moisten the flour particles properly. Then 25 ml of a watery solution of lactic acid and isopropyl alcohol is added and the cylinder is shaken again. Then the proteins of the flour start to swell. Finally, the cylinder is left standing

upright for 5 minutes in order to allow the suspended protein to precipitate. Then the volume of the sediment is read off the cylinder scale, giving the sedimentation value (Figure 1.11).
The higher the gluten content in the flour and the better the bread-making propertiesof the gluten are, the more sediment will be formed. Thus, the sedimentation value is a measure of both the quantity and quality of the gluten (Zeleny, 1947).

The SDS sedimentation test

The Zeleny sedimentation test and the SDS sedimentation test work on the same principle but differ in details. The Zeleny test was developed in the USA in the late 1940s and has been adopted in many European countries.

Fig. 1.11: The Zeleny Sedimentation Test; the level of the sediment is an empirical measure of the baking quality of the sample flour. Taken from Belderok (2000).

The SDS sedimentation test was devised at the Flour Milling and Baking Research Association, Chorleywood (UK), some 30 years later, to avoid the necessity of a flour produced by a special mill. It has been widely used in the UK. Although it can be performed with flour, the SDS test normally uses wheat ground on a disc-type mill with a 1 mm sieve. Six grams of ground wheat are added to 50 ml water in a 100 ml measuring cylinder, and the meal is dispersed by vigorously shaking. The contents are re-shaken twice at 2 minutes intervals. Following the last shaking, 50 ml of a SDS-lactic acid solution is added and mixed by inverting the cylinder. Inversion is repeated at intervals of 2, 4 and 6 minutes. After the last inversion the content of the cylinder is allowed to settle for 20 minutes before the sedimentation volume is read (Dick and Quick, 1983).
An equation predicting Zeleny volumes from SDS volumes was derived. The rough rule of thumb is *Zeleny = SDS - 20*.

The mixograph

As early as 1933, Swanson and Working published the description of a 'recording dough mixer' suitable for the analysis of the quality of wheat flour. Initially, this mixograph was used quite successfully for quality breeding in USA and Australia. It found its way to Europe much later. There are three versions: the macro-mixograph, with a capacity of 30–35 g flour, and two micro-mixograph with a capacity of 10 g and 2 g of flour. The 2 g-mixograph has been developed to test wheat quality from early generations, but can be used not only for screening, but also to study the effect of individual subunits on dough quality, by adding or incorporating small amounts of purified protein (Bekes et al. 1994a, b).

The heart of the mixograph is a regularly rotating pin-kneading machine. Ten grams of flour are kneaded for 7 minutes with a fixed amount of water (usually 5.5–6 ml). The kneading time is registered on the horizontal axis and the power exerted on the dough on the vertical axis of graph paper passing through a recording device. In the beginning, the mixograph curve increases to a maximum and then decreases.

Several parameters may be read from a mixogram:

- the height of the curve, mainly dependent on the protein content of the sample and the amount of water added;
- the developing time of the dough, i.e. the time span between starting the mixing process and reaching the maximum level of the curve;
- the dough relaxation, i.e. the difference in height of the curve at its maximum and 3 minutes later;
- the tenacity of the dough, measured as the bandwidth after a fixed time.

All heights are read from the centre of the band.

The mixograph curves allow a breeder to recognize samples with very weak doughs (often sticky) and samples with extremely stiff doughs Both types of dough are unsuitable for bread and biscuit making (Belderok, 1977).

Mixograph analysis has also been recently employed to assay the dough functionality of flours derived from transgenic wheat with altered gluten protein composition (Alvarez et al. 2001).

Fig. 1.12: Micro-mixograph for the analysis of dough characteristics. Kneading basin (1) , pens (2) and recorder (3) are indicated. Taken from Belderok (2000).

1.5 Genetic improvement of wheat

Since wheat is indisputably one of the major food crops of the world and the single most important source of plant protein in the human diet, it has always been a target of choice for improvement programs.

Traditionally, genetic improvement in wheat was generated by using extensive crossing program and then systematically selection of useful new combinations (McIntosh, 1998).

Plant breeders have tried for decades to improve the yield of wheat by using conventional methods of breeding, but their efforts were reaching plateau especially with respect to yield.

Hence the genetic transformation of wheat has received considerable attention worldwide over the years with the purpose of increasing the grain yield, to improve the quality of wheat end-use products, to minimize crop loss due to unfavourable environmental conditions and with the aim of introducing resistances against various pests and pathogens (Rakszegi et al. 2001, Sahrawat et al. 2003, Jones 2005, Shewry and Jones 2005).

The last decade has witnessed the extensive use of recombinant DNA technology for introduction of exogenous DNA into major cereal crops including wheat (Repellin et al., 2001). The technology is based on the delivery of defined foreign genes into plant cells, obtaining integration of the genes into plant genome and subsequently plant regeneration from transformed cells or tissues.

The efficiency of transformation is greatly influenced by genotype, explant source and also medium composition. Therefore, most of the approaches to transform wheat have attempted to develop a genotype independent and cost effective procedure for the introduction of alien genes. The first transgenic wheat plants were produced by Vasil et al. (1992, 1993), Weeks et al. (1993), Nehra et al. (1994), Becker et al. (1994) and Altpeter et al. (1996a) employing microprojectile bombardment as a method of DNA delivery. Subsequently, the development of

methodology for the delivery of genes into intact plant tissues by bombardment of DNA-coated gold or tungsten particles has revolutionized the field of wheat transformation.

The first report of an agronomically trait related to plant defence (the coat protein of barley yellow mosaic virus) transferred to wheat was published in 1996 (Karunarante et al. 1996). Wheat is attacked by a number of viral, bacterial and fungal pathogens and also by insect and nematode pest which severely affect grain yield. 5-10% losses of total wheat production is reported just because of fungal pathogen (Pellegrineschi et al. 2001). Therefore, most of the work on genetic engineering of wheat for resistance against biotic stress has focused on developing protection against fungal pathogen (Dahleen et al. 2001). Chitinases, for example, a group of PR proteins, have always been regarded as agronomically important genes, since they are able to catalyze the degradation of chitin, an important component of the cell wall of many filamentous fungi. Chen et al. (1998) introduced a rice chitinase gene (*chi11*) under the control of constitutive CaMV 35S promoter into wheat employing particle bombardment and reported constitutive expression of the rice *chi11* gene in T_0 plants.

Bliffeld et al. (1999) introduced barley seed class II chitinase gene (*pr3*) driven by maize ubi promoter along with either a ribosome-inactivating protein (*rip*) gene or β-1,3-glucanase gene in Bobwhite cultivar of wheat using particle bombardment. Stable expression of transgenes was observed in successive three generations and transgenic plants showed increased resistance to infection with the powdery mildew-causing fungus *Erysiphe graminis*.

Bieri et al. (2000) introduced a barley ribosome-inactivating protein (RIP30) into wheat mediated by particle bombardment and reported the expression of RIP30 in transgenic wheat plants which resulted in moderate protection against *E. graminis* . Towards enhancing fungal resistance in transgenic wheat, Oldach et al. (2001) expressed three cDNAs encoding the antifungal protein AFP from the fungus *Aspergillus giganteus*, a barley class II chitinase and a barley type I RIP under the control of maize *ubiquitin1* gene promoter. This study showed not only the stable integration and inheritance of the chitinase and the *rip1* gene from barley and the fungal *afp* gene up to T3 generations, but also significant reduction of formation of powdery mildew or leaf rust colonies on transgenic wheat leaves expressing AFP or chitinase II when compared with control plants.

Zhang et al. (2001) introduced a mutant bacterial ribonuclease III gene (*rnc70*) in wheat using particle bombardment and reported that transgenic lines showed a high level of resistance to Barley Stripe Mosaic Virus infection in three progeny of transgenic plants.

As an alternative to the scheme based on utilization of Bt-endotoxin for creating insect resistant plants, plant or engineered inhibitors have been considered important tools for the building of resistance against insects in cereal including wheat (Jouanin et al 1998). Altpeter et al. 1999 transformed immature embryos of wheat with trypsin inhibitor gene (*itr1*) and reported increased insect resistance in transgenic wheat lines expressing trypsin inhibitor CMe.

In comparison to control, a significant reduction in the survival rate of the storage pest *Sitotroga cerealella* was also reported after rearing of larvae on transgenic wheat seeds expressing trypsin inhibitor CMe.

Transgenic wheat lines expressing resistance to wheat streak mosaic virus have also recently been achieved after introduction of viral coat protein gene and the replicase (NIb) gene of wheat mosaic virus (WSMV) into wheat via particle bombardment (Sivamani et al. 2000b, 2002). Transgenic wheat lines produced in this study showed various degree of resistance to WSMV.

Fusarium head blight (FHB) disease occurs in cereals throughout the world and causes necrosis of florets which results in moderate to severe reductions in grain yield (Dahleen et al. 2001). The primary causal agents of FHB are *F. graminearum* and *Fusarium culmorum* which produce deoxynivalenol (DON), a trichothecene mycotoxin that enhances disease severity and poses a health hazard to humans and monogastic animals. Enhanced trichothecene in tobacco was demonstrated by Muhitch et al. (2000) by expressing a trichothecene acetyltransferase (TRI101) gene from *Fusarium sporotrichioides*. Following this report, recently, Okubara et al. (2002) transformed Bobwhite cultivar of wheat with the *FsTRI101* gene driven by maize ubiquitin promoter via particle bombardment of immature embryos and reported partial protection against the spread of *F. graminearum* in inoculated wheat spikes.

As most agronomic traits are polygenic in nature, wheat genetic engineering will require the integration of multiple transgenes into the plant genome, while ensuring their stable inheritance and expression in succeeding generations.

Therefore, recent developments in wheat genetic engineering were aimed to the integration of multiple transgenes into the plant genome and coordinated expression of these transgenes in transformed plants.

In next decade, therefore, it is assumed that wheat transformation is going to play a very crucial role in complementing the conventional wheat breeding for generating novel cultivars with desirable characters.

Recently, Agrobacterium-mediated transformation has also emerged as a highly efficient alternative to direct gene delivery in a number of economically important crop plants, including wheat (Cheng et al. 1997).

However, irrespective of methods and genotypes used in attempts to transform wheat, the best results have been obtained by direct bombardment of scutellum of immature wheat embryos.

Target tissue	Source of the gene	Gene	marker	phenotype	reference
Immature embryo	Barley yellow mosaic virus	Coat protein (Cp)	*bar*	no data available	Karunarante et al. 1996
Immature embryo	*T. aestivum*	High molecular weight glutenin subunit 1Ax1	*bar*	Accumulation of glutenin subunit 1Ax1	Altpeter et al. 1996b
Immature embryo	*T. aestivum*	High molecular weight glutenin chimaeric subunit	*bar*	Accumulation of chimaeric glutenin subunit Dy10:Dx5	Blechl & Anderson, 1996
Embryogenic callus	*Bacillus amyloliguefaciens*	Barnase	*bar*	Nuclear male sterility	Sivamani et al. 2000a
Immature embryo	*T. aestivum*	High molecular weight glutenin subunits 1Dx5 and 1Ax1	*bar*	Increased dough elasticity	Barro et al. 1997
Immature embryo	*T. aestivum*	High molecular weight glutenin subunit 1Dx5	*bar*	Increased dough strength	Rooke et al. 1999
Immature embryo	*T. aestivum*	High molecular weight glutenin subunits 1Dx5 and 1Ax1	*bar*	Increased dough strength and stability	He et al. 1999
Immature embryo	*T. aestivum*	Natural occurring low molecular weight glutenin subunit	*bar*	Accumulation of low molecular weight glutenin subunit, alteration of dough functionality	Masci et al. 2003
Scutellum-derived calli	*T. turgidum*	Epitope -tagged Low molecular weight glutenin subunit	*bar, uid*	Accumulation of epitope-tagged low molecular weight glutenin subunit	Tosi et al. 2004
Embryogenic callus	*Hordeum vulgare*	Class II chitinase (*chiII*)	*bar*	Resistance to fungus *E. graminis*	Bliffeld et al. 1999
Immature embryo	*Oryza sativa*	Thaumatin-like protein (TLP), chitinase (chiII)	*bar, hpt*	Resistance to fungus *F. graminearum*	Chen et al. 1999
Immature embryo	*Hordeum vulgare*	Trypsin inhibitor (CMe)	*bar*	Increased insect resistance	Altpeter et al. 1999
Immature embryo	*Galanthus nivalis*	Agglutinin (gna)	*bar*	Decreased fecundity of aphids	Stöger et al. 1999
Immature embryo	Wheat streak mosaic virus	Replicase gene (NIb)	*bar*	Resistance to wheat streak mosaic virus	Sivamani et al. 2000b
Immature embryo	Monoclonal antibody	Single-chain Fv antibody (ScFvT.84.66)	*bar, hpt*	Production of functional recombinant antibody in the leaves	Stöger et al. 2000
Immature embryo	*Hordeum vulgare*	Ribosome inactivating protein (RIP)	*bar*	Moderate resistance to fungal pathogen *E. graminis*	Bieri et al. 2000
Immature embryo	*T. aestivum*	Protein puroindoline (PinB)	*bar*	Increased friabilin levels and decreased kernel hardness	Beecher et al. 2002
Embryogenic callus	*F. sporotrichioides*	*Fusarium sporotrichioides* gene (FsTRI101)	*bar*	Increased resistance to FHB (*F. graminearum*)	Okubara et al. 2002

Tab. 1.6: Some of the agronomically important genes transferred into wheat by particle bombardment. Redrawn from Sahrawat et al. (2003), modified.

1.5.1 Genetic manipulation of gluten protein composition

Good bread making quality is a key goal for wheat breeding. It is mainly determined by dough strength as a result of the interactions between gluten proteins.

In recent years, several efforts are being made to genetically transform wheat with different alien genes of agronomically importance and related to end-use quality (Blechl and Anderson, 1996; Altpeter et al. 1996b; Barro et al. 1997; He et al. 1999, Masci et al. 2003; Tosi et al. 2004). Of the many proteins that make up gluten, both the high molecular weight and the low molecular weight glutenin subunits (HMW-GS and LMW-GS, respectively) are particularly amenable to modification by transformation (Blechl and Anderson, 1996; Altpeter et al., 1996b, Masci et al., 2003). However, most of the published work to modify grain composition focused on the introduction of additional HMW-GS genes. For example, Blechl and Anderson (1996) demonstrated that a hybrid Dy10/Dx5 HMW glutenin subunit protein was over-expressed in the model wheat cultivar "Bobwhite" and accumulated to levels similar to native HMW glutenin subunits. The same year, Altpeter et al. (1996b) reported that a genomic 1Ax1 clone, also transgenically introduced into Bobwhite, resulting in a marked increase in total HMWG subunit protein. Mixograph studies on dough from transgenic lines expressing genes encoding 1Ax1 and 1Ax1 plus 1Dx5 showed a step-wise increase in both the peak dough resistance and the mixing time (Barro et al., 1997). In one particular transgenic line, highly overexpressing the 1Dx5 transgene, the proportion of HMWG subunit proteins in the gluten fraction was increased from 12% to over 20% with the dough proving too strong for conventional bread making (Rooke et al., 1999). The lines described by Barro et al. (1997) were also grown in replicate field trials and subjected to small-scale milling and baking tests which revealed changes in elastic modulus of the gel protein fraction, loaf volume and crumb structure between transgenic lines and controls (Darlington et al., 2003).

Since also LMW-GS are important determinants of the visco-elastic properties of the wheat dough, and their quantity has been correlated with the quality of wheat end-use products (Gupta et al. 1989, Payne 1987, Payne et al. 1987a, b, c) recent papers (Masci et al. 2003; Tosi et al. 2004) focused on transgenic approaches aimed at the introduction into bread and pasta wheat of additional copies of these genes. Masci et al. (2003) were the first to report the transformation of bread wheat cv. Bobwhite with a LMW-GS gene driven by its own promoter. They extensively characterized an independent line where the transgenic LMW-GS accumulated in the endosperm at very high levels. They showed that the transgenic polypeptides were incorporated into the glutenin polymers, nearly doubling the glutenin overall amount in the seeds. However, the SDS sedimentation test values were lower for the transgenic flour compared to a non-transgenic control, predicting a drastic change in the technological properties of the transgenic wheat flour. Masci et al. (2003) observed, too, that a high level of transgene(s) overexpression was constantly associated with lower levels of other storage proteins, including HMW-GS and gliadins.

The effects of the overexpression of the transgenic LMW-GS on the abundances of the other seed proteins, both in mature and developing caryopsis, is the main subject of this thesis.

1.6 Safety assessment for the biotech crops

Since this thesis deals with the transcriptional and proteomic differences existing between a particular transgenic wheat line and its non-transformed counterpart, the results achieved here permit an evaluation, yet preliminary, about their equivalence.

Many recent studies focused about the issue of "substantial equivalence" with the attempt to establish globally agreed guidelines for the safety assessment of foods and food ingredients derived from genetically modified (GM) organisms.

Since the first discussions on strategies to assess the food safety of GM crop plants, assessment of GM plants and derived tissues has been based on comparisons with their traditionally bred counterparts. This was termed the Principle of Substantial Equivalence (IFBC, International Food Biotechnology Council, 1990; Kok and Kuiper, 2003).

The underlying assumption was – and still is – that traditional crop plant varieties currently on the market have not been elaborately tested in the laboratory before being marketed.

However, because they have been consumed (after appropriate processing) for decades, they have gained a history of safe use. This history of safe use can be used as a baseline for the safety assessment of new GM plant varieties derived from established plant lines. The comparative concept for the safety evaluation of foods derived from GM crops has further been elaborated by the Organisation for Economic Cooperation and Development (OECD, 1993).

OECD proposes that food safety evaluation issues of foods derived from GM crops should comprise all of the following:

- molecular characterization of the introduced genetic fragment and resulting new proteins or metabolites (in addition, an increasing number of European member states routinely ask for characterization of the insertion point of the transgenic fragment);
- analysis of the composition of the relevant plant parts with respect to key nutrients and anti-nutrients, including natural toxins and potential allergens;
- potential for gene transfer of specific genes from the GM food to, particularly, microorganisms in the human and animal gastro-intestinal tract;
- potential allergenicity of the new gene products, or alteration of the intrinsic allergenicity of the GM food organism;
- estimated intake levels of the newly introduced proteins as well as of the final product, including any altered constituent;
- a toxicological and nutritional evaluation of the resulting data; and
- additional toxicity testing (of the whole food).

With regard to the last point, toxicity testing of the whole crop or derived plant products might be required, for example, in cases where the composition of the whole crop has been changed significantly compared with the traditional counterpart, or where there is a need to further investigate potential unintended side effects of the genetic modification.

Specific guidances on these issues has also been provided by the European Commission, Scientific Committee on Foodstuffs (EU 97/618/EC, 1997) and by the United Nations Food and Agriculture Organisation/World Health Organisation (FAO/WHO, 1996, 2000, 2001).
The Principle of Substantial Equivalence, as it was formalized by the OECD (basically, the evaluation of the equivalence based on the comparison of the GM line with the parent line and then with other traditionally bred varieties already on the market) was introduced with the aim of establishing a scientifically sound approach that would meet global acceptance.
However, it soon became clear that the principle left much scope for individual (and national) interpretations.
Further concerns established that the principle could only be applied on the basis of a compositional analysis of the varieties under scrutiny (the GM line and its traditional counterpart). In addition, the compositional comparison is the starting-point of the food safety evaluation and not – as an end-point in itself. Once differences in composition have been identified between the GM food plant and its appropriate comparator, targeted toxicological and nutritional studies should be carried out to assess the safety and nutritional impact on humans (Fig. 1.13) Thus, toxicological and nutritional testing is an essential part of the safety assessment model for foods derived from GM crops.

1.6.1 Methods to detect and assess unintended effects of a genetic modification

Concerns that unintended and unexpected side effects might occur in GM organisms (GMOs) as result of the genetic modification process, thereby impacting on human and animal health, has attracted attention from both scientific and public groups. However, the potential occurrence of side effects in non-GM organisms must also be highlighted (Kuiper et al. 2001). Compositional analyses of the GM plant and its traditional counterpart, in addition to the notion that relevant unintended side effects might remain undetected when analysing only specific compounds, are complicated issues. It was therefore encouraged that more general, unbiased methods of analysis be developed to detect relevant changes in a much larger part of the physiology of the plant (OECD, 1993 and European Commission Scientific Steering Committee, 2000). This could be of particular importance for GM plants that have multiple genes inserted, which possibly have a higher occurrence of unexpected and unintended effects.
Unbiased fingerprinting approaches at the level of DNA, gene expression, proteins, metabolites and their secondary structures, could potentially provide a more thorough insight into any unpredicted changes in the physiology of the plant that might go undetected when focusing on single compounds.

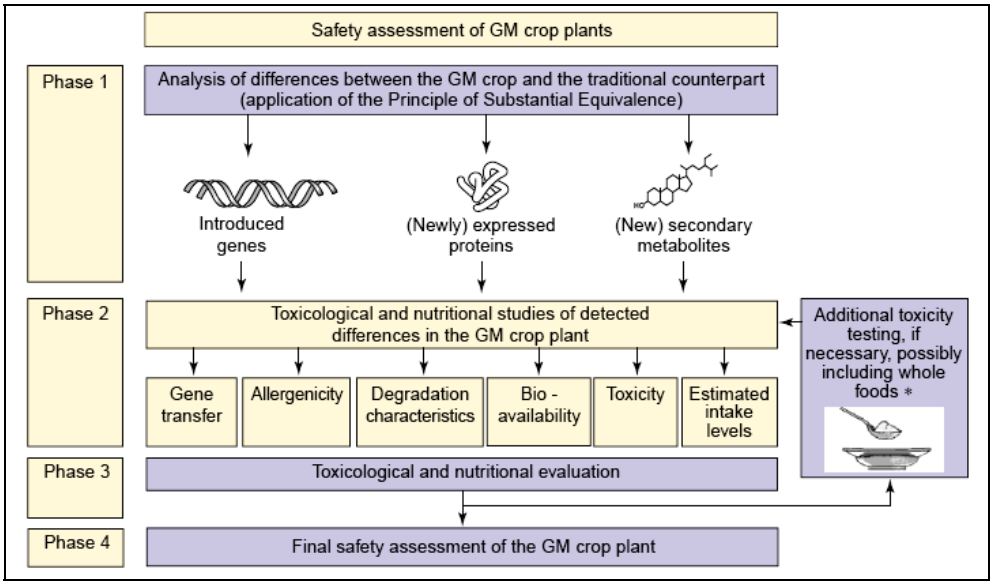


Fig. 1.13: A proposed pipeline for the safety assessment strategies for genetically modified (GM) - crop-derived foods. Taken from Kok and Kuiper (2003).

DNA analysis

Localization and characterization of the place(s) of insertion are the most direct approaches to predicting and identifying possible occurrence of (un-) intended effects due to transgene insertion in recipient plant DNA. Data for transgene-flanking regions will give leads for further analysis, in the case of a transgene insertion within or in the proximity of an endogenous gene. Transgene chromosomal location and structure can be detected by various methods such as genomic in situ hybridization (Iglesias et al., 1997) and fluorescence in situ hybridization (Pedersen et al., 1997), and by direct sequencing of flanking DNA (Spertini et al., 1999; Thomas et al., 1998).

Gene expression analysis

The DNA microarray technology is a powerful tool to study gene expression. The study of gene expression using microarray technology is based on hybridization of mRNA to a high-density array of immobilized sequences (immobilized probes), each corresponding to a specific gene. mRNAs from samples to be analysed are labelled by incorporation of a fluorescent dye and subsequently hybridized to the array. The fluorescence at each spot on the array is a quantitative measure corresponding to the expression level of the particular gene. The major advantage of the DNA microarray technology over conventional gene profiling techniques is that it allows small scale analysis of expression of a large number of genes at the same time, in a sensitive and quantitative manner (Schena et al., 1995, 1996). Furthermore, it allows

comparison of gene-expression profiles under different conditions. cDNA and oligo arrays may effectively be used to screen for altered gene expression and, at the same time, provide initial information on the nature of detected alterations, whether the observed alteration(s) may affect the safety or nutritional value of the food crop under investigation.

Proteomics

The most direct method of investigating unpredicted alterations is proteomic analysis of the tissue of interest. Considerable expertise in 2D gel electrophoresis has enabled the simultaneous screening of large number of proteins with subsequent characterization by mass spectrometry (MS) and/or N-terminal sequence data.

Proteomics can be divided into three main areas: (i) identification of proteins and their post-translational modifications; (ii) “differential display” proteomics for quantification of the variation in contents; and (iii) studies of protein-protein interactions.

The method most often used for analysing differences in protein pattern is 2D PAGE (mainly IEFxSDS-PAGE, i.e. isoelectrofocusing x sodium dodecyl sulphate polyacrylamide gel electrophoresis) followed by excision of

protein spots from the gel, digestion into fragments by specific proteases, and subsequently analysis by mass spectrometry (peptide mass fingerprinting). It allows the identification of proteins by comparing the mass of peptide fragments with data predicted by genetic or protein sequence information. When searching for unintended changes by 2-D PAGE, the first step is to compare proteomes of the lines under investigation. If differences in protein profiles are detected, normal variations should be evaluated. If the profiles are outside normal variations, identification of the protein must be carried out, which may lead to further toxicological studies. Moreover, metabolic changes may be looked at if the identified protein has a known enzymatic activity.

There are still few examples of the use of proteomics to study alterations in the composition of a genetically modified plant (FAO/WHO, 2000, Lehesranta et al. 2005 and this thesis). In the FAO/WHO report a targeted change in the level of a specific protein resulted in other proteins being affected. The improvement in rice storage proteins by antisense technology resulting in low-glutelin genetically modified rice for commercial brewing of sake has been associated with an unintended increase in the levels of prolamins (FAO/WHO, 2000).

2. AIM OF WORK

Wheat improvement by genetic transformation remains controversial. One of the major issues is the potential for unexpected side effects as a result of the genetic modification process. Comparative safety assessment should include extensive compositional analyses of the GM wheat lines and of its traditional parental controls. The large-scale technologies for expression profiling available today are promising tools for the screening of unintended effects.

The experiments described here were aimed at the comparative evaluation of the potential equivalence of a particular transgenic bread wheat line with its corresponding wild type genotype.

Since the transgenic wheat line here considered over-expresses at high levels a LMW-GS (Low-Molecular-Weight Glutenin Subunit), which is a seed protein correlated with the end-use quality of flours, we have carried out a comparative microarray and proteomic profiling, during the period of seed filling, to identify the differentially expressed genes and proteins, with the aim of both verifying the principle of substantial equivalence and testing the effect of transgene integration and expression over the expression of seed proteins.

During our initial characterization studies on the progeny of this transgenic line (Masci et al. 2003), we noticed that a high level of of transgene over-expression was constantly associated with lower levels of other storage proteins, we thus decided to broaden our evaluation of differential expression using large scale transcriptional and proteomic profiling.

3. MATERIALS AND METHODS

3.1 Genetic material

The UBI:BAR (Cornejo et al. 1993) and pLMWF23A plasmid DNA clones, the former conferring resistance to the herbicide BASTA, and the latter containing a LMW-GS gene coded at the *Glu-D3* locus isolated from the bread wheat cultivar Cheyenne (Cassidy et al. 1998), were previously used for wheat transformation. The pLMWF23A clone contains, in the *Eco*RI site of pBluescript (Stratagene, La Jolla, CA), the coding region of a LMW-GS gene flanked by about 1100 bp and 1600 bp of the 5' and 3' flanking regions, respectively (Cassidy et al. 1998). The coding region, including the signal peptide, is 921 bp and the molecular weight of the deduced peptide is 32,842 Da (Fig. 3.1).

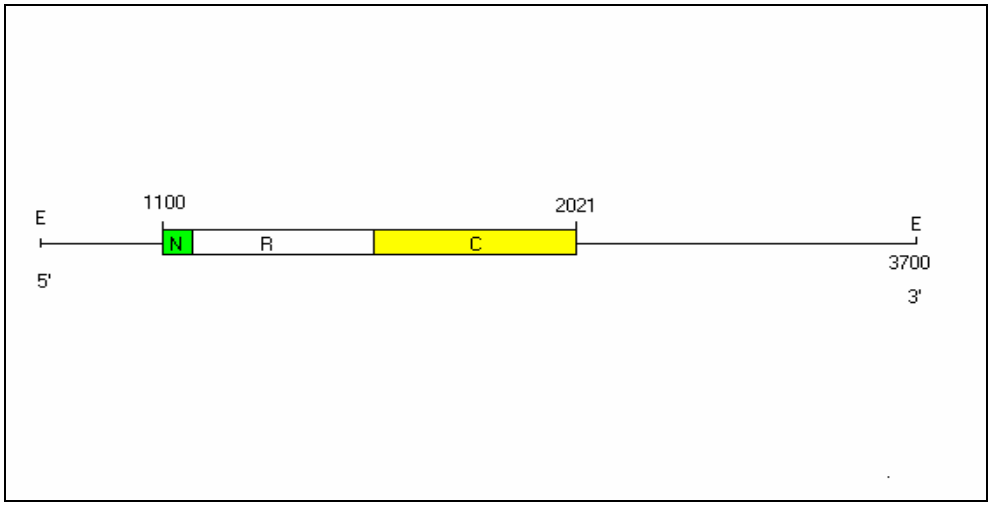


Fig. 3.1: Diagram representing the genomic clone F23A encoding the LMW-GS used for the genetic transformation experiments. N, R and C indicate the N-terminal, repetitive and C-terminal domain, respectively, within the LMW-GS coding sequence. E indicates the *Eco*RI sites used for cloning F23A into the pBluescript vector.

The UBI:BAR and pLMWF23A plasmid DNAs (12.5 μg each) were previously used to co-transform wheat using microprojectile bombardment (Masci et al., 2003).

Eleven independent lines were selected based on their ability to grow on culture media containing bialaphos. Because it was not possible to verify the presence of the transgene by PCR, due to its high sequence similarity with endogenous LMW-GS, all the selected lines were checked for the expression of the transgenic LMW-GS by SDS-PAGE analysis of the proteins extracted from endosperm tissue of T_1 seeds (Fig. 3.2). During our initial characterization studies (Masci et al., 2003) we have determined that, of the eleven bialaphos-resistant independent lines, only one showed detectable over-expression of the transgenic LMW-GS encoded by pLMWF23A. The transgene-encoded LMW-GS accumulated to very high levels in

seeds of this line (Fig. 3.2), and we have determined that it was incorporated into the glutenin polymers, nearly doubling their overall amount.
We thus decided to focus on the progeny of this single line to carry out the pilot comparative microarray and proteomic profiling described here.

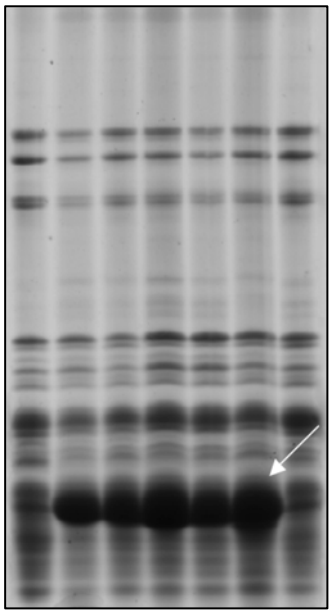


Fig. 3.2: SDS-PAGE of protein extracts from different T1 seeds belonging to the line showing over-expression of the transgenic LMW-GS. Seed proteins extracts from un-transformed cv. Bobwhite are included for comparison (first and last lane). The arrow shows the position of the transgenic LMW-GS.

3.2 Microarray profiling

3.2.1 Plant material and seed collection strategy

Triticum aestivum seeds (cv. Bobwhite), belonging to the transgene-overexpressing line (which has been selfed for eight generations after the transformation) and to the wild-type genotype were germinated and grown in a greenhouse under controlled, identical conditions.
Heads were tagged when the first anther was exposed (0 DPA). Developing seeds were then harvested at 10, 20, 30 and 35 DPA by flash-freezing the whole spikes in liquid nitrogen and collecting the caryopses on dry ice.
Several heads from different individual plants were tagged in the same day. Each collection tube, containing seeds belonging to the same timepoint and genotype, harvested in the same day, was kept separated. An individual plant, in which some of the spikes had already been

tagged for seed collection at a certain timepoint, was not used again for a second tagging-collection step at the same timepoint.

In our original experimental design, we planned to include four timepoints in the analysis (10, 20, 30 and 35 DPA). However, we observed that caryopses of different sizes were present when collecting seeds belonging to a single timepoint. We decided, in order to make comparisons from both genotypes between seeds at the same developmental stage, to re-classify and pool the seeds from a single timepoint according to morphological criteria (seed colour and dimensions). The seeds collected at 10 DPA were then splitted into two groups: “early” and “late” (according to the different length across the seed longitudinal crease, and thus to the volume of the endosperm), whereas only green seeds, yellow-green seeds and yellow seeds, of equal dimensions, were collected from 20, 30 and 35 DPA, respectively (Fig. 3.3).

a.

b.

c.

d.

e.

f.

Fig. 3.3: Samples of the developing wheat caryopses used for microarray and proteomic profiling. a) 10 DPA "early"; b) 10 DPA "late"; c) 20 DPA; d) 30 DPA; e) 35 DPA; f) comparison of the five morphologies.

At the end of the seed collection, tubes containing less than 1 g of caryopses belonging to the same genotype, timepoint and morphology were pooled together. We finally obtained two (10 DPA early) and three (10 late, 20, 30 and 35 DPA) unique sample pools, from which independent RNA isolations (biological replicates) have been performed. In this way, within each timepoint, each biological replicate effectively represents RNA deriving from different pools of seeds, each one coming from different groups of plants.

Frozen developing caryopses were stored at -80°C until RNA isolation.

3.2.2 Experimental design and replication

In this thesis we will conform to the nomenclature proposed by Duggan et al. (1999); we will thus refer to the solution containing the ssDNA retrotranscribed from the RNA samples as the target, and to the ssDNA printed on the solid substrate of the array as the probe.

A direct, two-sample, multiple dye swap comparison experimental design was applied (Kerr 2003). For each timepoint (10 early and late, 20, 30 and 35 DPA), RNA from both the wild-type and the transgenic genotype was reverse-transcribed, labelled (see protocols reported below) and the pooled target solution was then applied to the cDNA array. All hybridizations have been technically replicated (involving the same RNA samples) by swapping of the dyes. Each timepoint was represented by two (10 DPA early) or three (10 late, 20, 30 and 35 DPA) pooled biological replicates. In diagram 3.4, the microarray experimental design is illustrated according to the layout nomenclature proposed by Kerr and Churchill (2001).

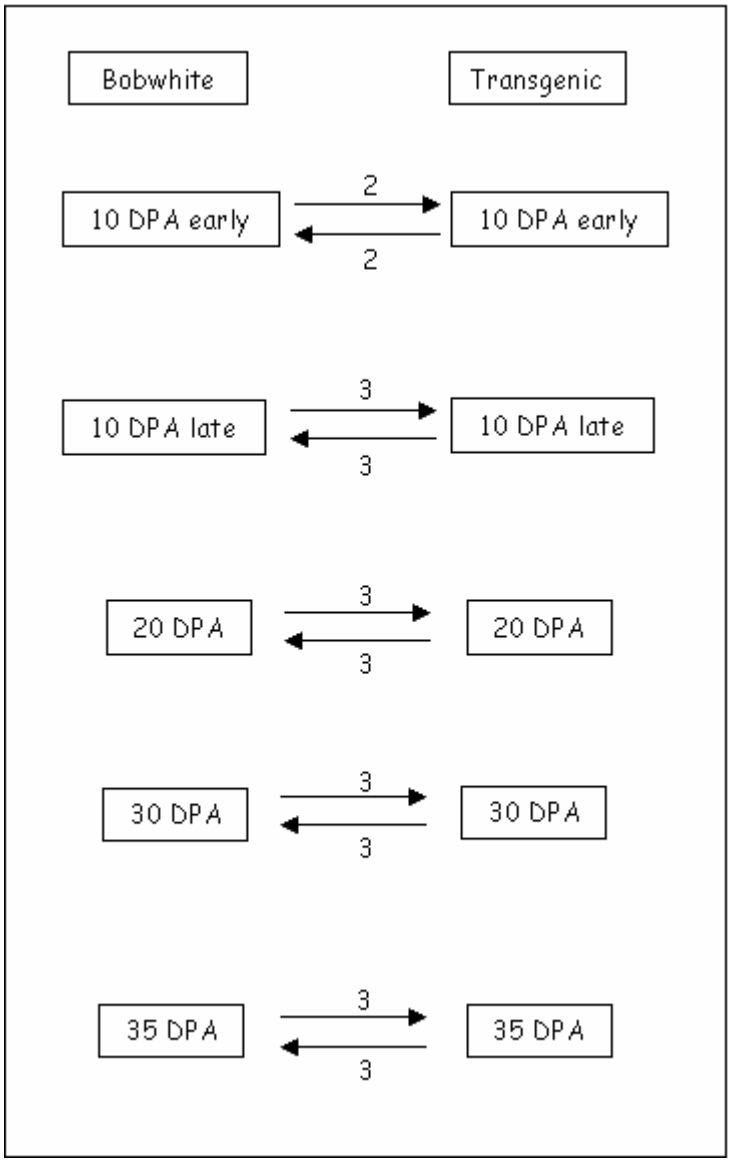


Fig. 3.4: Diagram showing the experimental design adopted for the microarray hybridizations. Boxes indicate RNAs sources (sample variety and timepoint). Arrows represent arrays. We assigned one dye to the tail and the other dye to the head of directed arrows. Numbers indicate replicated hybridizations.

To assess the array reproducibility, control experiments in which the same RNA sample was labelled with Alexa 647 and Alexa 555 (Molecular Probes) were performed ("yellow test", data not shown). Using the normalization and data analysis procedures described below, none of the clones represented on the array were found to be statistically differentially expressed (with a FDR-corrected p-value less than 0.01). Globally, only 2,4 % of the clones (260 clones out of 10800) showed > 1.5 fold-variation; and only 0.64 % (70 clones out of 10800) showed > 2 fold-variation. These data are comparable to those reported by Ruuska et al. (2002) regarding the technical degree of variation of a cDNA array.

3.2.3 RNA isolation and purification

Total RNA from developing caryopses was prepared using TRIzol reagent. A pre-extraction step, prior to the add of TRIzol, was performed in order to reduce the co-precipitation of starch and other polysaccharides with RNA. Briefly, 1 g of developing caryopses was ground to a fine powder with mortar and pestle in liquid nitrogen, transferred to a tube containing 5 ml of 50 mM Tris, 200 mM NaCl, 1% Sarcosyl, 20 mM EDTA, 5 mM DTT and extracted once with 5 ml of Phenol:Chloroform:Isoamyl alcohol (25:24:1 v/v). After adding 10 ml of TRIzol Reagent to the acqueous phase, all subsequent steps were performed according to the TRIzol manufacturer's instruction.
Isolated total RNA was then treated with RQ1 RNase-free DNase (Promega), followed by a clean-up step using RNeasy spin columns (Qiagen). RNA integrity and concentration were determined using the 2100 Agilent Bioanalyzer.

3.2.4 Target synthesis

10 ug of each RNA sample were first incubated at 70°C for 5 minutes in the presence of 0.5 ug of oligodT23 (Sigma), 5 ng of the spiking controls mix and 2 ul of the Lucidea Scorecard mRNA test/reference mix (Amersham), in a total volume of 9 ul. After chilling on ice for 1 minute, a reaction mix with a final composition of 0.5 mM each of dATP, dCTP, dGTP, 0.15 mM dTTP, 5 mM dithiothreitol, 200 U Superscript II (200 U/ul), 1x Superscript II buffer, 2 mM aminoallyl-dUTP (Molecular Probes) and 20 U RNAsin (40 U/ul) was added for a total reaction volume of 20 ul. The reaction mixture was incubated at 42°C for 90 minutes, followed by 70°C step for 15 minutes to inactivate the reverse transcriptase. Following addition of 1 ul 250 mM EDTA pH 8.0 and 4 ul 1 N NaOH, the reaction was incubated at 65°C for 20 minutes to degrade the RNA template, and then neutralized with 4 ul 1 N HCl. The samples were purified using the YM30 Microcon filters (Millipore) according to the instructions of the manufacturer, eluted in 50 ul of RNAse-free water and concentrated to a final volume of 5 ul using a speedvac concentrator. Purified amine-modified cDNA were then coupled independently to the fluorescent dyes Alexa 647 and Alexa 555 (Molecular Probes) according to the manufacturer's protocol. After incubation in the dark, at room temperature, for 1 hour, coupling reactions were quenched by adding 4.5 ul 4 M hydroxylamine and incubated again at room temperature for 15 minutes. Labeled cDNAs from the wild type and the transgenic genotype were pooled, according to the layout of the hybridizations, and further purified using the YM30 Microcon filters (Millipore) according to the instructions of the manufacturer. Targets were finally eluted in TE and completely dried in a speedvac concentrator.

3.2.5 Wheat cDNA Array construction

We used cDNA clones from 37 cDNA libraries generated by a consortium of U.S. and international researchers (Zhang et al. 2004) within the framework of a NSF funded project

("The structure and function of the expressed portion of the wheat genomes", NSF Award Abstract - #9975989). The libraries have been produced from Chinese Spring wheat (Triticum aestivum L.) and from other hexaploid (Cheyenne, Brevor, Butte 86, etc.), tetraploid (Triticum turgidum L.) and diploid (Triticum monococcum L.) wheat genotypes. The emphasis in the choice of plant materials for library construction was reproductive development (anther, embryo, endosperm and whole grain) subjected to environmental factors that ultimately affect grain quality and yield, but roots and other tissues were also included. The unique genes represented by the ESTs were determined by clustering the ESTs sequences using PHRAP (www.phrap.org). A clone representing each contig and the clone for each of the singletons were re-arrayed to use as probes for the arrays. Plasmid DNAs were isolated from each clone using the 96-Perfect Prep kit from Eppendorf AG (Hamburg, Germany). Clone inserts were amplified using the universal M13 forward and reverse primers. PCR amplifications were carried out in an MJ Research Tetrad Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) for 30 cycles with 57°C annealing temperature and 2.5 minutes extension time. Amplicon size, yield and integrity were determined by resolving 5 ul of the PCR product in a 1% agarose gel. Amplicons were purified using QIAquick 96 PCR purification kit (QIAGEN Inc., Valencia, CA), dried and resuspended in 50% DMSO as printing buffer. DNA probes (approximately 300 ng/ul) were single-spotted from 384-microtiter plates onto Corning UltraGAPS slides (Corning Inc., NY) using Omnigrid 100 robot (Genomics Solutions, Ann Arbor, MI) with CHP3 pins (Telechem, Sunnyvale, CA). The printed slides were UV-crosslinked at 300 mJ before use. Clone insert identities were verified by re-sequencing using the ABI Big Dye terminator mix (Perkin-Elmer, Wellesley, MA).

The wheat cDNA array used in the experiment contains 10800 features, with 7920 clones. Of these, 2304 are unique kernel clones, deriving from the developing endosperm libraries of bread cv. Bobwhite (5-30 DPA) and from the whole-kernel cDNA libraries of cv. Butte 86, which have been subjected to different biotic stresses and environmental regimes. The remaining 5616 clones constitute deletion-lines mapped probes and derive from many different cDNA libraries. To determine the number of unique genes encoded by the ESTs, the sequences were assembled using the gene clustering program PHRAP (www.phrap.org). Sequences that group together to form a consensus sequence are designated as tentative contig (TC); those that failed to assemble into contigs are designated tentative singletons (TS). Each TC and TS are assumed to encode a tentative unique gene. The sum of TC and TS gives an estimate number of tentative unique genes represented on the array. Together, the 7920 clones of the wheat cDNA array cluster into 7437 tentative unique genes. The putative identity of each EST was determined by searching the non-redundant database of NCBI using BlastN and BlastX (Altschul et al. 1997). The best annotation was extracted using an in-house written PERL script and used to annotate each gene.

3.2.6 Array hybridization

Prior to hybridization, cDNA microarray slides were pre-hybridized at 42°C, for 1 hour, with a in-house made solution containing 5x SSC, 0.2% SDS and 1% BSA. Slides were then washed in milli-q water for 1 minute and dried by centrifuge spinning for 2 minutes at 2000 RPM.
Dried fluorescent targets were first resuspended with 65 ul of Pronto! Universal Hybridization Solution (Corning), followed by incubation at 95°C for 5 minutes and cooling at room temperature. After a short centrifuge spin the resuspended target was deposited on the array and gently spread all over the printed surface applying the coverslip (22 mm x 60 mm, SIGMA).
Slides were sealed in humidified chambers and submerged in a pre-heated water bath at 42°C. Hybridizations were performed in the dark for 12-16 hours.
Slides were washed and dried according to the post-hybridization wash protocol of the Pronto! Universal Microarray Hybridization kit (Corning).

3.2.7 Scanning parameters, data analysis and presentation

Hybridized arrays were scanned simultaneously at 635 and 532 nm using a GenePix 4000B Scanner (Molecular Devices, Sunnyvale, CA), equipped with GenePix Pro 6.0 data acquisition software.
A first, preliminary, normalization was achieved adjusting the PMT voltages so that the fluorescent intensities curves in the high range of the red and green channels were almost overlapping (whole image intensity ratio around 1). Under these settings the color of the spiking control spots was roughly yellow.
For each slide in the experiment, a .gpr file, containing the raw signal and background intensities, was generated. In order to extract a list of differentially expressed genes for each timepoint between the wild-type and the transgenic genotype we considered each timepoint as a separate experiment ("single timepoint analysis"). The related .gpr files were then directly imported into the R language v. 2.1.0 for statistical computing (www.r-project.org) (Ihaka and Gentleman, 1996) for further normalization and analysis.
In the R environment, all analyses were done using the software package LIMMA (Linear Models for Microarray Analysis) (Smith 2005b; Smith and Speed 2003), freely available for download from the website of the Bioconductor project (www.bioconductor.org) (Gentleman et al. 2004).
For the single timepoint analysis, we first visualized the dye and spatial distortion of the raw data plotting, for each slide, the MA-plot (Dudoit et al., 2002), where:

$$M = \log_2\left(\frac{R}{G}\right)$$

Eq. 1.1

(R=red fluorescent intensity of the dye Alexa 647 and G=green fluorescent intensity of the dye Alexa 555) and:

$$A = \log_2 \sqrt{RG}$$

Eq. 1.2

After subtracting background intensities from all data, and in order to compensate for the dye biases and spatial distortions present, we applied a global (involving all genes), local (for each print-tip group of the array) and intensity-dependent lowess fit to the MA-plot (smoothing parameter=0.3) (Yang et al. 2002). M values were also scale-normalized between arrays (Yang et al. 2002; Smith and Speed 2003) thus providing better consistency for selecting the DE genes from the slides making up each single-timepoint experiment. In order to extract the lists of differentially expressed genes we used the multiple t-testing approach implemented in LIMMA (Smith 2004; Smith et al. 2005). Briefly, after specifying the technical/biological structure of the data, LIMMA performed, for the contrast of interest (wild-type Vs. transgenic) and for each gene, a one-sample t-test using a moderated t-statistic (Smith 2004; Smith et al. 2005; Lönnsted and Speed, 2001). The moderated, gene-specific t-statistic is the ratio of the normalized M value to its standard error:

$$t^* = \frac{\overline{M}}{\frac{s_p}{\sqrt{n}}}$$

Eq. 1.3

where: t^*= moderated t statistic;

$\overline{M}$ = average value of the normalized M;

s_p= pooled standard deviation;

n = number of measurements.

An extreme t-statistic, with its associated p-value, is a statistical evidence for differential expression. We based our selection of the differentially expressed genes on the P-values associated with the moderated t-statistics ($P<0.05$ or $P<0.01$, according to the selected timepoint), after FDR (False-Discovery-Rate) correction for multiple comparison (Benjamini and Hochberg, 1995).

Moreover, to generate the transcript profiles reported here ("single channel analysis of two-color data"), the raw data from all the slides in the experiment (stored as .gpr files) were imported once again into R and within-slide normalized in LIMMA using the same procedure as above.

However, in this case, the within-slide normalized data were also “quantile” normalized between arrays so that the same distribution of the dye intensities across all channels was achieved (Bolstad et al. 2003). The between-arrays normalized two-color intensities (Eq. 1.2) and ratios (Eq. 1.1) were converted to single-color intensity data and imported into Microsoft Excel for further analysis and data presentation. In Microsoft Excel the data related to the wild-type and to the transgenic genotype were separately combined and averaged to obtain, for each gene at each timepoint, a mean-absolute value of the expression level. For each gene, these values were then divided by the expression value of the first timepoint, and ratios were then rescaled by setting the lowest ratio to 1. The ratios thus obtained were used to plot the transcript profiles reported in this thesis. For the purposes of data presentation a clone was considered reliable if its average signal intensity was greater than 200 intensity units and more than twice the average background intensity, in at least one channel (Galbraith, 2003). This data presentation procedure was shown to better visualize the magnitudes of the variation in the gene expression levels (Ruuska et al., 2002). Up-regulated genes, with respect to the wild type genotype, were also analysed through the hierarchical clustering algorithm (distance measure: average linkage) using the Cluster and Treeview program (Eisen et al. 1998).

3.2.8 Classification of the differentially expressed genes

For each timepoint of the microarray experiment a list of differentially expressed ESTs was generated. The Genbank IDs of the ESTs from the five lists were then processed together using the gene clustering program PHRAP (www.phrap.org) to determine the number of differentially expressed (DE) unique genes. The putative identity of each DE unique gene was determined by searching the non-redundant database of NCBI using BlastN and BlastX (Altschul et al. 1997). The best annotation (the one with the lowest E-value) was extracted using an in-house written PERL script and used to annotate each differentially expressed unigene. The lists of the best BlastN and BlastX hits thus obtained, containing the GenBank IDs of the putative identities of the differentially expressed genes, were separately processed and submitted to the NMC/eGOn v 2.0 annotation database (www.genetools.no). The NMC Annotation Database v 2.0 collects and compiles data from UniGene (NCBI), EntrezGene, SwissProt and Gene Ontology (GO). The output data from the NMC database query were exported to Microsoft Excel for visualization.

3.3 Comparative analysis of seed-storage proteins

3.3.1 Isolation of albumins and globulins from wheat flours

Pools of seeds from the wild-type genotype and from its corresponding transgenic line were separately crushed and albumins were extracted with ultrapure water (1 mg: 5 μl) for ten minutes at room temperature; the samples were then centrifuged for 10 minutes at 13000 RPM; the supernatant thus obtained showed a strong enrichment for albumins. The remaining pellet was washed twice with ultrapure water, extracted with NaCl 0.5 M (1 mg: 5 μl according to the

initial weight) for ten minutes at room temperature and centrifuged for 10 minutes at 13000 RPM. The supernatant contained globulins (Singh and Skerritt, 2001).

3.3.2 Isolation of gliadins

Pools of immature caryopses or mature seeds from the wild-type genotype and from its corresponding transgenic line were separately crushed and gliadins were extracted for two hours at room temperature with a buffer containing 10% (v/v) dimethylformamide and 1x Protease Inhibitor Cocktail (SIGMA), in a 1 mg : 10 ul ratio. After a centrifugation at 13.000 RPM of 10 minutes, aliquots of the supernatant were loaded on a acid PAGE gel for immediate analysis or dried down in a Savant SpeedVac (Savant, Farmingdale, N.Y.) and stored at -20 °C.

3.3.3 Isolation of glutenins

Pools of seeds from the wild-type genotype and from its corresponding transgenic line were separately crushed and monomeric gliadins were removed by triple extraction with 1 ml of 50% (v/v) 1-propanol at room temperature for 30 min. The total glutenins were then extracted from the remaining pellet with a buffer containing 2 M Urea, 2 % (v/v) Triton X-100, 40 mM Tris-HCl pH 8.8, 1 mM EDTA, 10 mM iodoacetamide and 1% (w/v) DTT for 1 hour at room temperature, in a ratio 1 mg: 8 ul with respect to the dry weight. After a centrifugation of ten minutes at 13000 RPM, the supernatant contained the glutenins. Aliquots of the same volume, both from the wild-type genotype and from its corresponding transgenic line, were then analyzed by means of 2D-electrophoresis.

3.3.4 Enrichment procedure for the D-group of low-molecular-weight glutenin subunits

In order to obtain a fraction enriched in D-type low-molecular weight glutenin subunits, we used the procedure reported by Masci et al. (1999). Briefly, pools of seeds from the wild-type genotype and from its corresponding transgenic line were separately crushed and total glutenins were extracted with a buffer containing 50% (v/v) 1-propanol, 50 mm Tris-HCl pH 8.0 and 1% (w/v) DTT, for 30 minutes at room temperature. Solubilization of glutenin polymers was aided by sonication for 10 seconds at 50 W power setting. after a centrifugation of 10 minutes at 10000 g, pure 1-propanol was added to the supernatant to reach a final concentration of 60% (v/v) and HMW-GS were selectively precipitated at 4°C for 1 hour. After a centrifugation of 5 minutes at 10000 g, pure 1-propanol was again added to the supernatant to reach a final concentration of 70% (v/v) and precipitation was performed at 4°c for 1 hour. After a centrifugation of 5 minutes at 10000 g, a pellet enriched in D-type low-molecular-weight glutenin subunits and ω-gliadins was obtained.

3.3.5 Enrichment procedure for the B- and C-groups of low-molecular-weight glutenin subunits

In order to obtain fractions enriched in B or C subunits, we used different precipitation steps involving increasing concentrations of 1-propanol, based mainly on the methods described by Verbruggen et al. (1998). The procedure used was as follows: wheat seeds (from the wild-type genotype and from its corresponding transgenic line) were crushed with a mortar and pestle to obtain 130 mg of flour, and gliadins were removed by triple extraction with 1 ml of 50% (v/v) 1-propanol at room temperature for 30 min. Each step was followed by 5 min of centrifugation at 14000 g. Total glutenin subunits were extracted (1 mg:5 µl) with a buffer containing 50% (v/v) 1-propanol, 80 mM Tris-HCl, pH 8.5, 20 mM dithiothreitol (DTT) at 60°C for 30 min. After centrifugation at 14000 g for 10 min, a freshly prepared solution of pure 1-propanol (at room temperature) and 1% DTT was added to the supernatant containing the glutenin subunits to reach the final 1-propanol concentration of 60% (v/v). The precipitation was performed at 4°C for 1 h. After 20 min of centrifugation at 12,000 g, sequential precipitation was performed on the supernatants by adjusting the 1-propanol concentration first to 75%, then to 80% (this latter step resulted in pellets strongly enriched in B subunits) and finally to 85% in the same conditions as above. In this last step, the pellet was discarded, and the supernatant was stored at 4°C overnight, in order to allow specific precipitation of C subunits, which were obtained as a pellet after centrifugation for 20 min at 12000 g. Pellets containing either B or C subunits were dried-down in a Savant Speed-Vac (Savant, Farmingdale, N.Y.) concentrator and stored at −20°C until used.

3.3.6 Acid polyacrylamide gel electrophoresis (Acid-PAGE)

The gliadin extracts from developing caryopses or from mature seeds were analysed by polyacrylamide gel electrophoresis in aluminium lactate buffer, pH 3.2. In this system, migration is determined mainly by the net positive charge on the protein molecules. The procedure was those of Khan et al. (1985), with minor modifications.

3.3.7 Two-dimensional electrophoresis (Acid-PAGE x SDS-PAGE) of gliadins, B-, C- and D-type low-molecular-weight glutenin subunits

In order to analyse the pattern of gliadins, B-, C- and D-type low-molecular-weight glutenin subunits in more detail, two-dimensional electrophoresis (Acid-PAGE x SDS-PAGE) was performed on fractions obtained from the gliadin isolation or after the 1-propanol precipitation steps reported above. The procedure used, partly based on that proposed by Morel (1994), was as follows:

First dimension (Acid-PAGE)

The first-dimension electrophoresis was carried out in acidic solution with a Biorad (Hercules, CA) mini Apparatus for polyacrylamide gels (0.75 mm, T=6 and C=2.67) containing 4 M urea and 0.75% (v/v) acetic acid, and were polymerised by using 7.5 mM ammonium persulfate and 0.6% (v/v) TEMED. A pre-run with normal polarity at constant current (16 mA/gel) was performed with 0.75% (v/v) acetic acid for 50 minutes.

The dried samples (either enriched fractions of the B-, C- or D-type low-molecular-weight glutenin subunits or the dried gliadins extracts) were dissolved in 10 µl of a solution containing 0.14% acetic acid, 6 M urea, and methyl violet as a tracking dye (1% w/v DTT was added for the B- and C-type low-molecular-weight glutenin subunits). Solubilisation was improved by sonication (Branson 3200, Bransonic, Shelton, Conn.) for 5 min.

For the first-dimension electrophoresis, the upper chamber contained 0.14% (v/v) acetic acid and the lower contained 0.25% acetic acid. The run was carried out at constant current (12 mA/gel) with reversed polarity (upper electrode positive), and was stopped 5 min after the dye reached the bottom of the gel. The gels were stained according to Neuhoff et al. (1988) and de-stained in distilled water.

Second dimension (SDS-PAGE)

The second-dimension electrophoresis was performed in the same apparatus used for the first dimension, and gels were the same size. The main gel was T=12 and C=2, and contained 0.38 M Tris-HCl, pH 8.8 and 0.1% SDS. The stacking gel (T=3.75, C=2.67, 0.125 M Tris-HCl, pH 6.8, 0.1% SDS) was poured up to about 0.5 cm from the top of the glass plates, without a comb. Once the first-dimension gel had been stained, lanes containing samples to be submitted to the second-dimension separation were cut and equilibrated for 30 min at room temperature in a solution containing 10% glycerol, 2% SDS, 0.125 M Tris-HCl at pH 6.8, and 0.5% DTT. The equilibrated gels were placed on top of the second dimension gel without using agarose to seal them to the second dimension gel. The run was carried out at a constant voltage (200 V), and was stopped when the Coomassie Brilliant Blue used to stain the first dimension reached the bottom of the gel (about 50 minutes). The running buffer contained 0.2 M glycine, 25 mM Tris at pH 8.8 and 0.1% SDS. Gels were stained overnight according to Neuhoff et al. (1988) and de-stained in tap water.

3.3.8 Electroblotting and amino acid sequencing

Electroblotting of the 2-D patterns was performed using a X Cell II blot module (Invitrogen Carlsbad, CA). Sequi-blot PVDF (polyvinylidene difluoride) membranes (Bio-Rad, Hercules, CA) were wetted in methanol and rinsed with deionized water for 5 minutes before soaking in electroblot buffer (10 mM CAPS [3-cyclohexylamino-1-propanesulfonic acid], pH 11). Filter paper (Whatman 3 MM) was also soaked in electroblot buffer before use. After 2-D electrophoresis, unstained gels were soaked in electroblot buffer for 5-10 minutes. Transfer was performed for 1 hour at 4°C, at a constant voltage of 100 V. Transfer stack was then dismantled

and the membrane was rinsed with distilled water for 5 minutes before staining with coomassie blue (0,025 % (w/v) coomassie R-250, 40 % (v/v) methanol) for 5 minutes.. The membranes were then de-stained for 5 minutes in 50 % (v/v) methanol, briefly rinsed in distilled water and allowed to air dry at room temperature. Spots of interest were excised using a clean razor blade, and amino acid sequencinq was performed according to the procedure reported by Tao and Kasarda (1989).

4. RESULTS AND DISCUSSION

4.1 Overview

4.1.1 General considerations about the microarray experimental design

One major objective of this study was to describe the patterns of steady state mRNA abundances during the accumulation of storage reserves in developing wheat kernels. The patterns of mRNA abundances were compared between the wild type bread wheat cv Bobwhite and its corresponding transgenic line over-expressing a LMW-GS (low-molecular-weight glutenin subunit), a seed storage protein whose quantity is expected to be positively correlated with dough quality. In this study the term "gene expression" refers to these mRNA abundances. Transcriptional regulation demonstrated to provide strong control of major processes involved in seed development and metabolism, and the wheat cDNA microarray used in this study offered the opportunity to examine changes in mRNA levels for thousands of genes simultaneously.

The present study provides a description of the transcriptional differences induced by the integration and expression of a block of transgenes in an independent transgenic line. The "control" sample line we decided to use in our comparisons has been the wild type genotype; and thus the genetic transformation effects have been actually confounded with the variations induced in gene expression by the integration and expression of both the selectable marker gene UBI:BAR and the *lmw-gs* transgenes. Nevertheless, the use of the wild type variety as a control line offers the opportunity to monitor the global differences present between this particular transgenic wheat line and its non-transformed counterpart, thus allowing to verify the so-called *principle of substantial equivalence*, which clearly dictates the comparison with traditionally bred counterparts as the procedure for the safety assessment of GM plants and tissues (Kok and Kuiper, 2003). However, the microarray analysis showed that the UBI:BAR gene may not be expressed during the seed development. A probe encoding the BAR gene is present on the arrays, and no detectable signal was observed throughout the five timepoints analysed. Silencing of the UBI:BAR gene in wheat transgenic lines obtained by particle bombardment has often been reported in the literature (Srivastava et al. 1996, Cannel et al. 1999, Demeke at al. 1999, He et al. 1999 and A. E. Blechl, personal communication). Moreover, FISH analysis showed co-localization of the constructs encoding the UBI:BAR and *lmw-gs* transgenes within the pericentromeric region of chromosome 5D (Carozza et al. 2005). Monosomic analysis also showed that overexpression of the LMW-GS was associated to the presence of the 5D chromosome pair, thus confirming the 5D as the unique, or at least the main integration site responsible for the over-expression of the *lmw-gs* transgene(s).

Moreover, the pilot nature of this study needs to be highlighted. The experiment described here focused on a single independent transgenic line, which has already been characterized in detail (Masci et al. 2003). A study whose aim is the determination of the transcriptional and proteomic consequences of a genetic transformation event (and of the subsequent integration and expression of the transgene(s)) should include the parellel comparison of several independent

transgenic wheat lines; and this is advisable in order to draw stronger conclusions and give general insights about the issue of transgenic modification of crop plants.

The particular nature of this transgenic genotypes makes it difficult, if not impossible, to detect the transgene presence if the protein product is not strongly overexpressed, because actually the transgene corresponds to an endogenous gene, which is also controlled, like all other endogenous *lmw-gs* genes, by its own promoter. Actually, the initial aim of the transformation experiment was to obtain lines overexpressing a *lmw-gs* gene in order to understand its effect on qualitative properties of the derived flour (Masci et al. 2003). The transformation experiment at the origin of this work gave rise to only one transgenic line that strongly overexpressed the additional *lmw-gs* gene. For this reason we could focus only on this line. Thus, the microarray experiments described here involve RNA sources from a single independent transgenic wheat line. However, validation of the results here obtained about the differential expression will be compared with other independent lines, in which the same transformation procedure, along with the same marker gene and same genotype, have been used. . Nevertheless, we think that this approach is also valid and justified in the context of research based on microarrays, whose research-related costs, amount of data and related complexity of statistical analysis do not permit an extensive experimental design based on the comparisons from several independent transgenic lines.

With the aim of both extrapolating the effect of the overexpression of a single *lmw-gs* gene on the expression of the other endosperm genes, and to detect its effect on quality in different genetic backgrounds, we introgressed the *lmw-gs* transgenes on several bread wheat lines by means of classical breeding. The validation of the microarray and proteomic data on these materials will allow the separation of the genetic transformation effects, thus assessing the analytical contribution to the global genetic variation of the over-expression of the *lmw-gs* genes.

4.1.2 Dual nature of the microarray experiment and transformation of the dataset

Each of the arrays in the experiment described here makes a direct comparison between the wild type and transgenic RNA targets, in the five selected timepoints during seed development. In our original experimental design there were no arrays comparing the wild-type and transgenic targets within the timepoints (e.g.: a comparison of the wild type targets between 20 and 30 DPA). Since there are no arrays linking, independently, the wild-type and the transgenic targets *within* their respective timepoints, the experiment carried out here is an example of an *unconnected* design. In this situation it is not possible to make comparisons, on the basis of log-ratios alone, within the timepoints of the wild-type and transgenic targets in a separate way. However, the software packages described here allowed us to analyze the separate single-channel log-intensities instead of log-ratios, on the basis of a mixed model representation proposed by Wolfinger et al. (2001). For this purpose, LIMMA implemented several single-channel normalization algorithms (Smith, 2005a); making thus possible to correlate normalized gene intensity measurements even from arrays not linked by a direct hybridization (Fig. 4.1, red arrows). This has allowed us to transform *in silico* the design, and thus the dataset of the

experiment, from a direct two-sample comparison to a time-course experiment (Fig. 4.1). The availability of single-channel normalization algorithms for a two-color dataset made also possible the generation of the transcript profiles reported here.

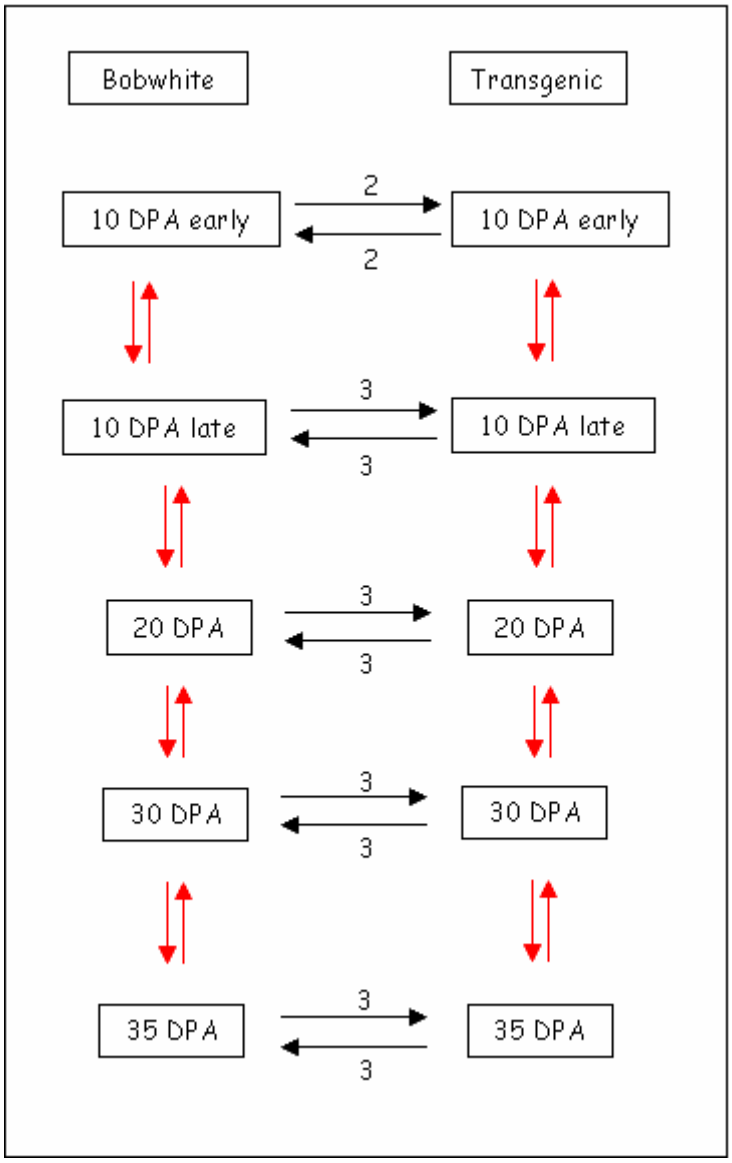


Fig. 4.1 Diagram showing the *in silico* transformation of the microarray experimental design. Boxes indicate RNAs sources (sample variety and timepoint). Black arrows represent arrays (directed hybridizations, where numbers indicate number of replicates). The single channel normalization procedure applied to the entire dataset allowed the correlation of the intensity signals even from the unconnected arrays (red arrows).

4.1.3 Nature of the proteomic profiling

It is clear that changes in gene expression are not always reflective of changes in protein levels; thus part of the transcriptional differences observed in this study during the period of seed filling

have also been confirmed by comparative proteomic profiling, on selected classes of seed storage proteins, at the level of mature grains. The proteomic comparisons described in this study involved the comparison between the wild type, the transgenic-overexpressing genotype and the null samples of the transgenic line, i.e. seeds from genetically transformed plants obtained through the segregation of transgenes from T_1 heterozygous individuals and thus negative for the presence of both *lmw-gs* and UBI:BAR transgenes (Carozza et al. 2005). Including also the null individuals in the proteomic analysis allowed us, in some cases, to separate the contribution of the genetic transformation effects from the variability induced by the integration and expression of the *lmw-gs* and UBI:BAR transgene(s). At the level of protein abundances, on mature grains, results showed that the null samples were indistinguishable from the wild type genotype; and the differences we observed at the protein level were then attributed to the joint effect of the integration and expression of the *lmw-gs* transgenes.

4.2 Identification of differentially expressed genes during the period of grain filling between the LMW-GS over-expressing line and the wild type genotype

Seed development from pollination to mature dessiccated grain generally occurs over 45 days in bread wheat plants grown in standard condition; proteins and starch, the main seed storage reserves, usually accumulate in a linear fashion with respect to the kernel dry weight, between 10 and 35 DPA (Altenbach et al. 2003). We chose to focus our study over this period, during which the transgenes are expected to be transcribed and thus most of the gene expression differences are likely to occur. To perform a detailed study we set up a kinetic analysis over five timepoints. A kinetic analysis with multiple sequential timepoints increases the temporal resolution and thus allows discrimination between early, medium and late responses to the transgene(s) expression. Through the analysis of comparative microarray experiments over the selected timepoints we identified the putative differentially expressed genes and generated their transcript profiles in developing grains.

4.2.1 Overall gene regulation throughout the seed development

The total number of statistically-significant differentially expressed (from now on indicated as DE) clones for each timepoint analysed according to the LIMMA analysis is shown in Tab 4.1. Considering an fdr-corrected P-value threshold of 0.05, a total number of 995 clones were called as significant by LIMMA: 98,6% (981 out of 995) of the clones occurred over four timepoints, from 10 DPA "late" to 35 DPA, with only 14 clones (1,4%) DE in the first timepoint analysed, 10 DPA "early" .

Timepoint	Replicated Hybs	fdr-corrected P-value threshold	Number of DE clones called by LIMMA analysis
10 DPA "early"	4(2)	0.05	14
		0.01	1
10 DPA "late"	6(3)	0.05	174
		0.01	69
20 DPA	6(3)	0.05	360
		0.01	142
30 DPA	6(3)	0.05	303
		0.01	174
35 DPA	6(3)	0.05	144
		0.01	19

Tab. 4.1.: The total number of differentially expressed clones identified from the microarray comparisons for each timepoint analysed. The number shown in parentheses in the "replicated hybs" column indicates the individual biological replicates.

The GenBank IDs of all the 995 significantly-regulated clones were submitted together to BlastX analysis (Altschul et al. 1997) and their putative identity (the BlastX hit with the lowest E-value) was extracted using an in-house PERL script program and used to annotate each clone. Tab. 3.2 reports all the recent annotations for each DE ESTs, with the corresponding BlastX E-value. Tab 4.2 does not include the redundant DE clones that occurred over the five timepoints analysed (the redundant clones are those that showed up as DE, in more than one timepoint, irrespective of the sign of their variation; these clones are listed only once in Tab. 4.2).

Out of 995 DE clones, only 542 are the unique, redundancies-free, DE clones. Among these, only those with a BlastX E-value < 10^{-5} are shown in Tab. 4.2. The remaining 167 DE clones (31%) had a very poor annotation by BlastX analysis; and some of them completely lacked any matches. However, among clones listed in Tab. 4.2, some functional classes were clearly over-represented (Tab. 4.3).

We have also tested the hypothesis of a position effect, induced by the *lmw-gs* or UBI:BAR transgene blocks, as a possible explanation to the large variation observed in mRNA

abundances, during seed development, between the transgenic and the wild type genotype. Almost half of the 995 significant DE clones are mapped probes, and their chromosomal location is known.

We thus downloaded from the wheat ESTs database (http://wheat.pw.usda.gov/wEST/) the mapped loci information regarding the DE clones, in order to check if a significant fraction of the clones were mapped on the 5D chromosome, thus strengthening the idea of a cis-acting effect responsible for differential expression, or on a specific chromosome different from the 5D, thus reinforcing the hypothesis of a preferential, trans-acting mechanism to explain differential expression of the geneset. However, among the significant DE clones for which map information was available, no particular trend was detected, and the clones were randomly distributed overall the wheat genome (data not shown).

In order to get additional information about the putative function of many differentially regulated genes, we submitted the lists of the Best BlastN and BlastX GenBank IDs hits to the GO database (www.geneontology.org) through the eGOn v. 2.0 interface hosted on the GeneTools website (www.genetools.no).

The eGOn v. 2.0 software retrieved current gene ontology (GO) terms for the BlastN and BlastX GenBank IDs submitted, for each of the GO categories: biological process, molecular function and cellular component.

Out of the 542 BlastN GeneBank IDs, corresponding to the best hits of the significant DE unique clones, only 9 (1,7%) had GO annotations; similar results were obtained submitting the 496 BlastX GenBank IDs, corresponding to the best hits of the significant DE unique clones, and only for 26 out of 496 genes GO annotations were available (5,2%).

Even lower results were obtained when both reporter lists were submitted, always through the GeneTools interface, against the NMC databases, comprising several general databases (Swissprot, NCBI HomoloGene, etc.) along with the Unigene assemblies of NCBI from several different organisms (including the Unigene build #44 of *Triticum aestivum*).

Globally, less than 10% of the significant DE unique clones matched a unigene name from the NCBI. This is consistent with the current status of genome annotation in wheat, and is of course a major issue in the biological interpretation of the gene expression dataset presented here.

GenBank ID	GenBank ID BlastX BestHit	BlastX E-val best hit	BlastX Best Hit (description)
BQ805672	NP_568742	4,00E-35	11-beta-hydroxysteroid dehydrogenase-like; protein id: At5g50600.1, supported by cDNA: gi_15450584, supported by cDNA: gi_17065383, supported by cDNA: gi_17386149, supported by cDNA: gi_20148648 [Arabidopsis thaliana]
BE591113	NP_177794	9,00E-66	12-oxophytodienoate reductase (OPR1); protein id: At1g76680.1, supported by cDNA: 38527., supported by cDNA: gi_18650649 [Arabidopsis thaliana]
BE423495	CAA54153	6,00E-53	12s globulin [Avena sativa]
BE605229	P31673	4,00E-15	17.4 KD CLASS I HEAT SHOCK PROTEIN
BE423111	BAB78490	1,00E-53	26S proteasome regulatory particle non-ATPase subunit12 [Oryza sativa (japonica cultivar-group)]
BE637521	BAB78495	1,00E-114	26S proteasome regulatory particle triple-A ATPase subunit4b [Oryza sativa (japonica cultivar-group)]
BE424523	NP_181528	2,00E-39	26S proteasome regulatory subunit; protein id: At2g39990.1,

			supported by cDNA: gi_15027934, supported by cDNA: gi_17064959, supported by cDNA: gi_20259172, supported by cDNA: gi_20260039, supported by cDNA: gi_2351375 [Arabidopsis thaliana]
BE398933	BAB92932	4,00E-38	40S ribosomal protein S23 [Oryza sativa (japonica cultivar-group)]
BE423428	O24573	2,00E-13	60S ACIDIC RIBOSOMAL PROTEIN P0
BE424723	NP_195865	1,00E-07	60S ribosomal protein - like; protein id: At5g02450.1, supported by cDNA: 30903., supported by cDNA: gi_11908069, supported by cDNA: gi_12642883, supported by cDNA: gi_13265482, supported by cDNA: gi_16323203 [Arabidopsis thaliana]
BE424414	Q40649	2,00E-61	60S RIBOSOMAL PROTEIN L10-3 (QM/R22)
BE424089	NP_200875	1,00E-47	60S ribosomal protein L12 - like; protein id: At5g60670.1, supported by cDNA: 42276., supported by cDNA: gi_14517557, supported by cDNA: gi_18700231 [Arabidopsis thaliana]
BE423964	P35684	8,00E-83	60S RIBOSOMAL PROTEIN L3
BE424257	NP_181471	3,00E-38	60S ribosomal protein L35; protein id: At2g39390.1, supported by cDNA: 11583., supported by cDNA: gi_14334861, supported by cDNA: gi_17104642 [Arabidopsis thaliana]
BE424340	P49210	2,00E-35	60S RIBOSOMAL PROTEIN L9
BQ804689	AAL85887	1,00E-109	70 kDa heat shock protein [Sandersonia aurantiaca]
BE443191	AAL85887	7,00E-64	70 kDa heat shock protein [Sandersonia aurantiaca]
BE423574	AAL85887	7,00E-52	70 kDa heat shock protein [Sandersonia aurantiaca]
BE352633	CAD32683	1,00E-105	acyl-[acyl-carrier protein] thioesterase [Triticum aestivum]
BE590521	AAO85795	2,00E-28	adenine phosphoribosyltransferase form 2 [Oryza sativa (indica cultivar-group)]
BE424657	EAA03396	9,00E-06	agCP3088 [Anopheles gambiae str. PEST]
BE499808	AAG42502	4,00E-97	alcohol dehydrogenase [Hordeum vulgare subsp. spontaneum]
BE424228	CAA65313	4,00E-65	alpha purothionin [Triticum aestivum]
BQ804641	AAN32704	3,00E-26	alpha/beta-gliadin [Triticum aestivum]
BE399836	P04724	1,00E-21	ALPHA/BETA-GLIADIN A-IV PRECURSOR (PROLAMIN)
BE399237	P04724	1,00E-05	ALPHA/BETA-GLIADIN A-IV PRECURSOR (PROLAMIN)
BE438402	P04726	5,00E-34	ALPHA/BETA-GLIADIN CLONE PW1215 PRECURSOR (PROLAMIN)
BE605158	P32032	2,00E-72	ALPHA-2-PUROTHIONIN PRECURSOR
BE399986	S16920	6,00E-29	alpha-amylase inhibitor - wheat
BE399060	P01085	3,00E-21	Alpha-amylase inhibitor 0.19 (0.19 AI) (0.19 alpha-AI)
BE425004	P01085	5,00E-14	Alpha-amylase inhibitor 0.19 (0.19 AI) (0.19 alpha-AI)
BE590835	P01085	1,00E-13	Alpha-amylase inhibitor 0.19 (0.19 AI) (0.19 alpha-AI)
BE399843	P01084	2,00E-12	ALPHA-AMYLASE INHIBITOR 0.53
BE590709	P10846	1,00E-06	ALPHA-AMYLASE INHIBITOR WDAI-3
BE424123	S18241	7,00E-72	alpha-amylase inhibitor, tetrameric, chain CM17 precursor - wheat
BE422447	P16850	3,00E-68	ALPHA-AMYLASE/TRYPSIN INHIBITOR CM1 PRECURSOR (CHLOROFORM/METHANOL-SOLUBLE PROTEIN CM1)
BF292893	P16159	6,00E-79	ALPHA-AMYLASE/TRYPSIN INHIBITOR CM16 PRECURSOR (CHLOROFORM/METHANOL-SOLUBLE PROTEIN CM16)
BE606678	P16851	6,00E-75	ALPHA-AMYLASE/TRYPSIN INHIBITOR CM2 PRECURSOR (CHLOROFORM/METHANOL-SOLUBLE PROTEIN CM2)
BE424957	P16851	8,00E-62	ALPHA-AMYLASE/TRYPSIN INHIBITOR CM2 PRECURSOR (CHLOROFORM/METHANOL-SOLUBLE PROTEIN CM2)
BE423717	P17314	2,00E-56	ALPHA-AMYLASE/TRYPSIN INHIBITOR CM3 PRECURSOR (CHLOROFORM/METHANOL-SOLUBLE PROTEIN CM3)
BE399348	P17314	6,00E-29	ALPHA-AMYLASE/TRYPSIN INHIBITOR CM3 PRECURSOR (CHLOROFORM/METHANOL-SOLUBLE PROTEIN CM3)
BE399372	T06564	1,00E-38	alpha-amylase/trypsin inhibitor homolog precursor - wheat
BE422742	AAA17741	2,00E-38	alpha-gliadin
BE422727	CAB76957	6,00E-29	alpha-gliadin [Triticum aestivum]
BE423527	CAB76957	3,00E-21	alpha-gliadin [Triticum aestivum]
BE422769	CAB76957	4,00E-10	alpha-gliadin [Triticum aestivum]
BE423477	CAB76959	3,00E-27	alpha-gliadin [Triticum aestivum]
BE424082	T06282	1,00E-07	alpha-gliadin precursor - wheat
BE438304	AAA96524	1,00E-12	alpha-gliadin storage protein
BG607362	AAG16905	8,00E-85	alpha-tubulin [Oryza sativa]
BE606407	NP_187733	1,00E-08	arginine-tRNA-protein transferase, putative; protein id: At3g11240.1, supported by cDNA: gi_13877874, supported by cDNA: gi_15810658 [Arabidopsis thaliana]

BE399819	NP_703259	9,00E-07	asparagine-rich antigen Pfa35-2 [Plasmodium falciparum 3D7]
BE398302	BAC41386	1,00E-48	asparaginyl endopeptidase REP-2 [Oryza sativa (japonica cultivar-group)]
BE423948	AAL09741	2,00E-88	AT4g05320/C17L7_240 [Arabidopsis thaliana]
BE399175	BAA20412	1,00E-52	A-type cyclin [Catharanthus roseus]
BG262302	JG0015	4,00E-16	avenin N9 - oat
BE637568	B36433	7,00E-21	avenin precursor - oat
BE590484	P80356	1,00E-15	Avenin-3 precursor (Prolamin)
BG262472	P80356	5,00E-15	Avenin-3 precursor (Prolamin)
BE399079	BAB91949	8,00E-30	B1003B09.3 [Oryza sativa (japonica cultivar-group)]
BF482436	BAB67981	1,00E-58	B1088D01.3 [Oryza sativa (japonica cultivar-group)]
BQ806155	T06486	4,00E-44	barwin homolog wheatwin2 precursor - wheat
BE424295	BAA96835	7,00E-48	beta 2 subunit of 20S proteasome [Oryza sativa (japonica cultivar-group)]
BE606197	P30271	2,00E-85	BETA-AMYLASE (1,4-ALPHA-D-GLUCAN MALTOHYDROLASE)
BQ805269	P93594	1,00E-127	BETA-AMYLASE (1,4-ALPHA-D-GLUCAN MALTOHYDROLASE)
BG274774	P93594	3,00E-75	BETA-AMYLASE (1,4-ALPHA-D-GLUCAN MALTOHYDROLASE)
BE426242	P93594	7,00E-41	BETA-AMYLASE (1,4-ALPHA-D-GLUCAN MALTOHYDROLASE)
BE590745	BAA04815	1,00E-108	beta-amylase [Hordeum vulgare subsp. vulgare]
BE423446	BAA04815	6,00E-82	beta-amylase [Hordeum vulgare subsp. vulgare]
BG262481	BAA04815	1,00E-81	beta-amylase [Hordeum vulgare subsp. vulgare]
BE423380	BAA04815	1,00E-67	beta-amylase [Hordeum vulgare subsp. vulgare]
BE424405	BAA04815	2,00E-55	beta-amylase [Hordeum vulgare subsp. vulgare]
BE422952	BAA04815	1,00E-49	beta-amylase [Hordeum vulgare subsp. vulgare]
BQ168043	AAG25638	2,00E-68	beta-amylase [Hordeum vulgare]
BE438336	AAG25638	6,00E-08	beta-amylase [Hordeum vulgare]
BE422773	AAM73779	1,00E-58	beta-expansin [Oryza sativa]
BE424562	O81395	5,00E-20	BIFUNCTIONAL DIHYDROFOLATE REDUCTASE-THYMIDYLATE SYNTHASE (DHFR-TS) [INCLUDES: DIHYDROFOLATE REDUCTASE ; THYMIDYLATE SYNTHASE]
BQ807006	AAC64163	2,00E-29	blue copper protein [Zea mays]
BE422743	P29518	9,00E-28	Brittle-1 protein, chloroplast precursor
BE424367	AAF06696	2,00E-11	bZIP transcription factor [Nicotiana tabacum]
BE590469	T02033	5,00E-70	calcium/calmodulin-dependent protein kinase homolog - maize
BE591089	AAK92225	2,00E-73	calcium-binding protein 1 [Hordeum vulgare]
BE424387	AAK92225	9,00E-55	calcium-binding protein 1 [Hordeum vulgare]
BE590695	A29639	1,00E-84	carboxypeptidase D (EC 3.4.16.6) - wheat
BQ804153	T05701	1,00E-127	carboxypeptidase D (EC 3.4.16.6) precursor - barley
BE406516	BAB56099	4,00E-64	casein kinase-like protein [Oryza sativa (japonica cultivar-group)]
BE590480	1QDM	4,00E-68	Chain A, Crystal Structure Of Prophytepsin, A Zymogen Of A Barley Vacuolar Aspartic Proteinase.
BE423878	JN0884	3,00E-40	chitinase (EC 3.2.1.14) C - rye
BE471317	P12329	3,00E-82	Chlorophyll A-B binding protein 1, chloroplast precursor (LHCII type I CAB-1) (LHCP)
BE424498	S18818	2,00E-26	chymotrypsin inhibitor 2 - barley
BE423430	AAP06900	6,00E-77	COG1208, GCD1, Nucleoside-diphosphate-sugar pyrophosphorylase involved in lipopolysaccharide biosynthesis/translation initiation factor 2B, gamma/epsilon subunits (eIF-2Bgamma/eIF-2Bepsilon) [Cell envelope biogenesis, outer membrane / Translation, ribosom
BE490613	BAB61215	6,00E-28	contains ESTs AU066201(S5593),AU032549(S10727),AU088748(S5593)~unknown protein [Oryza sativa (japonica cultivar-group)]
BE606865	BAC15988	7,00E-19	contains ESTs D47406(S12806),AU101546(E61380)~similar to copper chaperone (CCH)-related protein [Oryza sativa (japonica cultivar-group)]
BG604704	AAN62336	6,00E-73	CTV.2 [Poncirus trifoliata]
BE590822	NP_180557	9,00E-59	cyclophilin; protein id: At2g29960.1, supported by cDNA: 19211., supported by cDNA: gi_15451025 [Arabidopsis thaliana]
BF483930	AAB37233	2,00E-48	cysteine proteinase
BQ806242	BAC53923	3,00E-29	cytochrome P450 [Petunia x hybrida]
BQ806457	AAM12494	1,00E-31	cytochrome P450-like protein [Oryza sativa (japonica cultivar-group)]
BE494817	NP_568336	3,00E-36	dehydroascorbate reductase, putative; protein id: At5g16710.1, supported by cDNA: 16205., supported by cDNA: gi_17473686 [Arabidopsis thaliana]

BE423906	AAO72551	8,00E-40	DNAJ-like protein [Oryza sativa (japonica cultivar-group)]
BE498242	T05741	1,00E-112	dnaK-type molecular chaperone HSP70 - barley
BQ838739	T05741	1,00E-107	dnaK-type molecular chaperone HSP70 - barley
BQ805549	S53126	1,00E-118	dnaK-type molecular chaperone hsp70 - rice (fragment)
BE398573	NP_569038	6,00E-05	dolichyl-di-phosphooligosaccharide-protein glycotransferase (oligosaccharyltransferase)-like; protein id: At5g66680.1, supported by cDNA: 18419., supported by cDNA: gi_14334505, supported by cDNA: gi_16226811 [Arabidopsis thaliana]
BE398950	CAA93825	5,00E-22	dormancy related protein [Trollius ledebourii]
BE499685	AAM62421	4,00E-07	Drm3 [Pisum sativum]
BE499799	Q03033	1,00E-96	ELONGATION FACTOR 1-ALPHA (EF-1-ALPHA)
BE424386	BAA95821	4,00E-32	EST AU082567(S21715) corresponds to a region of the predicted gene.~Similar to S.tuberosum ubiquinol--cytochrome c reductase. (X79275) [Oryza sativa (japonica cultivar-group)]
BE424036	BAA83566	8,00E-32	ESTs C98280(C1391),D15843(C1391) correspond to a region of the predicted gene.~Similar to OsENOD93a gene for early nodulin (AB018375) [Oryza sativa (japonica cultivar-group)]
BE399192	CAD56466	1,00E-08	ethylene response element binding protein [Triticum aestivum]
BQ806718	P55876	1,00E-54	Eukaryotic translation initiation factor 5 (eIF-5)
BE422660	NP_563851	1,00E-24	expressed protein; protein id: At1g09750.1, supported by cDNA: 6295. [Arabidopsis thaliana]
BE591531	NP_563986	8,00E-22	expressed protein; protein id: At1g15980.1, supported by cDNA: 122986., supported by cDNA: gi_16323197 [Arabidopsis thaliana]
BG262882	NP_566171	3,00E-54	expressed protein; protein id: At3g02420.1, supported by cDNA: 160403., supported by cDNA: gi_14190450, supported by cDNA: gi_15810092 [Arabidopsis thaliana]
BE606843	NP_566959	5,00E-32	Expressed protein; protein id: At3g52155.1, supported by cDNA: gi_14517465 [Arabidopsis thaliana]
BE422624	NP_567046	6,00E-10	expressed protein; protein id: At3g57280.1, supported by cDNA: 14487., supported by cDNA: gi_14517380, supported by cDNA: gi_15450538 [Arabidopsis thaliana]
BE422852	NP_568707	1,00E-08	expressed protein; protein id: At5g49210.1, supported by cDNA: 31573., supported by cDNA: gi_15724194, supported by cDNA: gi_20453328 [Arabidopsis thaliana]
BE438481	AAF79517	2,00E-05	F21D18.7 [Arabidopsis thaliana]
BQ805072	CAD30024	2,00E-77	ferredoxin-NADP(H) oxidoreductase [Triticum aestivum]
BE424294	AAF74220	4,00E-57	fructose 1,6-bisphosphate aldolase precursor [Avena sativa]
BE488825	T02057	1,00E-55	fructose-bisphosphate aldolase (EC 4.1.2.13) - rice
BQ804629	P20158	1,00E-24	Gamma-1 purothionin
BQ806422	P20159	5,00E-23	Gamma-2 purothionin
BE423114	AAD30440	6,00E-18	gamma-gliadin [Triticum aestivum subsp. spelta]
BE422980	AAD30556	2,00E-28	gamma-gliadin [Triticum aestivum]
BE424159	AAD30556	2,00E-17	gamma-gliadin [Triticum aestivum]
BE399706	AAF42989	3,00E-10	gamma-gliadin [Triticum aestivum]
BE423485	AAK84776	6,00E-32	gamma-gliadin [Triticum aestivum]
BE422970	AAK84778	4,00E-05	gamma-gliadin [Triticum aestivum]
BE424439	P04729	4,00E-11	GAMMA-GLIADIN B-I PRECURSOR
BE423843	BAB08988	3,00E-16	gene_id:MUK11.16~unknown protein [Arabidopsis thaliana]
BE490147	AAD28640	7,00E-94	geranylgeranyl hydrogenase [Glycine max]
BE590748	S35221	2,00E-68	globulin Beg1 precursor - barley
BE422452	S05078	2,00E-46	glucose-1-phosphate adenylyltransferase (EC 2.7.7.27) (clone AGA.3) - wheat (fragment)
BE399537	P12299	2,00E-56	Glucose-1-phosphate adenylyltransferase large subunit, chloroplast precursor (ADP-glucose synthase) (ADP-glucose pyrophosphorylase) (AGPASE S) (Alpha-D-glucose-1-phosphate adenyl transferase)
BE405260	AAK49456	1,00E-120	glutamine-dependent asparagine synthetase 1 [Hordeum vulgare]
BE423270	A30843	2,00E-09	glutenin high molecular weight chain Bx7 precursor - wheat
BE590674	S57655	9,00E-31	glutenin low molecular weight - wheat (fragment)
BE423268	T06980	5,00E-22	glutenin low molecular weight chain (clone F24B) precursor - wheat
BE398271	JN0689	2,00E-06	glutenin, high-molecular-weight Ax2* chain precursor - wheat
BE398985	JN0690	7,00E-57	glutenin, high-molecular-weight Bx7 chain precursor - wheat
BE398979	NP_192138	8,00E-19	glycosyl hydrolase family 9; protein id: At4g02290.1, supported by cDNA: gi_19310535, supported by cDNA: gi_20855940 [Arabidopsis thaliana]

BE637485	S48186	2,00E-69	grain softness protein 1a, 15K (clone TSF69) - wheat
BE398235	S48187	3,00E-31	grain softness protein 1b, 15K - wheat
BE399689	S48188	1,00E-17	grain softness protein 1c, 15K - wheat (fragment)
BE606698	AAK15703	9,00E-72	GTP-binding protein [Oryza sativa]
BE422736	AAD11549	3,00E-80	heat shock protein 80 [Triticum aestivum]
BG314518	AAD11549	3,00E-67	heat shock protein 80 [Triticum aestivum]
BE423029	AAO65876	5,00E-27	heat shock protein cognate 70 [Oryza sativa (japonica cultivar-group)]
BE399200	A54523	3,00E-05	histidine-rich protein - Plasmodium lophurae (fragment)
BE499931	NP_200914	2,00E-19	histone deacetylase - like; protein id: At5g61060.1, supported by cDNA: gi_20259319 [Arabidopsis thaliana]
BE424424	AAM93216	3,00E-10	histone H1-like protein HON101 [Zea mays]
BE423003	S53519	9,00E-22	histone H2A.9 - wheat
BE423289	P27807	1,00E-46	Histone H2B
BE424708	HSPM3	2,00E-41	histone H3 - garden pea (tentative sequence)
BE423622	CAA31966	2,00E-25	histone H3 (AA 1-123) [Medicago sativa]
BE425155	HSWT41	2,00E-39	histone H4 (TH091) - wheat
BE423060	HSWT41	4,00E-39	histone H4 (TH091) - wheat
BE423924	HSWT41	3,00E-38	histone H4 (TH091) - wheat
BE423864	HSWT41	7,00E-35	histone H4 (TH091) - wheat
BE423800	HSWT41	1,00E-31	histone H4 (TH091) - wheat
BE422698	P40621	1,00E-39	HMG1/2-like protein
BE423264	CAC83003	3,00E-20	HMW glutenin [Triticum aestivum]
BG604586	NP_567510	1,00E-68	HSP like protein; protein id: At4g16660.1, supported by cDNA: gi_15293148 [Arabidopsis thaliana]
BQ805925	AAB99745	1,00E-106	HSP70 [Triticum aestivum]
BE422622	AAB99745	2,00E-10	HSP70 [Triticum aestivum]
BE399823	CAA67191	6,00E-24	HSP80-2 [Triticum aestivum]
BE444111	T03274	1,00E-07	hsr201 protein, hypersensitivity-related - common tobacco
BE399887	NP_799506	3,00E-13	hypothetical protein [Bacillus megaterium]
BE422896	CAA04664	8,00E-17	hypothetical protein [Citrus x paradisi]
BE423436	CAC84115	8,00E-15	hypothetical protein [Gossypium hirsutum]
BE398483	NP_084748	1,00E-13	hypothetical protein [Oenothera elata subsp. hookeri]
BF474165	AAL34122	5,00E-09	hypothetical protein [Oryza sativa (japonica cultivar-group)]
BE422953	AAM08574	2,00E-31	Hypothetical protein [Oryza sativa (japonica cultivar-group)]
BF474092	AAM93693	4,00E-17	hypothetical protein [Oryza sativa (japonica cultivar-group)]
BG262212	AAK92630	6,00E-25	Hypothetical protein [Oryza sativa]
BE398351	NP_700950	3,00E-07	hypothetical protein [Plasmodium falciparum 3D7]
BE438330	NP_142215	3,00E-10	hypothetical protein [Pyrococcus horikoshii]
BG262851	D86261	6,00E-10	hypothetical protein F13K23.5 - Arabidopsis thaliana
BE590579	T04509	7,00E-34	hypothetical protein F8F16.240 - Arabidopsis thaliana
BF292996	T06986	4,00E-18	hypothetical protein wali6 - wheat
BE590870	NP_563869	1,00E-49	hypothetical protein; protein id: At1g10385.1 [Arabidopsis thaliana]
BG262275	NP_173164	9,00E-27	hypothetical protein; protein id: At1g17210.1 [Arabidopsis thaliana]
BF472999	NP_173925	6,00E-39	hypothetical protein; protein id: At1g25540.1 [Arabidopsis thaliana]
BF203031	NP_173925	1,00E-10	hypothetical protein; protein id: At1g25540.1 [Arabidopsis thaliana]
BE590730	NP_175266	1,00E-31	hypothetical protein; protein id: At1g48320.1 [Arabidopsis thaliana]
BE493784	NP_180596	6,00E-05	hypothetical protein; protein id: At2g30370.1 [Arabidopsis thaliana]
BE424411	BAC10812	1,00E-37	hypothetical protein~similar to Arabidopsis thaliana chromosome1, At1g09310 [Oryza sativa (japonica cultivar-group)]
BE590684	BAC10812	1,00E-34	hypothetical protein~similar to Arabidopsis thaliana chromosome1, At1g09310 [Oryza sativa (japonica cultivar-group)]
BE403898	BAC07067	2,00E-33	hypothetical protein~similar to Arabidopsis thaliana chromosome4,At4g08540 [Oryza sativa (japonica cultivar-group)]
BE424142	BAB61039	2,00E-10	iron-deficiency induced gene [Hordeum vulgare]
BE425124	CAB55554	2,00E-50	Isovaleryl-CoA Dehydrogenase; auxin binding protein (ABP44) [Pisum sativum]
BF474461	NP_568304	1,00E-19	L-aspartate oxidase -like protein; protein id: At5g14760.1, supported by cDNA: gi_15010649 [Arabidopsis thaliana]
BE590485	S21757	2,00E-49	lipid transfer protein - wheat
BE590609	CAC13149	8,00E-34	lipid transfer protein [Triticum turgidum subsp. durum]
BE425907	T05950	7,00E-48	lipid transfer protein 7a2b - barley
BQ806793	T05950	9,00E-48	lipid transfer protein 7a2b - barley
BQ804735	T05950	2,00E-43	lipid transfer protein 7a2b - barley

BE399577	T05947	4,00E-05	lipid transfer protein precursor 1 - barley (fragment)
BE399444	AAO53262	1,00E-05	low-molecular weight glutenin protein 1Agi [Thinopyrum intermedium]
BQ804218	AAO53266	4,00E-12	low-molecular weight glutenin protein 1Agi [Triticum aestivum/Thinopyrum intermedium alien addition line]
BE423334	AAO53266	2,00E-08	low-molecular weight glutenin protein 1Agi [Triticum aestivum/Thinopyrum intermedium alien addition line]
BE424629	BAB78750	1,00E-27	low-molecular-weight glutenin subunit group 4 type II [Triticum aestivum]
BE423321	BAB78750	7,00E-22	low-molecular-weight glutenin subunit group 4 type II [Triticum aestivum]
BE424477	BAB78753	2,00E-12	low-molecular-weight glutenin subunit group 5 type III [Triticum aestivum]
BE399055	BAB78760	1,00E-40	low-molecular-weight glutenin subunit group 9 type IV [Triticum aestivum]
BG262706	BAB78760	3,00E-40	low-molecular-weight glutenin subunit group 9 type IV [Triticum aestivum]
BG262868	NP_394937	7,00E-06	mannose-1-phosphate guanyltransferase related protein [Thermoplasma acidophilum]
BE423904	AAK38824	4,00E-08	metallothionein-like protein type 2 [Poa secunda]
BE422469	NP_201056	4,00E-19	microtubule-associated protein EB1-like protein; protein id: At5g62500.1, supported by cDNA: 112876. [Arabidopsis thaliana]
BE422529	AAG42149	4,00E-14	mitochondrial processing peptidase alpha-chain precursor [Dactylis glomerata]
BE398404	NP_191771	3,00E-47	multifunctional aminoacyl-tRNA ligase-like protein; protein id: At3g62120.1, supported by cDNA: gi_16648884 [Arabidopsis thaliana]
BE424200	T06179	2,00E-89	myb-related protein - barley
BE403860	AAK84883	4,00E-32	NAC domain protein NAC1 [Phaseolus vulgaris]
BE422717	CAA63101	1,00E-53	NAM [Petunia x hybrida]
BF429069	CAD45027	7,00E-05	NBS-LRR disease resistance protein homologue [Hordeum vulgare]
BG262713	AAL09398	5,00E-09	non-cell-autonomous protein pathway2; plasmodesmal receptor [Nicotiana tabacum]
BE498742	BAA75495	1,00E-18	NtEPc [Nicotiana tabacum]
BG312759	BAC55689	2,00E-16	OJ1066_B03.23 [Oryza sativa (japonica cultivar-group)]
BG262465	BAC55636	2,00E-41	OJ1081_B12.17 [Oryza sativa (japonica cultivar-group)]
BE423064	BAC55625	6,00E-38	OJ1081_B12.5 [Oryza sativa (japonica cultivar-group)]
BE399889	NP_569013	3,00E-32	oligopeptidase A; protein id: At5g65620.1, supported by cDNA: gi_15028226 [Arabidopsis thaliana]
BE516904	AAG12204	2,00E-23	Orf122 [Chlorobium tepidum]
BF292678	CAD39524	2,00E-50	OSJNBa0027O01.12 [Oryza sativa (japonica cultivar-group)]
BE637490	BAC57848	5,00E-44	OSJNBa0040K22.13 [Oryza sativa (japonica cultivar-group)]
BE406509	CAD40856	3,00E-24	OSJNBa0064H22.1 [Oryza sativa (japonica cultivar-group)]
BE399391	CAD41249	4,00E-31	OSJNBa0067K08.13 [Oryza sativa (japonica cultivar-group)]
BE498010	CAD40835	8,00E-05	OSJNBa0086B14.7 [Oryza sativa (japonica cultivar-group)]
BE490365	CAD40786	2,00E-92	OSJNBb0012E08.10 [Oryza sativa (japonica cultivar-group)]
BE424931	CAD40667	2,00E-60	OSJNBb0118P14.5 [Oryza sativa (japonica cultivar-group)]
BE399781	CAD40667	3,00E-36	OSJNBb0118P14.5 [Oryza sativa (japonica cultivar-group)]
BE424318	CAD40670	2,00E-23	OSJNBb0118P14.8 [Oryza sativa (japonica cultivar-group)]
BE497083	Q41048	3,00E-39	Oxygen-evolving enhancer protein 3-1, chloroplast precursor (OEE3) (16 kDa subunit of oxygen evolving system of photosystem II) (OEC 16 kDa subunit)
BG262899	BAB63766	2,00E-37	P0025A05.29 [Oryza sativa (japonica cultivar-group)]
BF473001	BAB93196	1,00E-17	P0031D02.25 [Oryza sativa (japonica cultivar-group)]
BE423985	BAC07416	2,00E-81	P0039H02.3 [Oryza sativa (japonica cultivar-group)]
BE424415	BAB63534	4,00E-30	P0435H01.30 [Oryza sativa (japonica cultivar-group)]
BE423484	BAB39879	1,00E-17	P0439B06.14 [Oryza sativa (japonica cultivar-group)]
BE499422	BAB20639	2,00E-28	P0475H04.5 [Oryza sativa (japonica cultivar-group)]
BQ804864	BAB89741	2,00E-35	P0506B12.13 [Oryza sativa (japonica cultivar-group)]
BG262893	BAB89731	5,00E-18	P0506B12.3 [Oryza sativa (japonica cultivar-group)]
BE497804	BAB78658	4,00E-50	P0681B11.25 [Oryza sativa (japonica cultivar-group)]
BE638140	BAC56799	3,00E-07	P0686C03.43 [Oryza sativa (japonica cultivar-group)]
BF474428	BAB63724	8,00E-32	P0694A04.6 [Oryza sativa (japonica cultivar-group)]
BE398411	AAB02259	6,00E-10	permatin precursor
BQ805616	S61408	2,00E-73	peroxidase (EC 1.11.1.7) 4 precursor - wheat

BE590763	AAM88383	9,00E-51	peroxidase 1 [Triticum aestivum]
BE424106	T03585	1,00E-38	probable chaperonin 10 - rice
BE424384	T06489	1,00E-91	probable peptidylprolyl isomerase (EC 5.2.1.8) FKBP77 - wheat
BE496834	T06489	7,00E-52	probable peptidylprolyl isomerase (EC 5.2.1.8) FKBP77 - wheat
BE590765	BAA36699	9,00E-11	prolamin [Oryza sativa]
BF484528	F86281	7,00E-56	protein F10B6.14 [imported] - Arabidopsis thaliana
BE606619	BAB88944	6,00E-19	protein phosphatase 2C [Mesembryanthemum crystallinum]
BE604924	P22244	5,00E-25	Protein synthesis inhibitor I (Ribosome-inactivating protein I) (rRNA N-glycosidase)
BF473546	NP_200854	7,00E-15	protein transport protein subunit - like; protein id: At5g60460.1 [Arabidopsis thaliana]
BE424229	T02667	3,00E-15	proteinase inhibitor - rice
BE590621	Q10464	4,00E-64	Puroindoline-B precursor
BE423187	Q10464	7,00E-62	Puroindoline-B precursor
BF145691	BAB86551	3,00E-76	putative 3-phosphoinositide-dependent protein kinase-1 [Oryza sativa (japonica cultivar-group)]
BE423340	BAB90039	3,00E-47	putative 60S ribosomal protein L37a [Oryza sativa (japonica cultivar-group)]
BE403438	AAL31061	2,00E-13	putative ABA-responsive protein [Oryza sativa]
BE424519	CAB71336	4,00E-54	putative acid phosphatase [Hordeum vulgare subsp. vulgare]
BE637511	AAO65864	1,00E-48	putative actin depolymerizing factor [Oryza sativa (japonica cultivar-group)]
BE500162	NP_192813	2,00E-30	putative aldolase; protein id: At4g10750.1, supported by cDNA: 35159. [Arabidopsis thaliana]
BM140436	BAB63594	3,00E-36	putative auxin-responsive GH3 protein [Oryza sativa (japonica cultivar-group)]
BE423926	BAB63673	1,00E-64	putative cytochrome B5 [Oryza sativa (japonica cultivar-group)]
BE606653	NP_191965	1,00E-55	putative dTDP-6-deoxy-L-mannose-dehydrogenase; protein id: At4g00560.1 [Arabidopsis thaliana]
BF429057	AAK98696	1,00E-19	Putative epsin [Oryza sativa]
BF473139	AAN05527	4,00E-69	putative glutamine synthetase [Oryza sativa (japonica cultivar-group)]
BG262235	AAK18848	2,00E-86	putative glutamine synthetase [Oryza sativa]
BQ838646	BAC22346	1,00E-103	putative GTP-binding protein [Oryza sativa (japonica cultivar-group)]
BE606912	BAC45183	5,00E-16	putative hageman factor inhibitor [Oryza sativa (japonica cultivar-group)]
BE403409	CAC80823	2,00E-57	putative IAA1 protein [Oryza sativa (indica cultivar-group)]
BE590752	AAN06944	7,00E-10	putative low temperature and salt responsive protein [Triticum aestivum]
BE423472	BAB55686	1,00E-44	putative malate dehydrogenase [Oryza sativa (japonica cultivar-group)]
BF472998	AAL84297	3,00E-27	putative male sterility protein [Oryza sativa (japonica cultivar-group)]
BE398492	CAD12837	2,00E-10	putative metallophosphatase [Lupinus luteus]
BE606262	AAK50423	2,00E-55	Putative Mic1 homolog [Oryza sativa]
BE398439	BAC16718	2,00E-58	putative NADH dehydrogenase [Oryza sativa (japonica cultivar-group)]
BE399809	BAB16335	2,00E-24	putative NAM protein [Oryza sativa (japonica cultivar-group)]
BG262836	AAO41132	1,00E-102	putative nicotinate phosphoribosyltransferase [Oryza sativa (japonica cultivar-group)]
BE424493	NP_567219	7,00E-22	putative NifU-like metallocluster assembly factor; protein id: At4g01940.1, supported by cDNA: gi_14517433, supported by cDNA: gi_15215669, supported by cDNA: gi_20908089 [Arabidopsis thaliana]
BE423328	BAB84432	3,00E-13	putative nuclear RNA binding protein A [Oryza sativa (japonica cultivar-group)]
BE488992	BAB67963	2,00E-11	putative polygalacturonase [Oryza sativa (japonica cultivar-group)]
BE422730	AAO37493	1,00E-64	putative polyprotein [Oryza sativa (japonica cultivar-group)]
BE423738	CAD21002	2,00E-68	putative potasium transporter [Oryza sativa (japonica cultivar-group)]
BE422649	BAB32709	6,00E-14	putative PRLI-interacting factor N [Oryza sativa (japonica cultivar-group)]
BE398361	CAB81548	7,00E-14	putative proline-rich protein APG isolog [Cicer arietinum]
BG263083	CAC39046	2,00E-11	putative protein [Oryza sativa]
BE591982	BAC10827	3,00E-07	putative protein kinase Xa21, receptor type precursor [Oryza sativa (japonica cultivar-group)]
BF201002	AAK20060	1,00E-11	putative protein phosphatase 2C [Oryza sativa (japonica cultivar-

			group)]
BF473933	NP_195409	1,00E-46	putative protein; protein id: At4g36910.1 [Arabidopsis thaliana]
BE499677	NP_197490	1,00E-22	putative protein; protein id: At5g19900.1 [Arabidopsis thaliana]
BE498001	NP_568618	5,00E-20	putative protein; protein id: At5g42960.1, supported by cDNA: gi_15451133 [Arabidopsis thaliana]
BE423282	NP_199210	1,00E-15	putative protein; protein id: At5g43970.1, supported by cDNA: 777., supported by cDNA: gi_15529291, supported by cDNA: gi_16974414 [Arabidopsis thaliana]
BE490662	BAC10762	1,00E-84	putative Pro-X carboxypeptidase homolog [Oryza sativa (japonica cultivar-group)]
BE403881	AAL34937	2,00E-91	Putative purple acid phosphatase [Oryza sativa]
BE423194	AAK98702	9,00E-53	Putative quinone oxidoreductase [Oryza sativa]
BE422811	NP_565733	1,00E-44	putative RAD50 DNA repair protein; protein id: At2g31970.1, supported by cDNA: gi_7110147 [Arabidopsis thaliana]
BF473100	NP_564940	1,00E-15	putative receptor protein; protein id: At1g68740.1, supported by cDNA: gi_15982932 [Arabidopsis thaliana]
BE490790	AAO37979	2,00E-43	putative replication factor [Oryza sativa (japonica cultivar-group)]
BE426625	AAL79739	3,00E-59	putative ribosomal protein L18 [Oryza sativa]
BE498782	NP_194629	7,00E-39	putative transcription factor; protein id: At4g29000.1 [Arabidopsis thaliana]
BG262915	AAL58240	2,00E-46	putative transposon protein [Oryza sativa]
BE424253	BAB90507	2,00E-13	putative U2 snRNP auxiliary factor [Oryza sativa (japonica cultivar-group)]
BE499948	NP_179544	4,00E-40	putative WD-40 repeat protein; protein id: At2g19540.1, supported by cDNA: gi_13877610 [Arabidopsis thaliana]
BE398343	CAC87260	8,00E-23	putative xylanase inhibitor protein [Triticum turgidum subsp. durum]
BE406691	BAC07099	1,00E-16	putative zinc-finger helicase [Oryza sativa (japonica cultivar-group)]
BE424870	T03960	2,00E-11	r40g2 protein - rice (fragment)
BE424975	P40393	3,00E-54	Ras-related protein RIC2
BF473126	BAA96958	2,00E-75	receptor-like protein kinase [Arabidopsis thaliana]
BE424939	BAB84843	5,00E-05	receptor-like protein kinase-like [Oryza sativa (japonica cultivar-group)]
BE518393	AAK25758	2,00E-70	ribosomal protein L17 [Castanea sativa]
BE423561	T50602	5,00E-65	ribosomal protein L21 [imported] - rice
BE423336	1909359B	8,00E-37	ribosomal protein L7
BE399912	AAA32866	3,00E-36	ribosomal protein S11 (probable start codon at bp 67)
BE424825	NP_189984	1,00E-27	ribosomal S29 -like protein; protein id: At3g43980.1, supported by cDNA: 473., supported by cDNA: gi_13194825, supported by cDNA: gi_13605739 [Arabidopsis thaliana]
BE591671	P08823	5,00E-45	RuBisCO subunit binding-protein alpha subunit, chloroplast precursor (60 kDa chaperonin alpha subunit) (CPN-60 alpha)
BQ805284	AAC49135	4,00E-39	SAG12 protein
BE590546	AAO72573	2,00E-86	sarcoplasmic reticulum protein-like protein [Oryza sativa (japonica cultivar-group)]
BE398314	AAM77580	2,00E-08	seed globulin [Aegilops tauschii]
BF293157	S65782	2,00E-84	serpin - wheat
BQ805944	T06597	1,00E-134	serpin homolog WZS3 - wheat
BE590622	T06597	5,00E-65	serpin homolog WZS3 - wheat
BE423505	T06488	2,00E-57	serpin WZS2 - wheat
BE423473	T06488	1,00E-53	serpin WZS2 - wheat
BE424965	BAA95872	5,00E-13	Similar to Arabidopsis thaliana chromosome 4 BAC F6I18; membrane-associated salt-inducible protein. (AL022198) [Oryza sativa (japonica cultivar-group)]
BE424925	XP_135742	8,00E-14	similar to integral membrane transporter protein [Homo sapiens] [Mus musculus]
BE423288	BAA95829	2,00E-19	Similar to Prunus armeniaca ethylene-forming-enzyme-like dioxygenase. (U97530) [Oryza sativa (japonica cultivar-group)]
BE606222	AAO86692	9,00E-14	small blue copper protein Bcp1 [Boea crassifolia]
BQ805498	AAK51797	7,00E-49	small heat shock protein HSP17.8 [Triticum aestivum]
BE590490	NP_199894	2,00E-20	small zinc finger-like protein; protein id: At5g50810.1, supported by cDNA: 33833., supported by cDNA: gi_5107154 [Arabidopsis thaliana]
BF473824	CAD59409	2,00E-84	SMC1 protein [Oryza sativa]
BF474164	AAL58968	7,00E-33	SRPK4 [Oryza sativa]
BQ804420	AAL23749	2,00E-65	stress-inducible membrane pore protein [Bromus inermis]

BE422842	P82977	1,00E-29	Subtilisin-chymotrypsin inhibitor WSCI
BE424574	P01053	4,00E-32	Subtilisin-chymotrypsin inhibitor-2A (CI-2A)
BF473090	AAO61749	2,00E-11	subtilisin-like seed-specific protein [Arachis hypogaea]
BE442676	AAL48317	7,00E-91	succinoaminoimidazolecarboximide ribonucleotide synthetase [Vigna unguiculata]
BE498428	AAL50570	2,00E-86	sucrose synthase 2 [Bambusa oldhamii]
BQ806964	BAB82469	1,00E-128	sucrose:fructan 6-fructosyltransferase [Triticum aestivum]
BE499337	BAB82469	2,00E-40	sucrose:fructan 6-fructosyltransferase [Triticum aestivum]
BE424581	NP_567642	5,00E-46	symbiosis-related like protein; protein id: At4g21980.1, supported by cDNA: 14759., supported by cDNA: gi_19912150 [Arabidopsis thaliana]
BE606193	NP_173250	3,00E-47	terminal Flower 1 (TFL1), putative; protein id: At1g18100.1, supported by cDNA: gi_12083219, supported by cDNA: gi_5002245 [Arabidopsis thaliana]
BE399084	NP_567708	4,00E-20	topoisomerase, putative; protein id: At4g24800.1, supported by cDNA: gi_17063162 [Arabidopsis thaliana]
BE424255	NP_193152	7,00E-65	transport protein; protein id: At4g14160.1, supported by cDNA: gi_18389287, supported by cDNA: gi_20147366 [Arabidopsis thaliana]
BF428943	AAB27108	1,00E-102	triticin precursor [Triticum aestivum]
BG604768	AAB27108	4,00E-82	triticin precursor [Triticum aestivum]
BE399248	S33631	7,00E-15	tritin - wheat
BE591070	S51811	6,00E-28	trypsin inhibitor CMx precursor - wheat
BE424622	S51810	4,00E-29	trypsin inhibitor CMx precursor (clones pCMx1 and pCMx3) - wheat
BE400000	Q05806	6,00E-09	Type V thionin precursor
BE422877	BAB93371	5,00E-84	ubiquitin conjugating enzyme [Oryza sativa (japonica cultivar-group)]
BE423297	AAB88617	2,00E-51	ubiquitin conjugating enzyme [Zea mays]
BE590865	NP_177189	7,00E-81	ubiquitin-protein ligase 2 (UPL2), putative; protein id: At1g70320.1 [Arabidopsis thaliana]
BE406580	AAL87666	2,00E-55	uncoupling protein [Zea mays]
BE498706	AAM62916	6,00E-06	unknown [Arabidopsis thaliana]
BQ806630	AAD02494	8,00E-12	unknown [Oryza sativa]
BE500311	AAK43900	2,00E-27	Unknown protein [Arabidopsis thaliana]
BG604574	AAO42097	1,00E-68	unknown protein [Arabidopsis thaliana]
BE590506	AAP04148	2,00E-36	unknown protein [Arabidopsis thaliana]
BG604593	BAC42600	2,00E-53	unknown protein [Arabidopsis thaliana]
BE500191	AAO60033	7,00E-07	unknown protein [Oryza sativa (japonica cultivar-group)]
BQ805916	AAG13582	3,00E-05	unknown protein [Oryza sativa]
BE495560	AAK50579	6,00E-59	unknown protein [Oryza sativa]
BF473539	NP_173582	4,00E-33	unknown protein; protein id: At1g21630.1 [Arabidopsis thaliana]
BE606728	NP_175574	2,00E-13	unknown protein; protein id: At1g51630.1 [Arabidopsis thaliana]
BF473825	NP_565378	7,00E-29	unknown protein; protein id: At2g15695.1 [Arabidopsis thaliana]
BE591325	NP_189197	4,00E-37	unknown protein; protein id: At3g25690.1 [Arabidopsis thaliana]
BM140569	BAA88176	1,00E-44	unnamed protein product [Oryza sativa (japonica cultivar-group)]
BE399952	BAA88176	5,00E-12	unnamed protein product [Oryza sativa (japonica cultivar-group)]
BE590756	BAA89546	1,00E-101	unnamed protein product [Oryza sativa (japonica cultivar-group)]
BE424566	BAA99386	2,00E-37	unnamed protein product [Oryza sativa (japonica cultivar-group)]
BF428535	CAD27730	6,00E-41	xylanase inhibitor [Triticum aestivum]
BE424902	CAD19479	2,00E-59	xylanase inhibitor protein I [Triticum aestivum]

Tab 4.2.: List of the statistically-significant DE genes throughout the five timepoints included in the microarray experiment. The genes listed above may vary in opposite directions (up-regulation or down-regulation with respect to the wild type), with a variable (but always significant) degree of magnitude, in at least one of the five timepoints analysed. Of the 542 tentative DE unique genes identified, only those with a BlastX E-value < 10^{-5} are shown in the table (375 genes). The list has been sorted according to the best BlastX hit description, thus providing a preliminary functional clustering. The remaining 167 DE genes identified as significant by statistical analysis (not shown) have 0,0001<E-value<9,9, or completely lack annotation by BlastX analysis.

Functional category	Number of clones in the functional category	Representative clone names
Seed-Storage Proteins	34	seed globulin, low-molecular-weight glutenin subunit, alpha/beta-gliadin, prolamin, gamma-gliadin, HMW glutenin
Ribosomal Proteins	14	ribosomal protein L17, ribosomal protein L7, 60S ribosomal protein L35, 40S ribosomal protein S23, 60S ACIDIC RIBOSOMAL PROTEIN P0
Heat-Shock Proteins & Chaperones, Transport	16	sarcoplasmic reticulum protein-like protein, small heat shock protein HSP17.8, transport protein, HSP70 [Triticum aestivum], HSP80-2 [Triticum aestivum]
α/β-amylase/trypsin inhibitors, Chloroform/methanol soluble proteins (CM proteins), other protein inhibitors	16	trypsin inhibitor CMx precursor, alpha-amylase inhibitor - wheat, ALPHA-AMYLASE INHIBITOR WDAI-3, alpha-amylase inhibitor, tetrameric, chain CM17 precursor - wheat, alpha-amylase/trypsin inhibitor homolog precursor - wheat
β-amylases	12	BETA-AMYLASE (1,4-ALPHA-D-GLUCAN MALTOHYDROLASE), beta-amylase [Hordeum vulgare subsp. vulgare]
Histones	9	histone H4 (TH091) - wheat, histone H2A.9 - wheat, Histone H2B
Lipid-Transfer-Proteins (LTP)	6	lipid transfer protein precursor 1 - barley (fragment), lipid transfer protein [Triticum turgidum subsp. durum]
Transcription Factors	8	small zinc finger-like protein, putative transcription factor, bZIP transcription factor

Tab 4.3.: Major functional classes of some of the DE unique clones identified by statistical analysis. The clones listed above may be up- or down-regulated in one or more of the five selected timepoints of the microarray experiment. Of the 375 DE unique clones listed in Tab. 4.2, 34 (9%) belong to the seed-storage proteins class.

4.2.2 Differentially-expressed genes at 10 DPA "early"

The list of statistically significant DE genes obtained from the LIMMA analysis is shown in Tab. 4.4. Sensitivity of statistical analysis and our deliberate decision not to apply a simple fold-change criteria, resulted in clones with a low fold-change (M) to be listed as significantly DE. Among recent papers on the subject, Salzmann et al. (2005) adopted both a 1.5-fold response cutoff and a P-value threshold (0.05) for differential expression; but our intention was to look globally at the significant clones, since many genes could still represent a biologically-relevant differential expression with a limited fold-change response.

The M range, for the DE clones at 10 DPA "early" , spans from -0,56 (equivalent to a fold-change variation of 0,68) for BF483930 (*Hordeum vulgare* cysteine proteinase precursor, mRNA, complete cds) to 0,65 (equivalent to a fold-change variation of 1,57) for the clone BE423864 (Wheat histone H4 gene).

At 10 DPA "early", the transcripts of the *lmw-gs* transgenes are not yet over-represented with respect to the wild type genotype (the onset of up-regulation of the *lmw-gs* transgenes is clearly evident only from 10 DPA "late"), and this could explain the lack of a large number of DE clones at this timepoint.

However, it is worth to notice that six different clones, among the 14 reported as DE, encode histones (highlighted in red in Tab. 4.4), and are all significantly up-regulated in the transgenic genotype. Histone clones were included in the Wheat cDNA array as control clones, and they are thus technically replicated; their differential expression occurs over several clones, thus limiting the possibility of false-positives due to a spot-to-spot variation.

ID	Name	M	A	t	P-value
BE499337	Agropyron cristatum sucrose:fructan 6-fructosyltransferase (6-SFT) mRNA, complete cds	0,60	8,21	14,38	0,0076
BE423436	beta3-glucuronyltransferase [Triticum aestivum]	0,63	9,73	12,80	0,0118
BE405260	Hordeum vulgare glutamine-dependent asparagine synthetase 1 mRNA, complete cds	-0,51	7,01	-11,42	0,0161
BE423622	Oryza sativa histone 3 mRNA, complete cds	0,44	8,16	10,69	0,0208
BF483930	Hordeum vulgare cysteine proteinase precursor, mRNA, complete cds	-0,56	7,59	-9,80	0,0294
BG607362	Hordeum vulgare mRNA for alpha-tubulin 3	0,38	8,46	9,56	0,0327
BE423060	Wheat histone H4 gene	0,49	9,35	9,49	0,0331
BE423864	Wheat histone H4 gene	0,65	9,53	9,26	0,0367
BE424653	Wheat histone H4 gene	0,48	9,23	9,09	0,0386
BE398313	Wheat histone H4 TH091 gene, complete cds	0,43	8,40	8,90	0,0422
BE499574	PREDICTED: similar to hypothetical protein FLJ30707 Canis familiaris, cd00172: SERPIN; SERine Proteinase INhibitors (serpins)	0,59	9,78	8,69	0,0466
BG605575	T.aestivum L mRNA for histone H2B	0,36	8,16	8,54	0,0491
BE423003	Wheat mRNA for protein H2A, complete cds, clone wcH2A-9	0,45	8,51	8,53	0,0491
BE423851	T.aestivum (subclone pAWJL175) AWJL175 gene	0,41	8,47	8,49	0,0493

Tab. 4.4: List of the differentially expressed clones between the wild type and the transgenic genotype obtained from the analysis of the microarray experiment at 10 DPA "early". The column "Name" reports the putative clone identity by BLAST analysis. "M" is the normalized $\log_2$ ratio of the channel intensities (transgenic/wild type). A positive M value indicates a clone up-regulated in the transgenic genotype with respect to the wild type; a negative M value indicates a clone down-regulated in the transgenic genotype with respect to the wild type. "A" is the normalized $\log_2$ product of the channel intensities, averaged on the measurements from the replicated hybridizations. "t" is the moderated t statistic associated with each clone; the fdr-corrected "P-value" associated with each t-test is also indicated. In this experiment, the corrected P-value threshold for differential expression was set to 0.05.

4.2.3 Differentially-expressed genes at 10 DPA "late"

The list of statistically significant DE genes obtained from the LIMMA analysis is shown in Tab. 4.5. A schematic representation of the output data from the microarray comparison can be seen in the so-called volcano plot in Fig. 4.2. Volcano plots have the advantage to give a global image of the experiment, and they thus reveal the overall gene-expression changes within all the clones represented on the array.

It is evident from Fig. 4.2 that, at 10 DPA "late", major changes in gene expression levels are occurring between the transgenic and the wild-type genotype, for a large number of clones. Many clones are strongly up-regulated, with up to a 4 Log_2 fold-change variation (≅ 16 times over-expression); while some show heavy down-regulation, with a -3 Log_2 fold-variation (≅ 10 times down-regulation). The total number of clones significantly DE, within a threshold of fdr-corrected P-value of 0,05, are 174. Tab. 4.5 only reports only the top 50 clones, in order of decreasing statistical evidence.

As expected, several clones for *lmw-gs* are strongly up-regulated, and are those at the upper far-right of the volcano plot. Within the top 50 significant DE clones, 9 different clones represent *lmw-gs* genes and show an average Log_2 fold change variation of 3,44. The over-expression of the *lmw-gs* genes thus start at this stage of the seed development, and some clones will continue to be significantly up-regulated until 30 DPA.

The higher abundance of *lmw-gs* transcripts with respect to the wild type genotype is most probably due to a multiple genetic insertion event leading to a high *lmw-gs* transgene copy number (Masci et al. 2003, Carozza et al. 2005), rather than to an early transcription or to a higher transcription rate within the *lmw-gs* transgene(s) block. The subsequent transcription from a larger number of genes would thus result in higher *lmw-gs* mRNA abundances in developing seeds..

Interestingly, among the top 50 up-regulated clones, some of them show a Log_2 fold-change variation of the same magnitude of the over-expressed *lmw-gs* clones. These are BQ804784 (*Mus musculus* zinc finger protein regulator of apoptosis and cell cycle arrest (Zac1), mRNA) and BE423328 (*Zea mays* putative transcription factor mRNA sequence). The E-value for BQ804784 is rather high, (0,5) and the clone putative identity is thus unknown; but both likely represent transcription factors or RNA-binding proteins involved in the expression and/or stability of *lmw-gs* genes and related transcripts.

It is also interesting to notice the strong up-regulation of a clone (BE591113, E-value=9,00E-66, double-spotted on the array, which again showed a Log_2 fold-change variation of the same order of magnitude to those of the up-regulated *lmw-gs* clones) encoding the enzyme 12-oxophytodienoate reductase (OPR), which is responsible for the conversion of 12-oxophytodienoic acid (OPDA) to jasmonic acid (JA). OPR from *Lycopersicon esculentum* was found to be up-regulated after wounding (Strassner et al., 2003), and JA is a plant growth regulator known to affect a variety of developmental processes (root growth, senescence, fruit ripening, pollen development, etc.), but has also been recently recognized as a signal molecule in plant defence reactions (for review see Parthier, 1991 and Blée, 1998). If the over- expression

of OPR would then result in higher JA content in developing seeds, we could speculate the activation, in the transgenic genotype, of a defence mechanism in response to the high over-expression of the *lmw-gs* transgene(s). Miersch and Wasternack (2000) already demonstrated a direct correlation between the amount of JA and the degree of defense gene activation. The high number of mRNA molecules encoding LMW-GS, which, as will be showed later, accumulate with different magnitude and kinetics from the endogenous *lmw-gs* transcripts, could be interpreted by the cells as a signal indicating a putative pathogen infection.

Moreover, recently, an *Arabidopsis* mutant (*opr3*) lacking OPR activity, showed male-sterility because of shortened anther filaments, delayed dehiscence of the anther locule and reduced pollen viability (Stintzi and Browse, 2000).

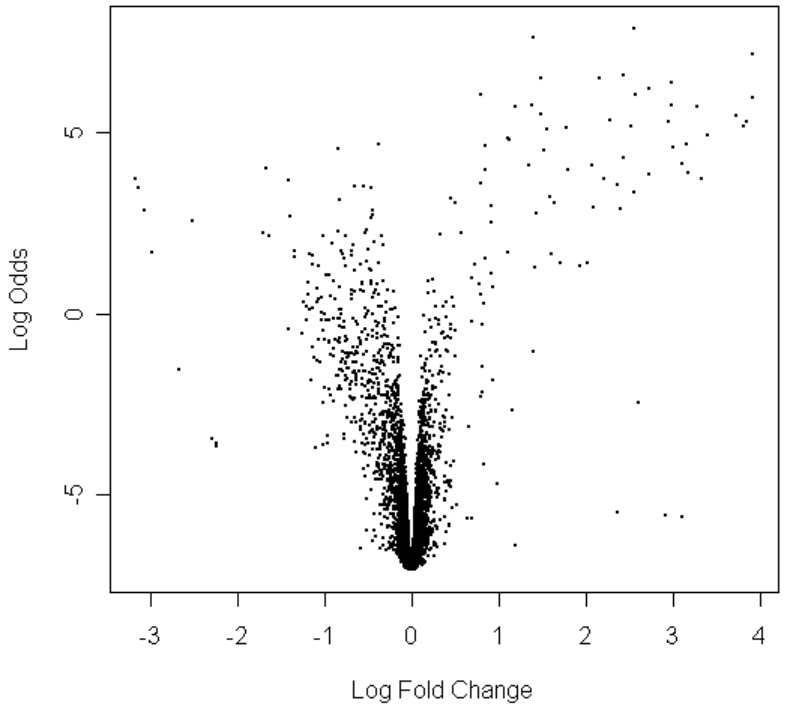


Fig. 4.2 Volcano plot for the microarray comparison at 10 DPA "late". Each point represents a clone. On the x-axis the Log Fold Change (M) is a measure of the magnitude of differential expression. Clones with high fold-change variation are at the far left and right of the plot. On the y-axis, the Log Odds are a measurement of statistical significance of differential expression. To be called as differentially regulated a clone should be characterized by a significant Log Fold Change (the M value, on the x-axis) across all the replicates (high Log Odds value, or, equivalently, an fdr-corrected P-value below the set threshold, e.g.: 0,05). The DE clones are thus represented by points at the far upper-left and upper right of the volcanoplot. Those at the right (with positive Log Fold Change value) are clones up-regulated with respect to the wild type, while those at the left (with negative Log Fold Change value) are clones down-regulated in the transgenic with respect to the wild-type genotype.

ID	Name	M	A	t	P-value
BE423878	Hordeum vulgare chitinase (CHI26) gene, complete cds	1,18	8,19	18,35	0,0012
BE590622	T.aestivum mRNA for serpin WZS3	0,79	8,15	18,30	0,0012
BE605124	Homo sapiens LOC158818 (LOC158818), mRNA	2,43	9,77	17,71	0,0012
BF473651	Drosophila melanogaster 3L BAC RP98-14E19 (Roswell Park Cancer Institute Drosophila BAC Library) complete sequence	2,28	11,78	17,40	0,0012
BE606843	Oryza sativa chromosome 9 clone PAC0663H05, complete sequence	1,56	8,57	15,93	0,0016
BE399480	Triticum aestivum low-molecular-weight glutenin storage protein mRNA, complete cds	3,92	11,49	15,48	0,0016
BQ806242	Oryza sativa chromosome 10 clone nbxb0046P18, complete sequence	1,78	7,97	15,35	0,0016
BQ806630	Oryza sativa genomic DNA, chromosome 1, PAC clone:P0431G06	0,84	8,37	14,81	0,0017
BE399060	Triticum aestivum mRNA for monomeric alpha-amylase inhibitor (WAI-0.28)	-0,84	10,35	-14,79	0,0017
BQ804784	Mus musculus zinc finger protein regulator of apoptosis and cell cycle arrest (Zac1), mRNA	3,91	11,08	14,20	0,0019
BQ804864	Oryza sativa genomic DNA, chromosome 1, PAC clone:P0506B12, complete sequence	1,40	7,57	13,87	0,0019
BE591113	12-oxophytodienoate reductase (OPR1) [Arabidopsis thaliana], jasmonic acid biosynthesis, Oxidored_FMN; NADH:flavin oxidoreductase / NADH oxidase family	2,44	11,12	13,77	0,0019
BE606262	Genomic Sequence For Oryza sativa, Nipponbare Strain, Chromosome X, Clone OSJNBa0050N08, complete sequence	2,16	10,72	13,71	0,0019
BE423321	Triticum aestivum low-molecular-weight glutenin storage protein mRNA, complete cds	3,81	11,07	13,40	0,0021
BE590546	Arabidopsis thaliana chromosome 5 CHR5v12152001 genomic sequence	2,99	11,01	13,09	0,0021
BE424477	Wheat (T.aestivum) gamma-gliadin class B-I mRNA, complete cds, clone pB11-33	2,51	9,42	13,08	0,0021
BE399271	Homo sapiens BAC clone RP11-174L18 from 2, complete sequence	1,80	10,81	12,97	0,0021
BE591113	12-oxophytodienoate reductase (OPR1) [Arabidopsis thaliana], jasmonic acid biosynthesis, Oxidored_FMN; NADH:flavin oxidoreductase / NADH oxidase family	2,96	11,55	12,62	0,0023
BE424142	Hordeum vulgare IDI1 mRNA, complete cds	1,49	8,50	12,14	0,0026
BG262893	S.cerevisiae SEC1 gene, involved in protein secretion	2,08	9,80	12,11	0,0026
BE399942	Triticum monococcum BAC clones 116F2 and 115G1 gene sequence	-1,66	9,23	-12,03	0,0026
BG262706	Triticum aestivum low-molecular-	2,72	11,36	11,95	0,0026

	weight glutenin storage protein mRNA, complete cds				
BE424629	Triticum aestivum low-molecular-weight glutenin storage protein mRNA, complete cds	3,84	10,81	11,83	0,0026
BE423328	Zea mays putative transcription factor mRNA sequence	3,73	9,15	11,79	0,0026
BE423268	Triticum aestivum low-molecular-weight glutenin storage protein mRNA, complete cds	2,56	11,55	11,74	0,0026
BE399055	Triticum aestivum low-molecular-weight glutenin storage protein mRNA, complete cds	2,98	10,93	11,69	0,0026
BE398672	Homo sapiens calcium binding protein (ALG-2) mRNA, complete cds	-0,83	10,16	-11,65	0,0026
BE398446	Triticum aestivum low-molecular-weight glutenin storage protein mRNA, complete cds	2,55	9,67	11,30	0,0028
BE422961	Triticum monococcum BAC clones 116F2 and 115G1 gene sequence	2,57	10,11	11,28	0,0028
BE398933	Rice mRNA for ribosomal protein S12 (320 gene), partial sequence	-0,55	9,14	-11,23	0,0028
BE424414	O.sativa R22 mRNA	1,52	9,34	11,04	0,0031
BE590546	Arabidopsis thaliana chromosome 5 CHR5v12152001 genomic sequence	2,40	10,94	10,94	0,0031
BE399079	Human DNA sequence from clone RP3-468K3 on chromosome 6q25-26. Contains a beta tubulin pseudogene, STSs and GSSs, complete sequence [Homo sapiens]	2,73	8,97	10,86	0,0031
BE424439	Triticum aestivum low-molecular-weight glutenin storage protein mRNA, complete cds	3,10	11,37	10,78	0,0032
BE424439	Triticum aestivum low-molecular-weight glutenin storage protein mRNA, complete cds	3,17	11,97	10,63	0,0034
BE424386	Oryza sativa genomic DNA, chromosome 6, PAC clone:P0015E04	0,79	8,70	10,36	0,0038
BE438245	Triticum monococcum actin (ACT-1) gene, partial cds; putative chromosome condensation factor (CCF), putative resistance protein (RGA-2), putative resistance protein (RGA2) and putative nodulin-like-like protein (NLL) gene, complete cds	-3,17	9,35	-10,22	0,0040
BQ805925	Triticum aestivum 70 kDa heat shock protein (TaHSP70d) mRNA, complete cds	-0,45	8,68	-10,07	0,0043
BG314518	Triticum aestivum heat shock protein 80 mRNA, complete cds	-0,46	8,35	-10,03	0,0043
BQ805944	T.aestivum mRNA for serpin WZS3	1,10	8,25	9,89	0,0044
BE399173	Oryza sativa genomic DNA, chromosome 1, PAC clone:P0410E01	-0,85	8,99	-9,82	0,0046
BE591089	Hordeum vulgare calcium-binding protein 1 (CaBP1) mRNA, complete cds	0,57	7,68	9,59	0,0052
BE590709	Triticum aestivum mRNA for monomeric alpha-amylase inhibitor (WAI-0.28)	-0,75	10,02	-9,47	0,0054
BE590674	low molecular weight glutenin [Triticum aestivum]	2,37	10,51	9,46	0,0054

BF473910	Oryza sativa genomic DNA, chromosome 1, PAC clone:P0468B07, complete sequence	1,64	10,32	9,46	0,0054
BE398649	Triticum aestivum mRNA for sucrose synthase type 2	-0,37	7,97	-9,42	0,0055
BE398375	Triticum monococcum BAC clones 116F2 and 115G1 gene sequence	-2,50	8,56	-9,29	0,0059
BE423717	T.durum mRNA for CM3 protein	-0,69	9,41	-9,16	0,0064
BE424110	Triticum monococcum BAC clones 116F2 and 115G1 gene sequence	-1,00	7,26	-9,05	0,0067
BE423473	T.aestivum WZCI mRNA for serpin	0,91	8,77	9,04	0,0067

Tab. 4.5: List of the differentially expressed clones between the wild type and the transgenic genotype obtained from the analysis of the microarray experiment at 10 DPA "late". Only the top 50 clones, in order of decreasing statistical evidence, are shown. Clones in red are discussed in the text.

4.2.4 Differentially-expressed genes at 20 DPA

The list of statistically significant DE genes obtained from the LIMMA analysis is shown in Tab. 4.6. The volcano plot (Fig. 4.3) shows that large variation in gene expression levels are occurring also at this timepoint, with a considerable increase in the number of significant DE down-regulated clones with respect to 10 DPA "late"; this is suggested by the crowded region of points (clones) at the upper-left part of the volcano plot. Similarly to the previous timepoint, several *lmw-gs* clones (3 *lmw-gs* clones within the top 50), along with their associated putative transcription factors-encoding clones (BQ804784 *Mus musculus* zinc finger protein regulator of apoptosis and cell cycle arrest (Zac1), and BE423328 (*Zea mays* putative transcription factor mRNA sequence), are all significantly up-regulated, with a Log_2 fold change variation in the range 1,2-1,5.

The over-expression of the *lmw-gs* genes is associated, from now on, to a vast down-regulation of many different genes, belonging to a wide range of functional categories. Within the 360 significant DE clones at 20 DPA (only the top 50 clones are shown in Tab. 4.6), and excluding those mentioned above, only 17 clones (4,7%, mostly unknown) are significantly up-regulated in the transgenic genotype with respect to the wild type. Among the down-regulated genes, many of them encode the storage proteins of the endosperm. Except for the *lmw-gs* clones, which are logically over-expressed, very likely because they all hybridize with the *lmw-gs* transgenic transcripts, several other classes of genes encoding seed storage proteins are heavily down-regulated.

Within the top 50 significant DE clones, we found BE422727 (Wheat gene for alpha/beta-gliadin storage protein with a Log_2 fold change variation of -1,24), BE398271 (*Triticum aestivum* HMW glutenin subunit Ax2* gene, complete cds, with a Log_2 fold change variation of -1,82) and BE399706 (Wheat *T. aestivum* gamma-gliadin gene, complete cds with a Log_2 fold change variation of -1,71).

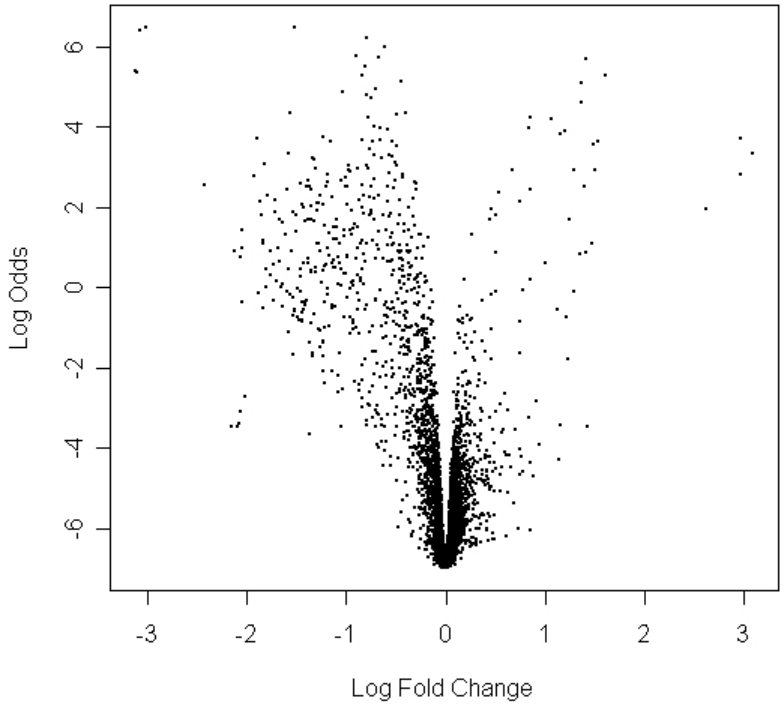


Fig. 4.3 Volcanoplot for the microarray comparison at 20 DPA. See Fig. 4.2 for details.

ID	Name	M	A	t	P-value
BE399173	Oryza sativa genomic DNA, chromosome 1, PAC clone:P0410E01	-0,81	8,27	-17,59	0,0008
BE423328	Zea mays putative transcription factor mRNA sequence	1,37	8,62	16,50	0,0008
BE424757	Homo sapiens 3 BAC RP11-88H10 (Roswell Park Cancer Institute Human BAC Library) complete sequence	-0,90	7,48	-15,84	0,0009
BE423245	Human DNA sequence from clone RP11-103A2 on chromosome 10, complete sequence [Homo sapiens]	-0,61	7,20	-15,40	0,0009
BE606698	Oryza sativa GTP-binding protein mRNA, complete cds	-0,80	9,05	-15,36	0,0009
BQ804784	Mus musculus zinc finger protein regulator of apoptosis and cell cycle arrest (Zac1), mRNA	1,36	11,76	15,27	0,0009
BE498001	Oryza sativa genomic DNA, chromosome 1, PAC clone:P0504E02, complete sequence	-0,75	7,68	-15,05	0,0009
BQ804864	Oryza sativa genomic DNA, chromosome 1, PAC clone:P0506B12, complete sequence	0,83	7,29	17,87	0,0012
BE637490	Homo sapiens cosmid clone U39B3 from Xp22.1-22.2, complete sequence	-0,67	8,67	-14,19	0,0012
BE399079	Human DNA sequence from clone RP3-468K3 on chromosome 6q25-26. Contains a beta tubulin	1,07	9,24	14,02	0,0012

	pseudogene, STSs and GSSs, complete sequence [Homo sapiens]				
BE398235	T.aestivum GSP-1b mRNA for grain softness protein	-1,56	8,68	-13,66	0,0013
BE424629	Triticum aestivum low-molecular-weight glutenin storage protein mRNA, complete cds	1,20	11,75	13,52	0,0014
BQ805498	Triticum aestivum small heat shock protein HSP17.8 mRNA, complete cds	-0,50	7,44	-13,27	0,0015
BE423328	Zea mays putative transcription factor mRNA sequence	1,53	9,64	12,95	0,0016
BE590709	Triticum aestivum mRNA for monomeric alpha-amylase inhibitor (WAI-0.28)	-1,90	9,44	-12,89	0,0016
BE606912	Mus musculus homeodomain protein RINX (Rinx) gene, complete cds	-1,04	7,18	-12,65	0,0017
BE590765	Human chromosome 14 DNA sequence BAC R-817G13 of library RPCI-11 from chromosome 14 of Homo sapiens (Human), complete sequence	-0,73	10,79	-12,58	0,0017
BE605124	Homo sapiens LOC158818 (LOC158818), mRNA	1,48	9,99	12,49	0,0017
BE604780	Homo sapiens clone RP11-69N1, complete sequence	-0,84	7,94	-12,42	0,0017
BE423289	T.aestivum L mRNA for histone H2B	-0,54	7,61	-12,19	0,0018
BE398645	chromosome VI of strain GB-M1 of Encephalitozoon cuniculi (Microspora)	-0,76	7,92	-12,11	0,0019
BE398172	Oryza sativa genomic DNA, chromosome 1, PAC clone:P0492G09	-1,34	10,71	-11,91	0,0020
BE422727	Wheat gene for alpha/beta-gliadin storage protein	-1,24	12,05	-11,60	0,0023
BE399372	T.aestivum mRNA for PUP88 protein	-0,72	7,16	-11,24	0,0027
BE590822	Arabidopsis thaliana chromosome 2 CHR2v12152001 genomic sequence	-0,54	11,50	-11,17	0,0027
BE424388	Wheat mRNA for chloroform/methanol-soluble (CM16) protein	-0,98	7,21	-11,14	0,0027
BE424082	Triticum spelta alpha-gliadin gene, partial	-1,15	13,63	-10,99	0,0028
BE424439	Triticum aestivum low-molecular-weight glutenin storage protein mRNA, complete cds	1,16	11,38	10,98	0,0028
BE426625	Oryza sativa chromosome 3 BAC OSJNBa0010E04 genomic sequence, complete sequence	-0,83	8,85	-10,89	0,0029
BE498242	Hordeum vulgare HSP70 mRNA, complete cds	0,66	7,28	10,89	0,0029
BE398585	Homo sapiens BAC clone RP11-1146E4 from 2, complete sequence	-0,72	7,75	-10,78	0,0029
BE423717	T.durum mRNA for CM3 protein	-1,93	7,95	-10,64	0,0029
BE398271	Triticum aestivum HMW glutenin subunit Ax2* gene, complete cds	-1,82	11,65	-10,64	0,0029
BE422971	Homo sapiens chromosome 2 clone RP11-737H13, complete sequence	-0,51	7,23	-10,62	0,0029
BQ806457	Oryza sativa genomic DNA, chromosome 1, PAC clone:P0034E02, complete sequence	-1,46	8,18	-10,62	0,0029
BE424512	Homo sapiens PAC clone RP1-15I23 from 22, complete sequence	-1,31	7,57	-10,60	0,0029
BE424523	Castanea sativa 26S proteasome regulatory subunit S12 isolog-like	-1,20	7,29	-10,49	0,0030

	protein mRNA, partial cds				
BE424384	T.aestivum mRNA for peptidylprolyl isomerase	-0,41	7,17	-10,41	0,0031
BF472998	Arabidopsis thaliana chromosome 3 CHR3v12152001 genomic sequence	-0,84	7,73	-10,40	0,0031
BE499422	Oryza sativa genomic DNA, chromosome 1, PAC clone:P0475H04	-0,97	8,60	-10,36	0,0031
BE637485	Ha=GSP-1a [Triticum=wheat, cv. Timgalen, endosperm, mRNA Partial, 540 nt]	-1,79	9,52	-10,26	0,0032
BE422622	Triticum aestivum 70 kDa heat shock protein (TaHSP70d) mRNA, complete cds	-0,57	7,35	-10,25	0,0032
BE606924	Mycobacterium tuberculosis CDC1551, section 67 of 280 of the complete genome	-1,35	7,97	-10,16	0,0032
BE399480	Triticum aestivum low-molecular-weight glutenin storage protein mRNA, complete cds	1,30	12,09	10,15	0,0032
BE499948	Homo sapiens glutamate rich WD repeat protein GRWD (GRWD), mRNA	-0,79	9,31	-10,09	0,0032
BF473100	Blastochloris viridis CheY (cheY) gene, partial cds; and RecA protein (recA) gene, complete cds	-0,57	7,91	-10,08	0,0032
BE399706	Wheat (T.aestivum) gamma-gliadin gene, complete cds	-1,71	11,05	-10,07	0,0032
BQ838739	Hordeum vulgare HSP70 mRNA, complete cds	0,86	7,54	10,00	0,0032
BE590469	calcium/calmodulin-dependent protein kinase homolog|CaM kinase homolog|MCK1 [Zea mays=maize, cv. Merit, root caps, mRNA, 2483 nt]	-0,66	8,98	-10,00	0,0032
BE606942	Haemophilus influenzae Rd section 125 of 163 of the complete genome	-1,87	9,53	-9,98	0,0032

Tab. 4.6: List of the differentially expressed clones between the wild type and the transgenic genotype obtained from the analysis of the microarray experiment at 20 DPA. Only the top 50 clones, in order of decreasing statistical evidence, are shown Clones in red are discussed in the text.

4.2.5 Differentially-expressed genes at 30 and 35 DPA

The volcano plots related to the experiments carried out at 30 and 35 DPA are shown in Fig. 4.4 and 4.5, respectively. The lists of significant DE genes are similar both between the two timepoints and also to 20 DPA. They almost present the same up- and down-regulated clones and are thus not presented here.

Again, large variation in gene expression levels are occurring both at 30 and 35 DPA. It is interesting to notice a shift of the down-regulated clones towards the region at the left of the volcano; and this is even more evident at 35 DPA compared with 30 and 20 DPA. Conversely, the region of up-regulated clones, at the right of the volcano, becomes gradually less rich of significant clones; and actually at 30 DPA the unique significant over-expressed clones (within a fdr-corrected P-value threshold of 0,05) are again two *lmw-gs* encoding clones (with a Log_2 fold change variation of +1,37 and +1,21, respectively) along with their associated putative transcription factors-encoding clones (BQ804784 *Mus musculus* zinc finger protein regulator of

apoptosis and cell cycle arrest (Zac1), and BE423328 (*Zea mays* putative transcription factor mRNA sequence), which both show, as in the previous timepoints, a Log_2 fold change variation of the same order of magnitude of the *lmw-gs* clones.

Most of the significant down-regulated clones, for which a putative annotation was available, both at 30 and 35 DPA, are again clones encoding several classes of seed storage proteins (high-molecular-weight glutenin subunits, α/β-, γ- and ω-gliadins, globulins, etc.) as well as clones putatively encoding several ribosomal proteins or proteins generally involved in the transcription and translation processes, but also CM proteins, serpins (Serin Proteinase inhibitors), α-amylase/trypsin inhibitors, lipid transfer proteins (LTPs), grain softness proteins (GSPs), heat-shock proteins (HSPs), and histones were all heavily down-regulated.

Interestingly, at 35 DPA, the significant DE clones are all down-regulated clones. The absolute expression levels of the *lmw-gs* genes are comparable between the transgenic and the wild type genotype, and they are thus no longer listed as differentially expressed. The overall signals also declined for those transcripts associated with the *lmw-gs*; and their absolute signal intensities were not significantly different from the wild type.

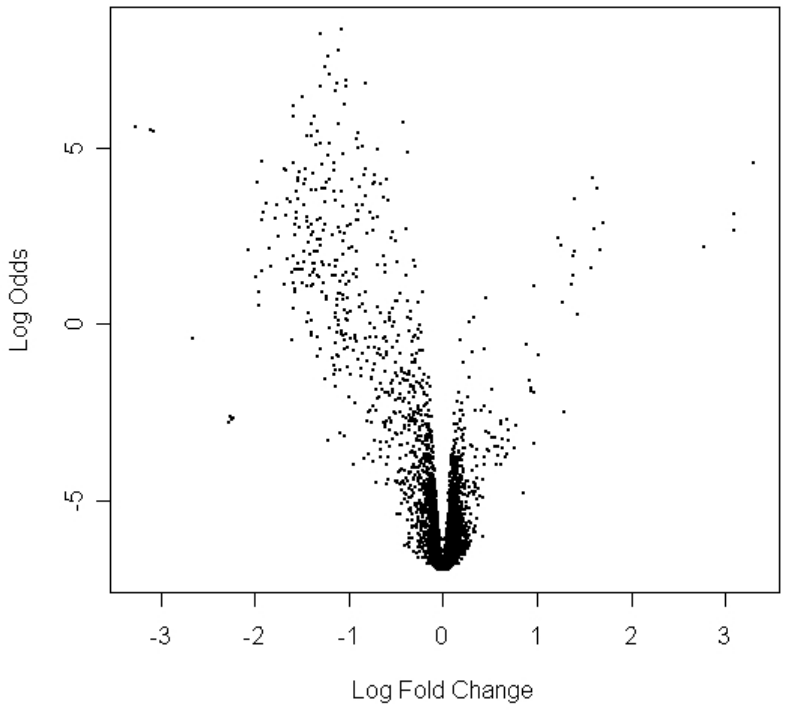


Fig. 4.4 Volcano plot for the microarray comparison at 30 DPA. See Fig. 4.2 for details.

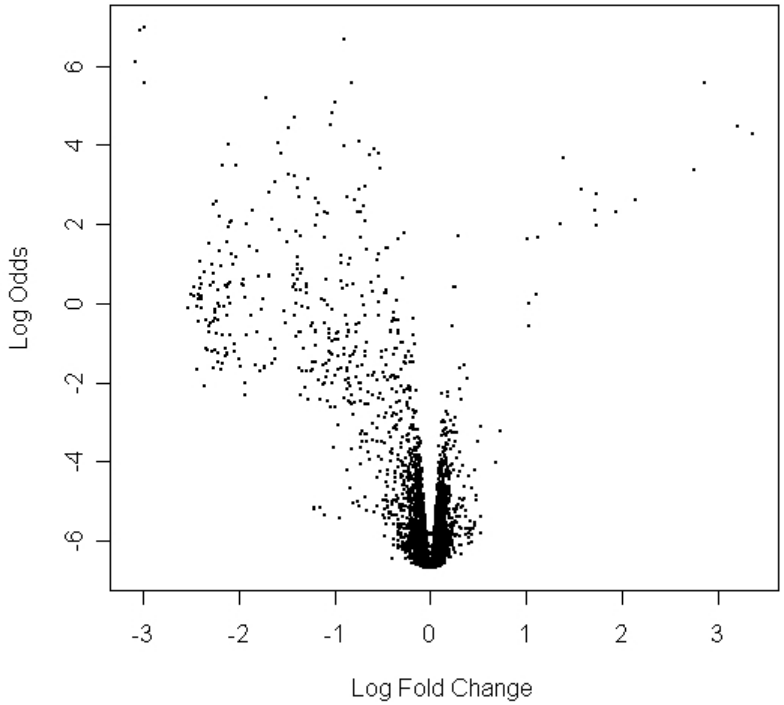


Fig. 4.5 Volcano plot for the microarray comparison at 35 DPA. See Fig. 4.2 for details.

4.3 Expression profiles of genes during seed maturation

The transformation of the entire microarray dataset from a direct comparison into a time-series design has allowed us to extract the single-channel normalized absolute intensities to plot temporal gene expression profiles during the period of seed filling. This has extended the information limited to the DE genes at each timepoint analysed to a more integrated vision of how expression levels change for a specific gene, *within* each genotype, across time. We thus decided to plot the transcript profiles of the most interesting genes previously identified.

4.3.1 The issue of cross-hybridization

Many of the significant DE clones identified actually belong to several multigene families, whose members share a high degree of similarity (and thus homology) at the level of nucleotide sequence. This is the case of low-molecular-weight glutenin subunit genes, whose total number of gene copies has been estimated by genomic Southern-hybridization analysis to be 20-40 (Sabelli and Shewry, 1991; Cassidy et al. 1998). At this regard, Kawaura et al. (2005), were able to define only 15 groups by clustering of ESTs homologous to LMW-GS. It is thus possible that only half of the *lmw-gs* gene copies reported previously are actually expressed in bread wheat. Anyway, cross-hybridization of all the *lmw-gs* transcripts to the different *lmw-gs* probes definitively occurs also in the context of cDNA microarrays.

Within all the other classes of seed-storage protein related genes (HMW-GS, α/β-, γ-, and ω-gliadins) we also have evidence of extensive cross-hybridization between the various class of transcripts to their relative array probes, and the wheat cDNA array is thus not able to discriminate within the single members of a multigene family.
Previous experiments involving the wheat cDNA array showed our estimates regarding the threshold of cross-hybridization to be in accordance to the data reported by Girke et al. (2000); we therefore know that over 80-90% of sequence similarity cross-hybridization occurs. This has to be kept in mind when looking at the gene expression profiles reported here.
We decided to plot the output data from each clone without any further transformation; and we thus superimposed gene expression profiles from the transgenic and the wild type genotype for several clones belonging to the same gene family (Fig. 4.7, 4.8, 4.9). This also confirmed the consistency of the intensity signals between different clones of the same family.
We anticipate that no speculation can be made, within each genotype, about the differential expression of clones belonging to the same multigene family

4.3.2 Storage proteins

Seed storage proteins related mRNAs were highly abundant, at least in the wild type, from 10 DPA "late" to 35 DPA; and their expression was clearly temporally regulated during seed development.
In the wild type, both the HMW-GS, the LMW-GS and gliadin genes had highly similar patterns of temporal expression; and this is also in agreement with previous RT-PCR results reported by Altenbach et al. (2002). Lmw-gs, hmw-gs and gliadins mRNAs were under strict temporal control and began accumulating and then decayed at precise intervals during development. For example, in Fig. 4.6, the expression of *lmw-gs* genes drastically increased between 10 DPA "late" and 20 DPA, to decrease then towards the end of the time series. In Fig. 4.7 the same clones, from the transgenic genotype, were superimposed on the profiles of the wild type genotype.
Globally, in terms of mean value, the lmw-gs mRNAs were 8-times more abundant in the transgenic genotype with respect to the wild-type at 10 DPA "late", but their levels of expression were significantly higher also at 20 and 30 DPA. Most strikingly, the peak of maximum quantity of lmw-gs mRNAs is different between the two genotypes: in the transgenic the lmw-gs transcripts had a 60-fold increase between 10 DPA "early" and 10 DPA "late", and actually 10 DPA "late" represents the timepoint of maximum accumulation of transcripts; in the wild type the increase between the same timepoints was only 4.5 fold; and only later, at 20 DPA, the lmw-gs transcripts reach their peak of maximum accumulation (Fig. 4.6). Thus, between the two genotypes, lmw-gs mRNAs were not only present in the developing seeds in different quantities (as expected), but they also accumulated and then decayed with different kinetics. This *shift* in the peak of maximum abundance is probably due the larger number of active lmw-gs gene copies (very likely corresponding to the *lmw-gs* transgene due to the cross-hybridization problem above mentioned) rather than to an altered transcription rate. Lmw-gs transcripts are in

fact detectable in the wild type since 10 DPA “late”, suggesting that the higher expression levels in the transgenic genotype are proportional to the number of transcriptionally active *lmw-gs* genes.

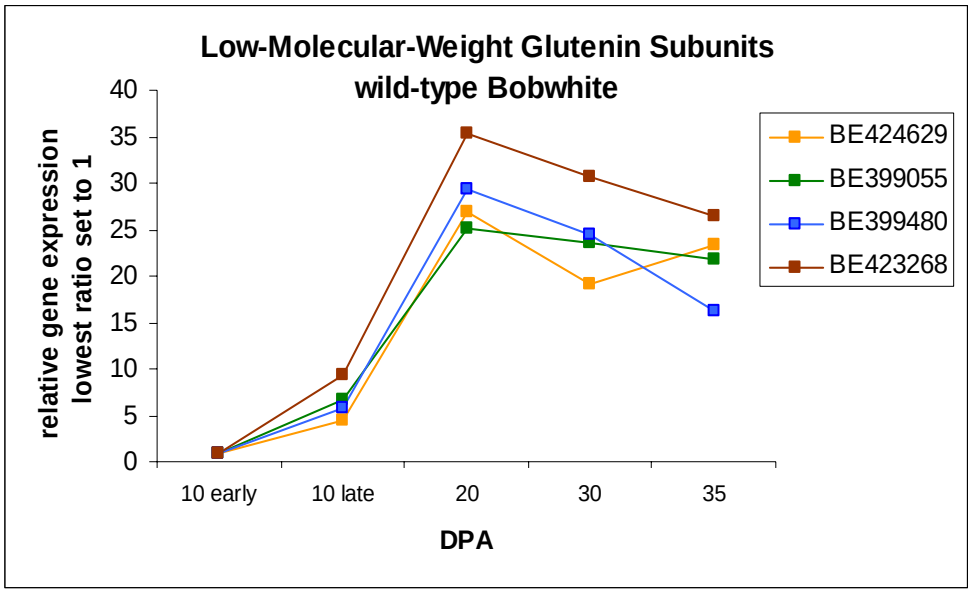


Fig. 4.6 Expression profiles of *lmw-gs* genes in the wild type genotype. Expression is related to 10 DPA “early”, and the ratios were rescaled by setting the lowest ratio to 1.

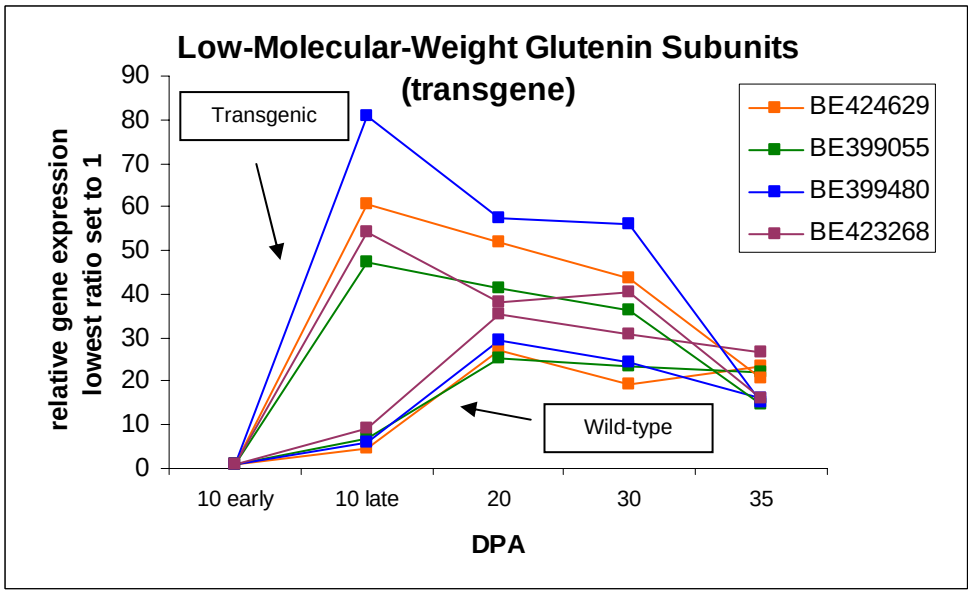


Fig. 4.7 Comparison between the expression profiles of *lmw-gs* genes in the wild type and in the transgenic genotype. Expression is related to 10 DPA “early”, and the ratios were rescaled by setting the lowest ratio to 1. Each clone should be looked at independently from the others. The ratio of the relative gene expression values (y-axis) for the same clone between the wild type and the transgenic line is the approx. fold-change for that clone at that timepoint. Some *lmw-gs* clones are significantly DE at 20 and 30 DPA, too.

As outlined in the previous sections, *hmw-gs* and gliadins genes were also differentially regulated. We report in Fig. 4.8 and 4.9 the comparison of the hmw-gs and α/β-gliadins transcript profiles between the wild type and the transgenic genotype. In the wild type, hmw-gs and α/β-gliadins transcripts have a similar pattern of expression to the *lmw-gs* genes: they show a drastic increase between 10 DPA "late" and 20 DPA, with a peak of maximum abundance at 20 DPA (*hmw* and *lmw-gs* genes) or at 30 DPA (α/β-gliadins) to decrease towards the end of the time series, when the seeds begin dessiccating. From 20 DPA, in the transgenic genotype, the transcripts for hmw-gs and α/β-gliadins are contantly down-regulated, and their mean absolute level of expression during the period of grain fill is almost constant (however, a modest peak of hmw-gs or α/β-gliadins maximum abundance is detectable around 20 or 30 DPA, respectively, and it is thus synchronous with those showed by the wild type).

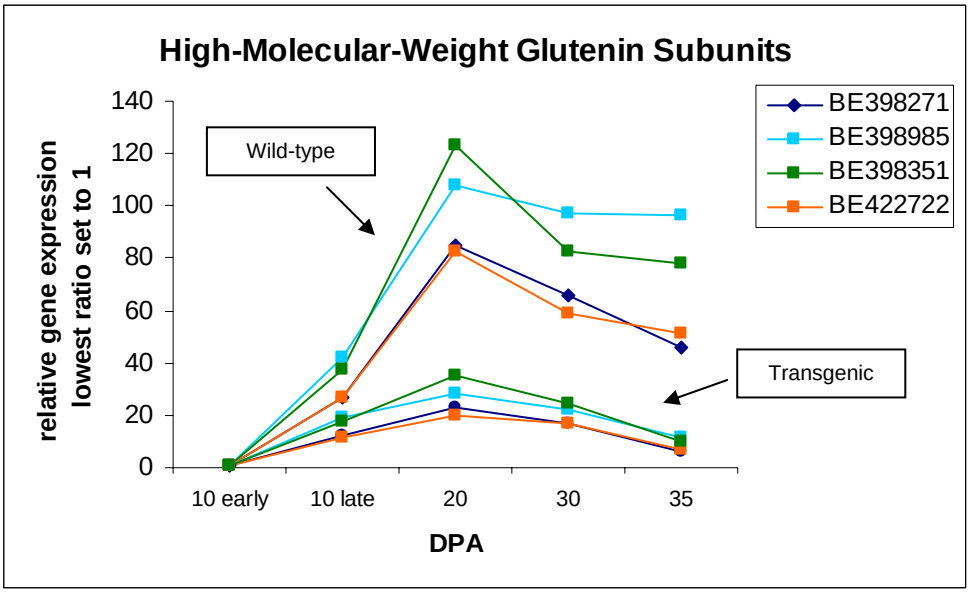


Fig. 4.8 Comparison between the expression profiles of *hmw-gs* genes in the wild type and in the transgenic genotype. Expression is related to 10 DPA "early", and the ratios were rescaled by setting the lowest ratio to 1. Each clone should be looked at independently from the others. The ratio of the relative gene expression values (y-axis) for the same clone between the wild type and the transgenic line is the approx. fold-change for that clone at that timepoint. *hmw-gs* genes are heavily down-regulated at 20, 30 and 35 DPA.

It is very likely that this extensive down-regulation of transcripts related to the seed-storage proteins is a response to the over-expression of the *lmw-gs* transgenes. Down-regulation of γ- and ω-gliadin genes was also detected, and their temporal patterns of expression are similar to those already showed regarding hmw-gs and α/β-gliadins: in the wild type, a peak of maximum abundance during the mid-stages of development with a decrease towards the end of the time-series; and in the transgenic genotype, a marked down-regulation from 20 DPA to 35 DPA, with almost a constant level of expression throughout the period of grain fill (data not shown). In the section "overall gene regulation" we already outlined that a consistent fraction of the total DE

clones identified actually represent genes encoding seed storage related-proteins (Tab. 4.3); and we thus have evidence of a vast differential expression directed, preferentially, towards the genes of the prolamin class. We postulate the existence of a negative feedback metabolic loop which maintains a homeostatic level of seed storage proteins compatible with seed development and germination.

It is known (Karchi et al. 1994) that plants might respond to a drastic increase in the production of nutrients or storage compounds (induced by over-expression of their encoding or biosynthetic genes), by activating a silencing/degradation pathway that negates the accumulation of storage reserves. This metabolic feedback seems definitively active in the seeds of the transgenic line (proteolytic peptides of LMW-GS have also been identified in mature grains of the transgenic line, see section 4.4.2); and the molecular signal responsible for this activation could reasonably be the number (per cell) of mRNA molecules encoding LMW-GS; which, in turn, could trigger a cascade of molecular events determining both the transcriptional down- or up-regulation of the many genes reported as significantly differentially expressed. Alternatively, a possible explanation of the vast down-regulation could simply be the titration of transcription- and translation-related proteins to the transcription, translation and processing of the transgenic LMW-GS; so that the cellular resources (transcription factors, RNA polymerases, ribosomes, ATP, etc.), almost all directed to the production of the transgenic polypeptides, would not be available in the transcription of the mRNAs corresponding to the genes identified as down-regulated. Of course this mechanism would not explain the molecular signals leading to the over-expression of the up-regulated genes identified through the period of seed development.

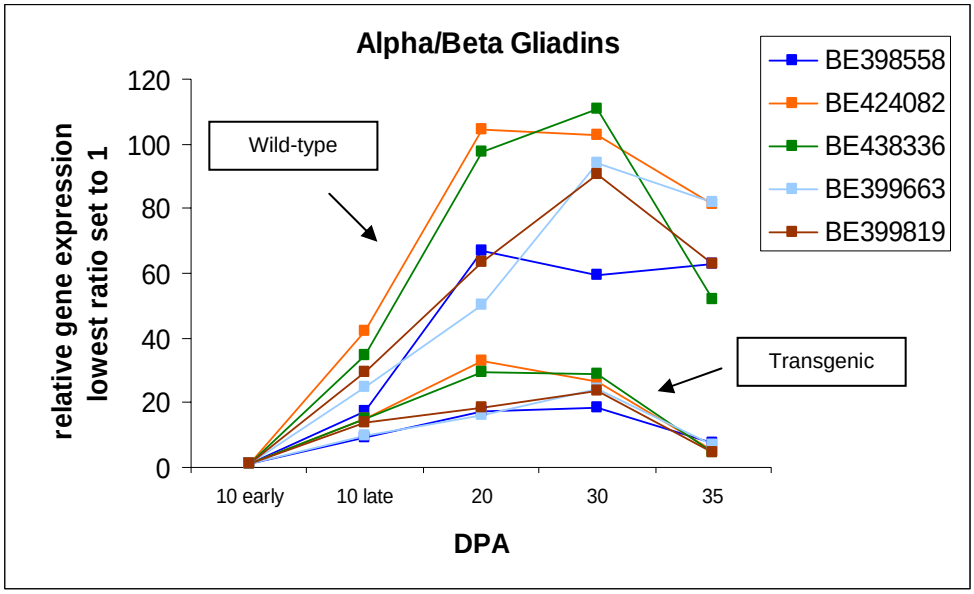


Fig. 4.9 Comparison between the expression profiles of α/β-gliadin genes in the wild type and in the transgenic genotype. Expression is related to 10 DPA "early", and the ratios were rescaled by setting the lowest ratio to 1. Each clone should be looked at independently from the others. The ratio of the relative gene expression values (y-axis) for the same clone between the wild type and the transgenic line is the approx. fold-change for that clone at that timepoint. α/β-gliadins are down-regulated at 20, 30 and 35 DPA.

4.3.3 Over-expressed genes have two different patterns of expression

To group the genes significantly up-regulated (with respect to the wild type genotype) with similar temporal expression patterns, we used the average linkage clustering algorithm of Eisen et al. (1998). The assumption behind clustering is that gene with similar expression profiles share a common biological function, and this could provide preliminary indications about their role in seed development.

The majority of the over-expressed genes during seed development could be divided in two subclusters, according to their trend of significant differential expression with respect to the wild type.

The absolute mean expression in first group (subcluster 1) followed a bell-shaped pattern that increased from 10 DPA "early" to 10 DPA "late", peaked around the middle stages of development (10 DPA "late" and 20 DPA) and then decreased from 30 DPA to 35 DPA. The significant over-expression of these genes persisted, with respect to the wild type, throughout 10 DPA "late", 20 and 30 DPA. Examples of genes belonging to this subcluster are shown in Fig. 4.10 and 4.11 and include, among others, the two putative transcription factors BQ804784 *Mus musculus* zinc finger protein regulator of apoptosis and cell cycle arrest (Zac1), and BE423328, *Zea mays* putative transcription factor mRNA sequence. Also several genes whose poor annotation does not permit to assign a putative identity to the clone belong to subcluster 1.

The temporal mean absolute expression profiles of genes belonging to subcluster 1 is similar, both in shape and relative magnitude of variation, to the expression profiles of several LMW-GS-encoding clones in the transgenic genotype (Fig. 4.7). Based on that, we propose a functional correlation of the genes included in the subcluster1 with genes encoding the LMW-GS, from 10 DPA "late" to 30 DPA. The high quantity of the lmw-gs transcripts may provoke a proportional shift and a subsequent, persistent up-regulation of several other related genes. This would be a strong indication about the functional role of those unknown genes.

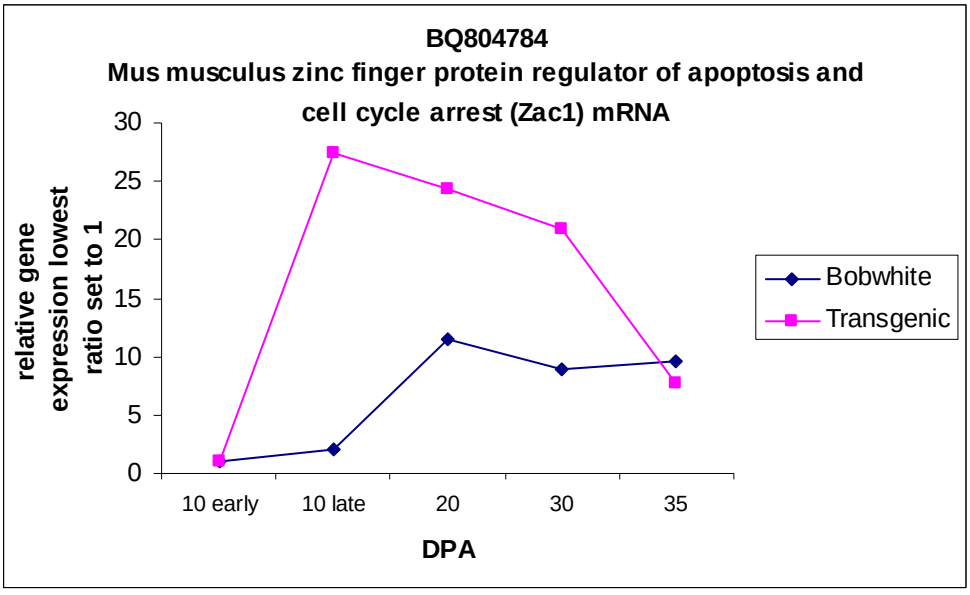


Fig. 4.10 (previous page). Comparison of the temporal gene expression profiles for the clone BQ804784. The ratio of the relative gene expression values (y-axis) between the wild type and the transgenic is the approx. fold-change for that clone at that timepoint. This clone is significantly up-regulated at 10 DPA "late", 20 and 30 DPA. The transgenic transcript profile is an example of a gene belonging to subcluster 1. The annotation reported is relative to the best BlastX hit, and has an E-value=0,5. The clone putative identity is thus unknown.

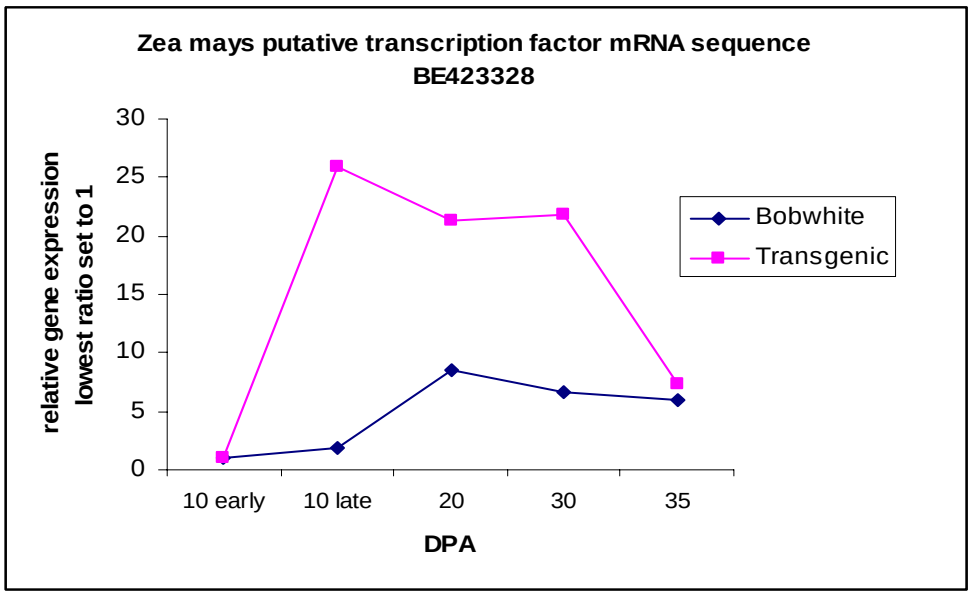


Fig. 4.11 Comparison of the temporal gene expression profiles for the clone BE423328. The ratio of the relative gene expression values (y-axis) between the wild type and the transgenic is the approx. fold-change for that clone at that timepoint. This clone is significantly up-regulated at 10 DPA "late", 20 and 30 DPA. The transgenic transcript profile is an example of a gene belonging to subcluster 1.

The second group (subcluster 2) was characterized by a distinctly different profile; genes belonging to this second group follow a pattern with a drastic increase from 10 DPA "early" to 10 DPA "late", as in subcluster 1, but 10 DPA "late" represents both the peak of maximum abundance and the only timepoint where a significant over-expression shows up. After 10 DPA "late", towards the end of the time-series, gene expression decreases. Genes belonging to subcluster 2 are those whose peak of maximum abundance, in the transgenic, is clearly shifted, from 20 DPA (wild type) to 10 DPA "late" (transgenic). Members of subcluster 2, at least those for which a putative identity is known, are generally clones encoding metabolic enzymes (BE591113, 12-oxophytodienoate reductase), kinases (BE606262), peptidase and transcription factors different from those belonging to subcluster1.

Fig. 4.12 and 4.13 represents examples of genes whose transgenic transcript profiles belong to subcluster 2.

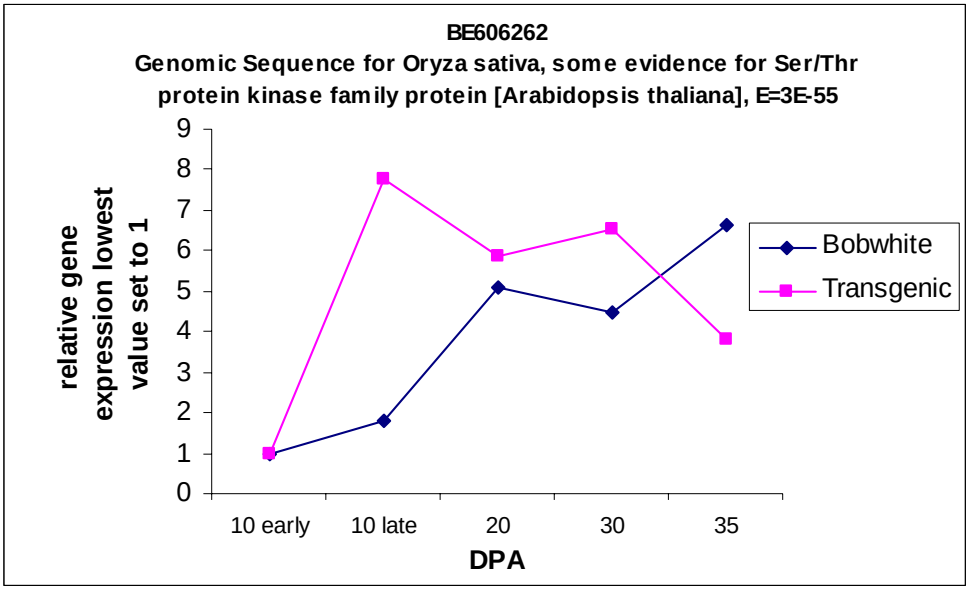


Fig. 4.12 Comparison of the temporal gene expression profiles for the clone BE606262. The ratio of the relative gene expression values (y-axis) between the wild type and the transgenic is the approx. fold-change for that clone at that timepoint. This clone is significantly up-regulated only at 10 DPA "late". The transgenic transcript profile is an example of a gene belonging to subcluster 2.

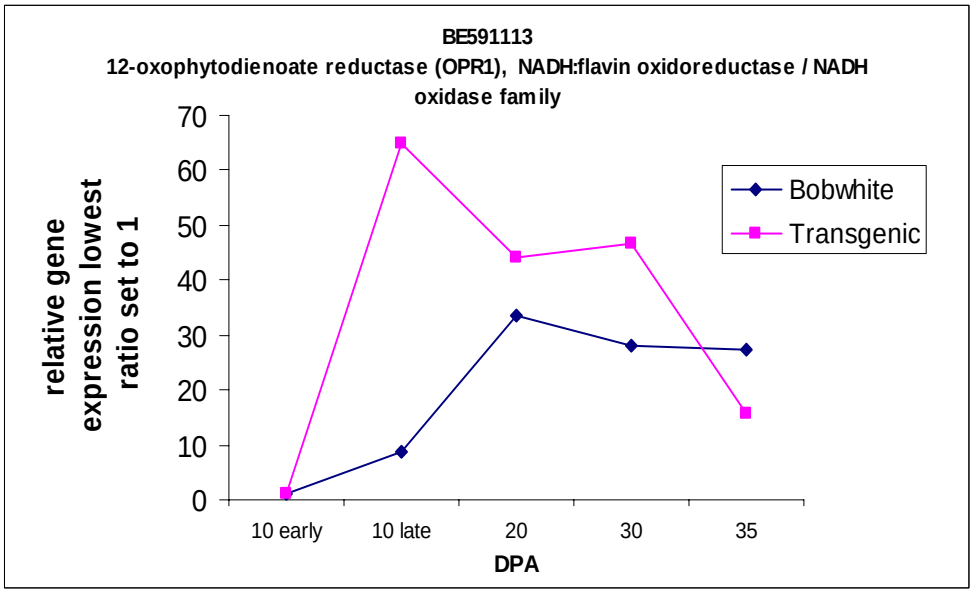


Fig. 4.13 Comparison of the temporal gene expression profiles for the clone BE591113. The ratio of the relative gene expression values (y-axis) between the wild type and the transgenic is the approx. fold-change for that clone at that timepoint. This clone is significantly up-regulated only at 10 DPA "late". The transgenic transcript profile is an example of a gene belonging to subcluster 2.

Since not all over-expressed genes had similar temporal patterns of absolute mean expression, we assume they respond to different induction mechanisms and controls during seed development (Goldberg et al. 1989, Ruuska et al. 2002, Zhu et al. 2003).
We speculate that genes belonging to subcluster 2 might actually constitute those responsible, in the transgenic genotype, to transduce the starting signal (reasonably, the number of mRNA molecules encoding LMW-GS) into the genome-wide transcriptional and chemical responses observed in the later developmental stages of the endosperm cells. This would imply that, once the molecular signal has been transmitted, expression of these putative related genes can be restored to steady-state levels, comparable to those present in the wild type genotype.

.4.3.4 Starch biosynthetic enzymes are not differentially expressed

On the wheat cDNA array, only three different clones involved in the starch biosynthetic pathway passed the threshold criteria of the quality of signal intensity set out in the materials & methods section,. These are: BE637556 (*Triticum aestivum* starch branching enzyme-I, SBE-I mRNA, Fig. 4.14), BM134764 (*Oryza sativa* putative starch synthase III gene, complete cds, Fig. 4.15) and BQ804548 (*O. sativa* starch synthase III, Fig. 4.16).
The temporal patterns of expression of genes involved in starch biosynthesis were clearly distinct from those encoding seed-storage proteins, suggesting that different networks of control separately regulate these pathways. Transcripts for starch synthesis and branching were already highly expressed at 10 DPA “early”, whereas intensity signals for seed-storage proteins, both in the wild type and in the transgenic genotype, at the same timepoint, were still undetectable. Seed storage proteins-encoding genes, with respect to the starch synthases and branching enzymes, were thus turned on at a later stage of seed development. Moreover, the starch biosynthetic enzymes were not significantly differentially expressed in any of the timepoints analysed, suggesting that starch content in the transgenic and wild type genotype does not change significantly.
The separate controls that characterize the starch and the seed storage protein pathways in developing seeds may explain why the increase in the amount of one particular storage protein (as in the case of our trangenic line), does not lead to a balancing increase in other non-protein storage compounds. This nonplasticity of the pathways is also in agreement with previous study on the *Arabidopsis* seed development, where a strong reduction in one class of storage reserves (proteins or oils) did not affect the content of other reserves (Finkelstein and Somerville, 1990; Focks and Benning, 1998).

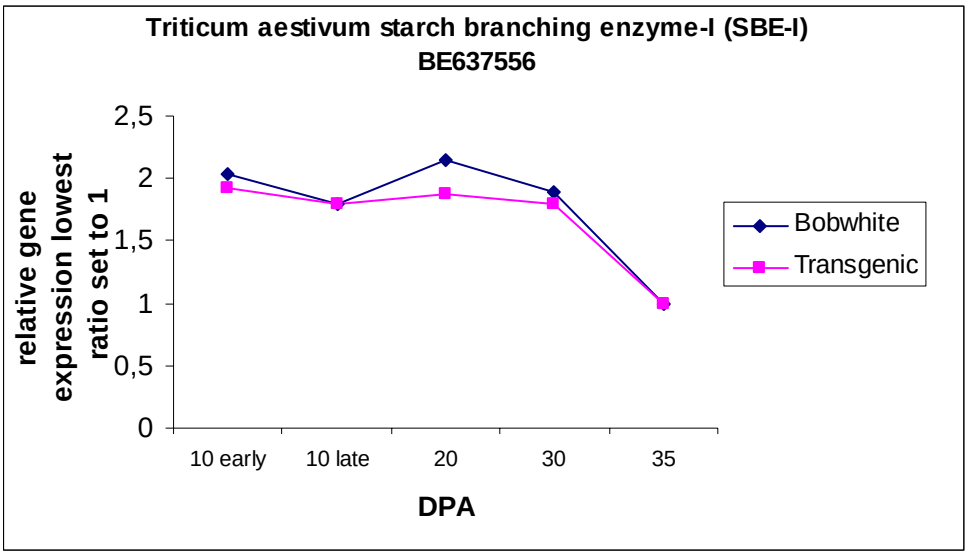


Fig. 4.14 Comparison of the temporal gene expression profiles for the clone BE637556. The ratio of the relative gene expression values (y-axis) between the wild type and the transgenic is the approx. fold-change for that clone at that timepoint. This clone is not significantly DE through seed development.

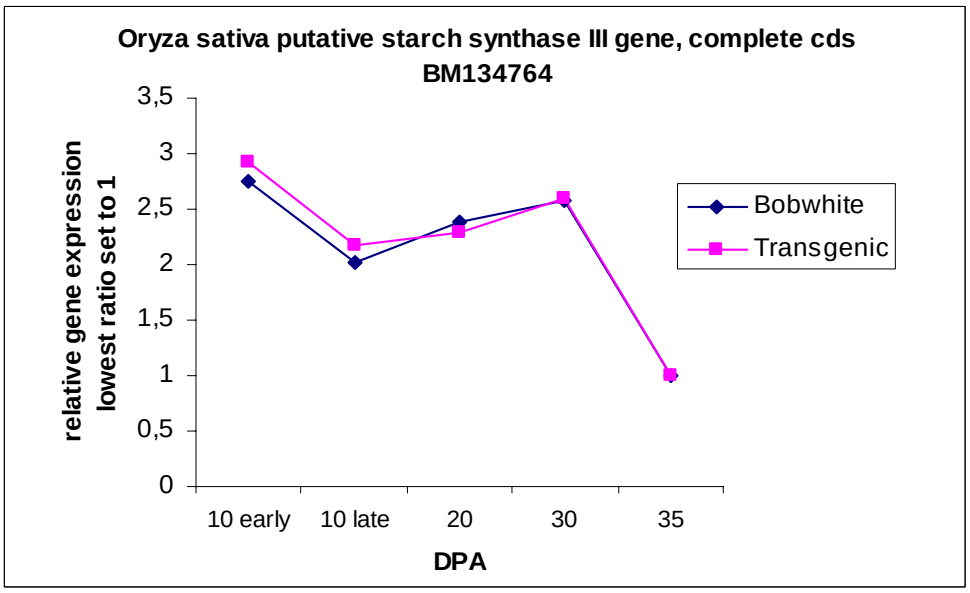


Fig. 4.15 Comparison of the temporal gene expression profiles for the clone BM134764. The ratio of the relative gene expression values (y-axis) between the wild type and the transgenic is the approx. fold-change for that clone at that timepoint. This clone is not significantly DE through seed development.

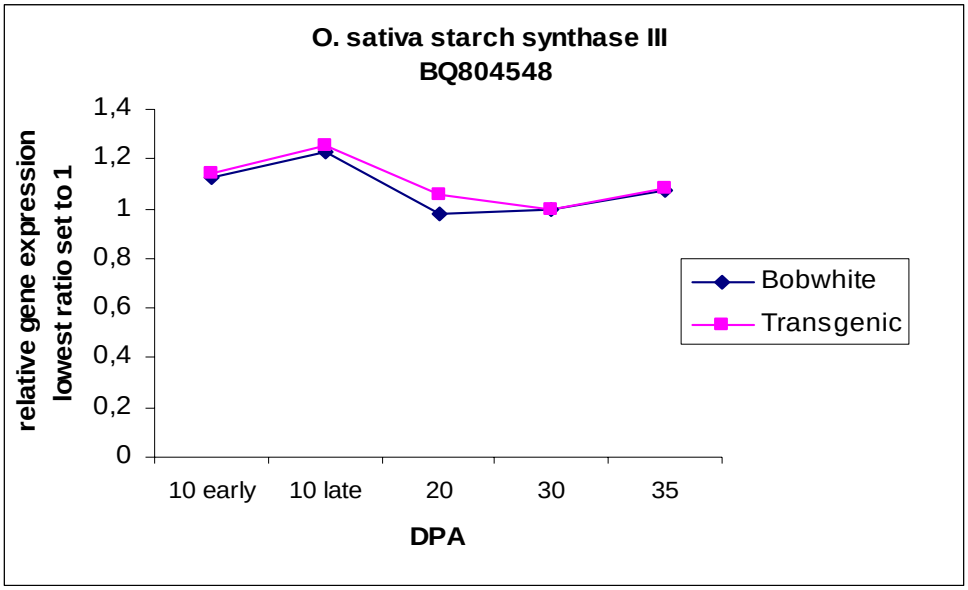


Fig. 4.16 Comparison of the temporal gene expression profiles for the clone BQ804548. The ratio of the relative gene expression values (y-axis) between the wild type and the transgenic is the approx. fold-change for that clone at that timepoint. This clone is not significantly DE through seed development.

4.4 Proteomic profiling

Since mRNAs abundances could not directly reflect the protein content in the seeds, and in order to, at least partially, verify the concordance of microarray data with protein levels, we also carried out a parallel proteomic profiling, on mature grains, of several classes of endosperm proteins. In particular, seed-storage proteins can be easily fractionated according to their differential solubility properties, and the availability of high resolution 2-DE (two-dimensional electrophoresis) techniques allowed us to determine and compare the relative abundance of specific protein sub-classes between the transgenic and the wild type genotype.

Because of the great abundance of seed storage proteins, that might hidden specific classes of proteins, we used a pre-fractionation procedure aimed to analyse, separately, the 2D gel patterns of gliadins and of B, C- and D-type LMW-GS protein fractions, both from the wild type, the transgenic and the "null" genotypes. The null seeds are those obtained by segregation of the T_1 heterozygous individuals and thus negative for the presence of *lmw-gs* and UBI:BAR transgenes. In all analyses performed, the proteome maps of the null individuals were indistinguishable from the wild type, and are thus not reported here.

4.4.1 Two-dimensional electrophoresis (Acid-PAGExSDS-PAGE) of B-type LMW-GS

The 2-D gels of B-type LMW-GS from the wild type and transgenic genotype are shown in Fig. 4.17.

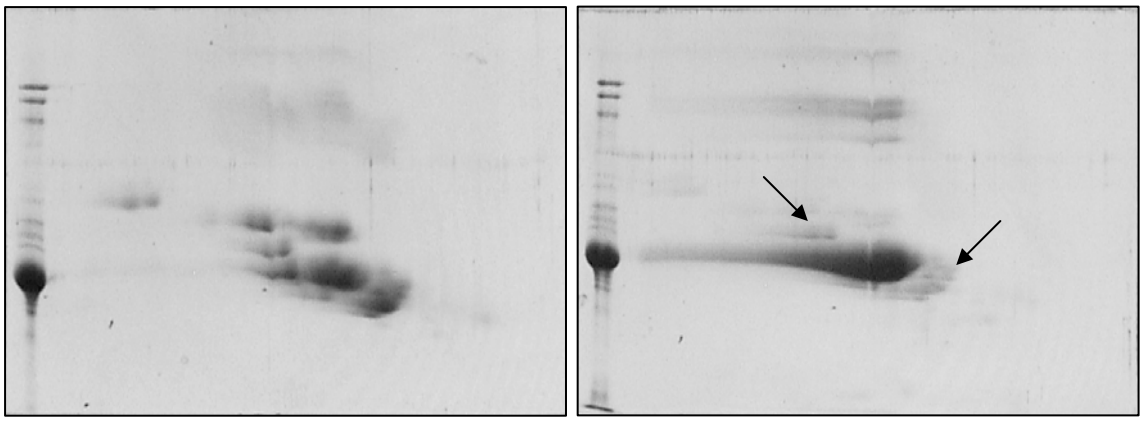


Fig. 4.17: 2-DE (Acid-PAGExSDS-PAGE) of B-type LMW-GS from the wild type (left) and from the transgenic genotype (right). A sample of total seed protein extract from the transgenic genotype (on the left of each image) was included for comparison.

B-type LMW-GS from the wild type genotype, that include also the transgenic protein, were resolved into several components with different net positive charges and molecular weights, confirming their extensive biochemical polymorphism already detected by Jackson et al. (1983), with non-equilibrium pH gradient electrophoresis (NEPHGE), and also reported by Tao and Kasarda (1989).

The two-dimensional pattern of B-type LMW-GS from the transgenic genotype was distinctly different (Fig. 4.17, right) from the wild type one: the transgenic LMW-GS is almost the only B-type subunit expressed in the endosperm of mature grains, although minor amounts of other components are detectable (Fig. 4.17, right, indicated by arrows).

The strong prevalence of the transgenic LMW-GS as the only B-type subunit expressed reflects the marked over-expression of several lmw-gs encoding clones detected by microarray analysis in developing seeds. This confirms the observed cross-hybridization in the microarray experiments. In fact, the different lmw-gs array probes, that showed over-expression during seed development, hybridized to the same mRNA type, derived from the transcription of the lmw-gs transgene blocks.

4.4.2 Two-dimensional electrophoresis (Acid-PAGExSDS-PAGE) of C- and D-type LMW-GS

C- an D-type LMW-GS are distinct sub-classes of LMW-GS. N-terminal amino acid sequencing showed that C-type LMW-GS are mainly formed by α/β- and γ-like gliadin components (Masci et al. 2002), whereas D-group is actually composed of modified ω-gliadin components that have acquired a single cysteine residue (Masci et al. 1993, 1999). C- and D-type LMW-GS can be separated from the bulk of LMW-GS by means of sequential extraction with increasing alcohol concentrations, according to the procedures reported by Masci et al. (1999, 2002).

It has been speculated that differences in the absolute content of C- and D-type LMW-GS would correlate with quality characteristics, since both C- and D-type LMW-GS, having an odd number of cysteine residues (just one for the D-type LMW-GS), would probably act, upon incorporation

into the glutenin polymers, as chain terminators (Tao and Kasarda, 1989; Masci et al. 1999, 2002).

The 2-D gels of C-type LMW-GS from the wild type and transgenic genotype are shown in Fig. 4.18.

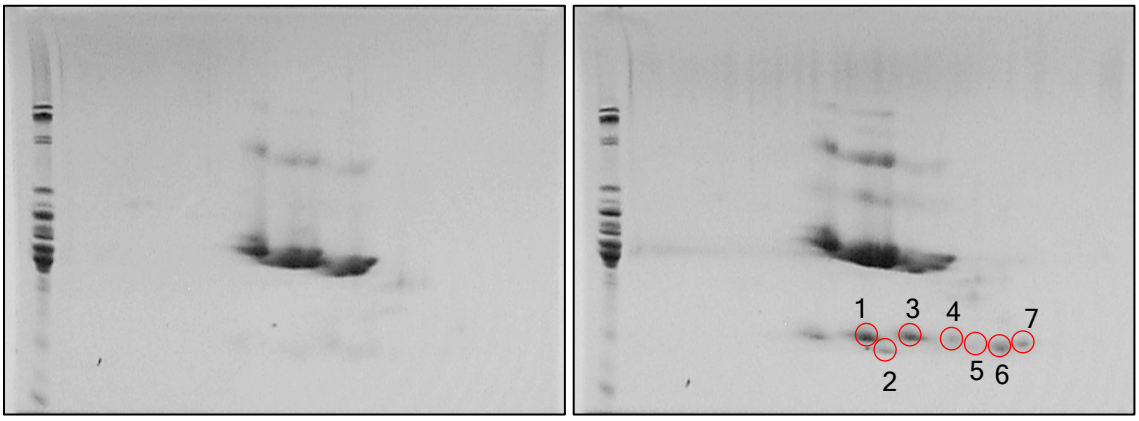


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Fig. 4.18 2-DE (Acid-PAGExSDS-PAGE) of C-type LMW-GS from the wild type (left) and from the transgenic genotype (right). A sample of total seed protein extract from the wild type genotype was included for comparison (on the left of each image). Circled spots (red, right image) are some of those exclusively expressed by the transgenic genotype which have been submitted to N-terminal sequencing.

The 2-DE Acid-PAGExSDS-PAGE allowed separation of the C-group of LMW-GS into several components with different net positive charges and molecular weights; a clearly evident group of low-molecular weight spots (≅ 15 kDa) were exclusively expressed by the transgenic genotype and some of them (Fig. 4.18, right image, red circles) were submitted to N-terminal amino acid sequencing after blotting of the 2D gel onto PVDF membrane. The N-terminal sequences obtained are reported in Tab. 4.7.

Spot number (Fig. 4.18)	N-terminal sequence
1	METRQIPGLE-
2	XXXXCIP-
3	METRQIPGLE-
4	XPTS(Q/P)I(Q/P)(Q/G)L-
5	XQRSQMLVQ-
6	LQQ-
7	LARSQML-

Tab. 4.7 N-terminal amino acid sequences obtained from the circled spots highlighted in Fig. 4.18.

Spots numbers 1, 2, 3, 6 and 7 have sequences typically found within the transgenic LMW-GS itself (Fig. 4.19), and actually spots number 1 and 3 have a 90 % similarity over 10 amino acids

to the N-terminal sequence of a mature LMW-GS. Actually Q could be a derivatized C, when C is not alkylated, as in this case, and thus the similarity would increase to 100 % over 10 amino acids. The presence of LMW-GS fragments could be interpreted as a post-translational mechanism to limit the quantity of the transgenic LMW-GS. However, fragments, putatively belonging to the transgene-encoded protein have also been reported in several wheat lines transformed, by means of particle bombardment, with extra copies of *hmw-gs* genes (Barro et al., 1997; He et al., 2005). As in our case, these extra bands might correspond to truncated forms of the transgenic protein itself. It is thus possible they could result from unequal recombination, at the level of the transgene(s) block, within the nucleotide coding sequences of the repetitive domains.

In addition, the process of particle bombardment could have determined the fragmentation of some of the constructs molecules, resulting in the insertion, and subsequent expression, of truncated transgenes (Blechl and Anderson, 1996; Cannell et al. 1999).

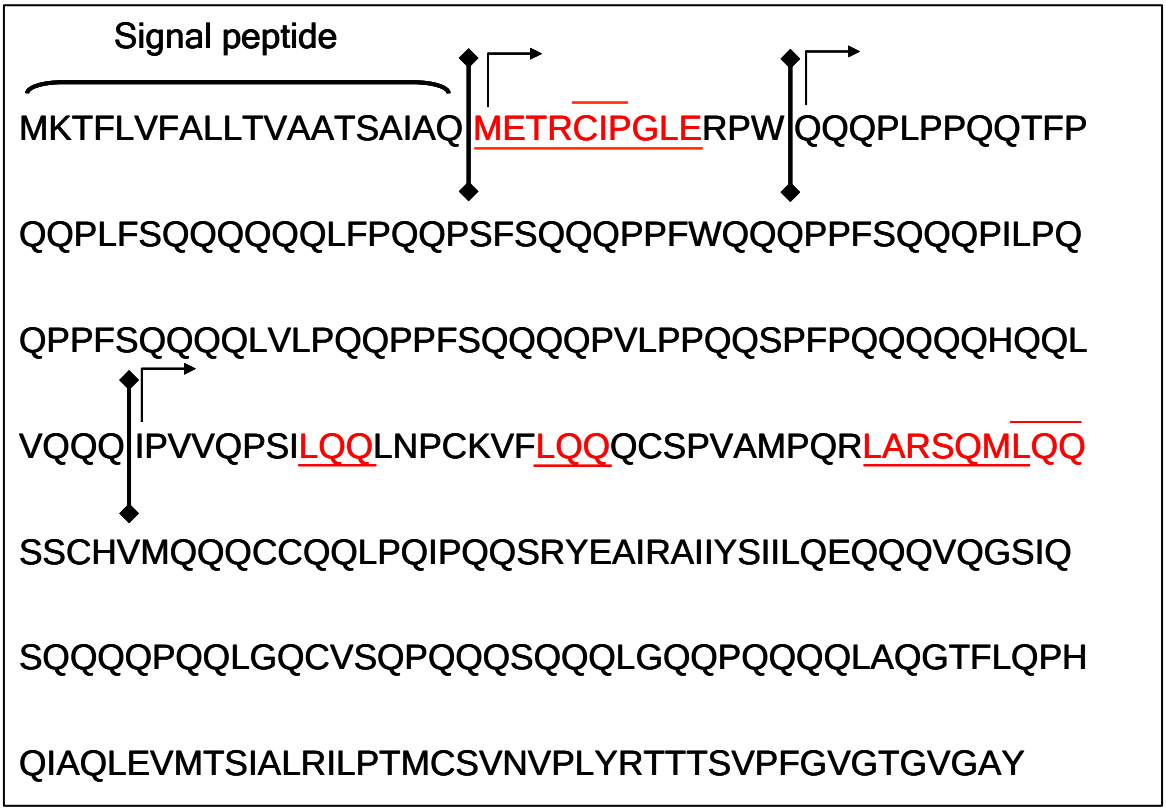

Fig. 4.19 Deduced amino acid sequence of the transgenic LMW-GS encoded by clone F23A (Cassidy et al. 1998). Underlined or overlined in red are fragments identified by N-terminal sequencing in C-type LMW-GS preparations from the transgenic genotype. Vertical brackets and directed arrows define the borders and the start positions of the N-terminal, central repetitive and C-terminal domains of a typical LMW-GS, respectively.

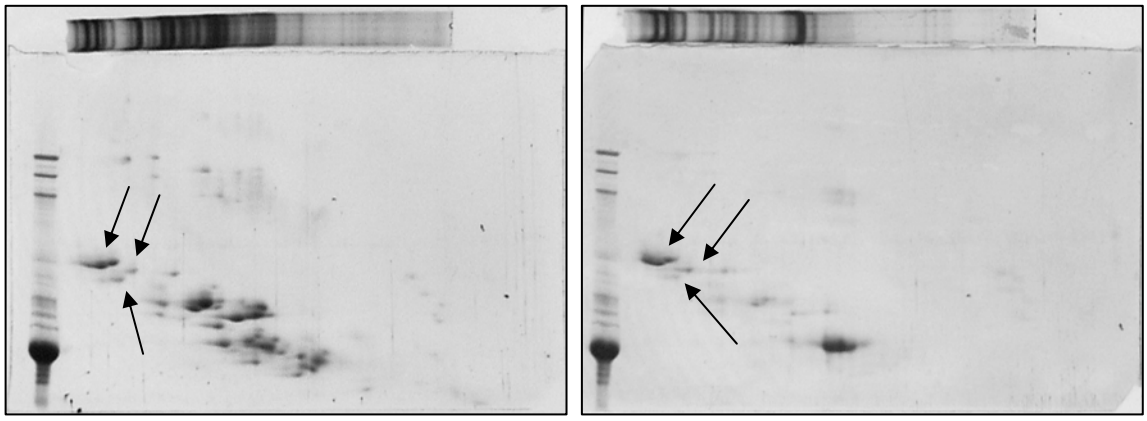

Fig. 4.20 2-DE (Acid-PAGExSDS-PAGE) of a D-type LMW-GS enriched fraction from the wild type (left) and from the transgenic genotype (right). A sample of total seed protein extract from the transgenic genotype (on the left of each image) and the first dimension sample lane (top) were included for comparison. D-type LMW-GS subunits are indicated.

The patterns of D-type LMW-GS were actually similar to those for the B-type LMW-GS. The procedure used to isolate D-type LMW-GS, based on that proposed by Masci et al. (1999), showed to be a fraction enriched in D-subunits, where a large fraction of typical B-type LMW-GS also co-precipitated. The 2D gel maps of the D-subunits-enriched fractions from the wild type and transgenic genotype are shown in Fig. 4.20.
Gel Image analysis of the two-dimensional patterns revealed only slight quantitative differences at the level of D-type LMW-GS, which were considered not worth of further investigation.

4.4.3 Gliadins analysis

According to the microarray data, all classes of gliadin genes were heavily down-regulated during seed development. We thus decided to analyse in detail the relative protein abundances both in developing and mature grains, and assess the concordance between the relative transcript levels from the microarray data with the specific protein abundances.
In Fig. 4.21 the 2-DE of total gliadin preparation from the wild type and transgenic genotype at the level of mature grains is shown. Acid-PAGExSDS-PAGE provided excellent resolution of the gliadin class, which were resolved into up to 35 components. Gliadins, of all sub-classes, were clearly much less abundant in the transgenic than in the wild type genotype. In particular, those with higher mobility (α/β-gliadins, boxed in Fig. 4.21) were absent, or at least expressed at an undetectable level by coomassie blue staining, in the transgenic genotype. After blotting of the 2D gel onto PVDF membrane, the differential spots were submitted to N-terminal sequencing; their identity with α/β-gliadins was then confirmed (100% similarity over 8 amino acids identified).

Since differential expression of gliadin genes established early during seed development (several gliadin-encoding clones were significantly down-regulated since 10 DPA "late") we also investigated the patterns of accumulation of gliadins during the period of seed maturation.

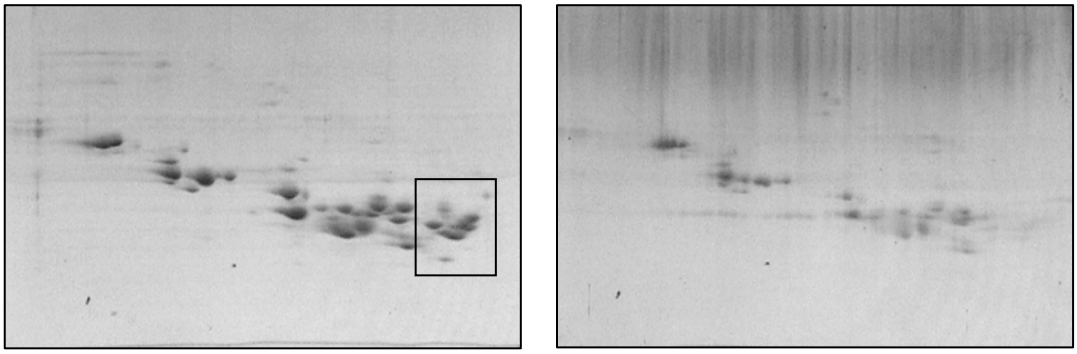

Fig. 4.21 2-DE (Acid-PAGExSDS-PAGE) of a gliadin preparation from mature grains of the wild type (left) and transgenic genotype (right). Boxed spots (left image) were exclusively expressed by the wild type genotype and have thus been submitted to N-terminal sequencing.

One-dimensional patterns of gliadin extracts from the wild type and transgenic genotype during grain development is illustrated in Fig. 4.22.

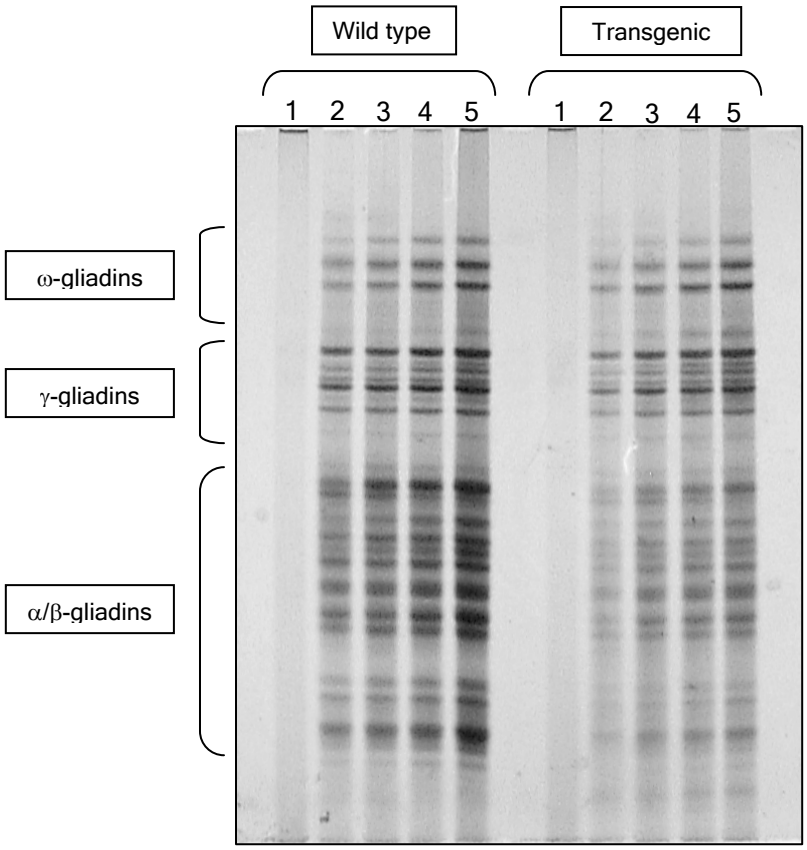

Fig. 4.22. One-dimensional Acid-PAGE separation of total gliadin extracts from the developing seeds of the wild type (left) and transgenic genotype (right). 1) 10 DPA "late"; 2) 20

DPA; 3) 30 DPA; 4) 35 DPA; 5) mature grain (around 45 DPA).

The results showed that, in the wild type, gliadins were first detectable at 10 DPA "late", and their synthesis thus begun between 10 DPA "early" and 10 DPA "late" (Fig. 4.22, wild type lanes 1 and 2). This confirmed what already indicated by microarray data, in which gliadin transcripts were also first detectable at 10 DPA "late".

The temporal patterns showed that ω-, γ- and α/β-gliadins were synthesized concurrently during grain development, and the abundances of all sub-classes increased gradually until seed maturation (Fig. 4.22, wild type lanes 2,3,4 and 5). This co-ordinated, simultaneous synthesis of gliadins is in agreement with data reported by Mecham et al. (1981) and Benetrix et al. (1994). The increase we observed in gliadins content during seed development was also parallel to the transcript abundancies detected by microarray analysis.

As in the wild type, in the transgenic genotype gliadins were first detectable at 10 DPA "late", and their synthesis begun, too, between 10 DPA "early" and 10 DPA "late". Establishment of differential expression is clearly evident since 10 DPA "late" (Fig. 4.22, transgenic lane 2), with marked lower abundancies of the α/β-gliadins, mainly, but also involving the other gliadin subclasses. Basically, gliadins accumulation, in the transgenic genotype, stopped at 20 DPA and differential expression thus persisted throughout the development of the seed. This is again in agreement with the gliadin transcripts abundancies detected by microarray analysis.

Thus, both in the wild type as in the transgenic genotype, during grain development, the accumulation of all gliadin sub-classes paralleled accumulation of their relative transcripts.

5. CONCLUSIONS

Genetic modification for crop improvement offers significant potential. Within Europe, at least, genetic modification still remains controversial. A major concern is the possibility of unintended effects caused, for example, by the site of transgene integration (e.g. interruption of important open reading frames or regulatory sequences), or strictly due to the consequences of transgene expression; all of these could result in modified metabolic pathways, novel fusion proteins, or other pleiotropic effects that could compromise safety.
The aim of this work was to gain insights about the transcriptome and proteome diversity existing between a bread wheat transgenic line and its corresponding wild type genotype. The approaches we used, basically, cDNA microarrays and two-dimensional electrophoresis (2-DE), demonstrated the high potential of these techniques to detect significant changes and assess the global effects of genetic modification. The experiments here described represent a pilot research in agreement with the assessment strategies dictated by the principle of *substantial equivalence* regarding genetically modified crops.

This study has provided a dataset of candidate differentially expressed genes between the wild type and a transgenic bread wheat line over-expressing a block of *lmw-gs* transgenes; and several conclusions have emerged from the data presented here.

First, and most importantly, the joint contribution of genetic biolistic transformation, presence of the selectable marker gene UBI:BAR, integration and over-expression of a block of *lmw-gs* transgenes had substantial consequences on the endosperm gene expression. We have used a comparative cDNA microarray profiling, between the wild type and the transgenic genotype, to detect the differentially expressed genes during seed development.
Statistical analysis gave evidence for differential expression of 542 unigenes; and those that were annotated have been clustered into functional categories to give a general overview of the transcriptional responses to transformation and transgene(s) over-expression.
We found that the majority of differences in mRNA abundances, detected by microarray analysis, emerged with the onset of up-regulation of the *lmw-gs* transgene(s) and persisted through the period of seed filling until maturation. Such changes would be caused specifically by the over-expression of the *lmw-gs* transgene(s) and not by the transformation process or putative integration or positional effects. Since most of the genes encoding seed storage-related proteins were heavily down-regulated in the seeds of the transgenic line, we have postulated the existence of a negative metabolic loop that would negate, beyond a certain threshold, the over-accumulation of storage proteins.

Second, by expression pattern matching, we identified several regulatory factors, whose transcript profiles well correlate with that of the *lmw-gs* transgenes; and thus they represent, potentially, the candidates involved in the metabolic networks of genome responses to the *lmw-gs* transgene(s) over-expression.

Third, the quantitative proteomic profiling on selected classes of seed storage proteins, both in mature and developing grains, confirmed the transcript abundancies data emerged from microarray analysis. In case of gliadins, their accumulation paralleled the corresponding transcript levels. The quantity of seed storage proteins thus showed to be mainly regulated by transcriptional processes.

Fourth, even if extensive transcriptional and proteomic differences were detected in the seeds of the transgenic genotype, these did not influenced the fundamental processes involved in seed viability and germination; and the general plant morphology of transgenic individuals was also indistinguishable from those of the wild type. Thus, the experimental evidences presented here regarding this particular transgenic bread wheat line do not constitute, in our opinion, a violation of the principle of *substantial equivalence*.

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