UNIVERSITÀ DEGLI STUDI DELLA TUSCIA DI VITERBO

DIPARTIMENTO DI SCIENZE E TECNOLOGIE PER L'AGRICOLTURA,
LE FORESTE, LA NATURA E L'ENERGIA
(**DAFNE**)

CORSO DI DOTTORATO DI RICERCA
BIOTECNOLOGIE VEGETALI XXIV CICLO

PROCESSING AND ACCUMULATION OF LOW MOLECULAR WEIGHT GLUTENIN SUBUNITS IN WHEAT ENDOSPERM

AGR-07

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A Sergio e alla
mia famiglia

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ABSTRACT

Among the various classes of LMW-GS, the most abundant are LMW-m and LMW-s, so called according to the first amino acid of mature sequence (Met and Ser respectively). We have hypothesized that the presence of the Asn residue in position 23 of LMW-s types determines a different maturation process, that might generate the cleavage of the peptide MEN by an Asparaginyl endoprotease. For this reason, the coding genes for these two protein types were mutated in position 23 in order to have Thr instead of Asn and *viceversa*. The mutated gene versions have been used to transform plants of durum wheat. The wild-type LMW-m type was used as control.

Our hypotheses was confirmed in both cases. In regard to the mutated LMW-m type, MS analysis and N-terminal sequencing demonstrated that the mature peptide began with SCISGLERP-, like LMW-s type. Similarly, for the mutated LMW-s type, the N-terminal mature polypeptide was METSHIP, as for LMW-m type.

Moreover, we set up a strategy that allows to study trafficking of LMW-GS and of other wheat storage proteins.No clear conclusion at this regard has in fact been reached yet. To work out this problem, constructs containing markers for cellular compartments (Vacuole, Golgi and ER) were used to transform wheat plants in order to monitor their intra-cellular trafficking, to backcross with other transgenic plants transformed with LMW-GS containing different tags, in order to eventually establish the precise route of deposition.

RIASSUNTO

Tra le varie classi di LMW-GS, le più abbondanti sono le LMW-m e LMW-s, così chiamate in base al primo aminoacido della sequenza matura (Met e Ser, rispettivamente). E' stato ipotizzato quindi che la presenza del residuo Asn in posizione 23 nelle LMW-s possa determinare una diversa maturazione, che potrebbe generare il taglio del peptide MEN da parte di un'asparaginil-endoproteasi. Per questo motivo, i geni codificanti per queste due proteine sono stati mutati in posizione 23 in modo da avere una Thr invece di un'Asn e viceversa. Le versioni del gene mutato sono state utilizzate per trasformare piante di frumento duro. La LMW-m *wild-type*(è stata utilizzata come controllo.

La nostra ipotesi è stata confermata in entrambi i casi. Infatti, analisi MS e sequenziamento N-terminale hanno mostrato che la LMW-m mutata comincia con SCISGLERP-, così come la LMW-s typecon METSHIP, come atteso.

Inoltre, è stata messa a punto una strategia per determinare il processo di smistamento delle LMW-GS e delle altre proteine di riserva, sul quale non esistono ancora dati certi. Allo scopo, abbiamo prodotto dei costrutti contenenti marcatori per i compartimenti cellulari (Vacuolo, Golgi e ER), che sono poi stati usati per trasformare piante di frumento, in modo da poter monitorare il processo di smistamento. In seguito, tali piante verranno incrociate con altre piante transgeniche trasformate con LMW-GS contenenti diversi tags, allo scopo si definire la precisa via di deposizione.

INTRODUCTION

1. INTRODUCTION

1.1 Why 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 tonnes are produced annually on 300 million hectares (Mha), (*http://faostat.fao.org/*) (Tab. 1.1). At present, wheat is the staple food in more than 40 countries for over 40% of the world's population and provides over 25% of the calories and proteins in human nutrition. Its key role is due to numerous 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, wheat is worldwide consumed at almost 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 (that are instead limited in wheat flours). 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. Additionally, the grain proteins determine the visco-elastic properties of dough, in particular, the storage proteins that form a network in the dough, called gluten (Beccari, 1728).

World wheat market						
	2007/08	2008/09	2009/10	2010/11 estimate	2011/12 forecast	
					Previous (03 Nov 2011)	Current (08 Dec 2011)
	(million tonnes)					
Production[1]	610.7	683.7	684.6	652.3	691.0	**694.8**
Supply[2]	768.8	823.9	860.1	850.8	872.8	**880.7**
Utilization	626.9	645.0	658.5	663.4	681.9	**682.8**
Trade[3]	111.5	139.8	130.4	125.0	131.0	**133.0**
Ending Stocks[4]	140.2	175.5	198.5	186.0	189.7	**194.8**
	(percent)					
World stock-to-use ratio	21.7	26.6	29.9	27.2	28.2	**29.0**
Major exporters' stock-to-disappearance ratio[5]	13.4	18.8	21.4	18.0	19.0	**18.9**

Tab. 1.1: The world wheat market (FAOSTAT data).The table reports the production and the utilization of wheat in the world in different years (2007-2011).

Gluten shows, upon mixing with water, unique rheological properties such as elasticity and extensibility. By modifying the technological 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 organization of the endosperm proteins into gluten.

On the other hand the gluten proteins are also responsible for triggering celiac disease, one of the most common and dangerous food intolerances, whereas the other proteins of the grain (albumins and globulins) can cause food and respiratory allergies in susceptible individuals.

1.2 The 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.

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 according to the donor species, each of which consists of seven pairs of chromosomes numbered 1 to 7. They are products of natural hybridization of ancestral types. In the hybridization 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).

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 Tauschii* 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 Italy) as durum or pasta wheat. 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 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 (Dvorak et al., 2006).

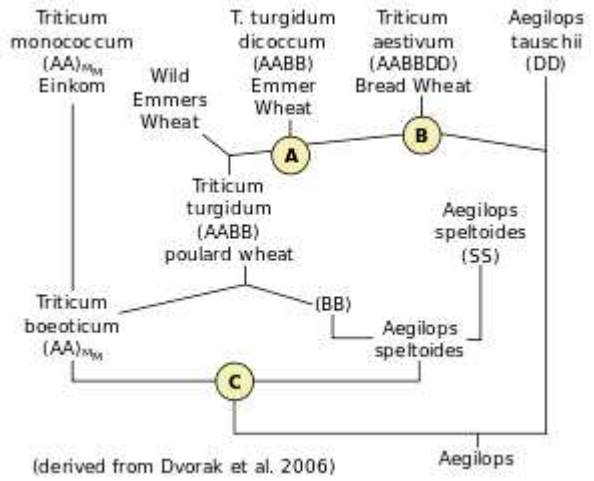


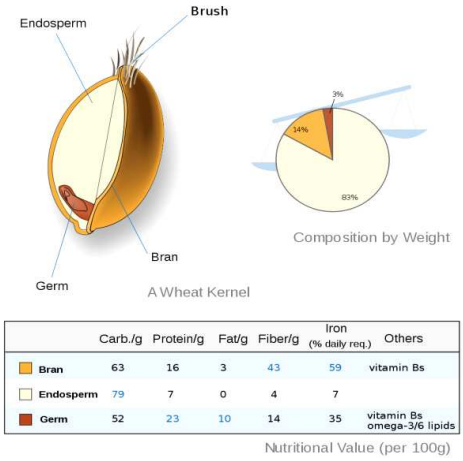
Fig. 1.1: A schematic representation of the evolution of the polyploidy wheats. The genomic composition of the species are indicated.

It should be noted that polyploidy definitely contributed to wheat wider adaptability; 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.

Currently, about 95% of the wheat grown worldwide is hexaploid bread wheat, with most of the remaining 5% being tetraploid durum wheat. The latter is more adapted to the dry Mediterranean climate than bread wheat and is often called pasta wheat to reflect its major end-use. Small amounts of other wheat species (einkorn, emmer, spelt) are still grown in some regions including Spain, Turkey, the Balkans, and the Indian subcontinent. In Italy, these hulled wheats are together called farro (Szabò and Hammer, 1996), while spelt continues to be grown in Europe, particularly in Alpine areas (Fossati and Ingold, 2001). The recent interest in spelt and other ancient wheats (including kamut, a tetraploid wheat of uncertain taxonomy, related to durum wheat) as healthy alternatives to bread wheat (Abdel-Aal et al., 1998) may also lead to wider growth for high value niche markets in the future (rev. in Shewry, 2009).

1.3 The wheat grain structure and composition

The wheat grain is botanically a dry one-seeded fruit, “caryopsis,” commonly called a kernel or berry (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 ~30-50 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. A simplified representation of the wheat grain chemical composition is reported in the Fig. 1.2.



	Carb./g	Protein/g	Fat/g	Fiber/g	Iron (% daily req.)	Others
Bran	63	16	3	43	59	vitamin Bs
Endosperm	79	7	0	4	7	
Germ	52	23	10	14	35	vitamin Bs omega-3/6 lipids

Nutritional Value (per 100g)

Fig. 1.2: The structure and the organization of the wheat kernel (bran, endosperm and germ). The table shows the chemical composition of the whole wheat grain with its various parts.

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 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 seed coat (also called “testa”) which is firmly joined to the tube cells. Grain color, usually red or white, is related to pigment in the seed coat. 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. Bran is particularly rich in dietary fiber and omegas and contains significant quantities of starch, protein, vitamins, and dietary minerals.

1.3.2 The endosperm and the embryo

The 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.

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: 79% carbohydrates (mainly starch), 7% proteins (Wieser et al., 1998). The content of mineral and dietary fibers is low, 7% and 4% respectively (Pomeranz, 1988) (Fig. 1.2).

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 and it also contains many enzymes. The germ is a rich source of protein (23%), sugar (52%), oil (10% of the embryonic axis and 32% of the scutellum are oil) (Fig 1.2). 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 industrial utilizations.

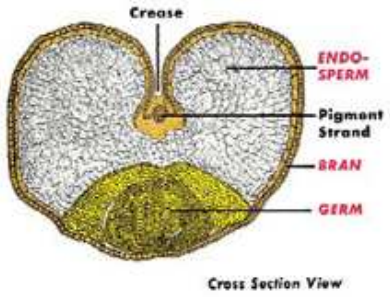


Fig. 1.3: Cross-section of the wheat kernel

1.3.3. Endosperm development

The growth 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, Dupont and Altenbach, 2003). 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. 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 extension (Chanda and Singh, 1998).

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.

1.3.4 The 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 using 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 105 to 107 and contains frequent branch points, on average approximately one branch for every 15–20 glucose units. Amylose has a lower degree of polymerisation (103 to 104) 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, 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. In all higher plants, starch is packaged into starch granules which are characterized by their density (1.5 – 1.6 g/cm^3) and by their semi-crystalline nature, as indicated by their characteristic birefringence under polarized light. The size distribution of wheat starch includes two, and sometimes three, size classes of granules, a feature shared with starches from other *Triticeae,* notably barley and rye. Additionally wheat starch granules contain a defined set of prominent proteins located within the interior of the starch granule, including Granule-Bound Starch Synthase (GBSS), Starch Synthase I (SSI), Starch Branching Enzymes IIa and IIb (SBE) and Starch Synthase IIa (SSIIa). Starch granules extracted from mature grain are associated with a range of surface located proteins which become bound as the maturation and desiccation of the grain leads to the disruption of the amyloplast membrane, largely described as the purindolines, friabilins and grain softness proteins (Darlington *et al.* 2000; Baldwin 2001).

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 identify 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 characteristics may be open to modification by molecular/genetic approaches; in recent literature there are a lot of examples about starch manipulation in wheat (Zeeman et al., 2010; Sestili et al., 2010; Botticella et al., 2011).

1.3.5 The seed proteins

Wheat grains contain relatively minute protein compared to legume seeds, with an average of about 16–23% dry weight. Nevertheless, they provide over 200 million tonnes (mt) of protein for the nutrition of humans and livestock, which is about two times the amount derived from the more protein-rich (20–40%) legume seeds. In addition to their nutritional value, 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 depends primarily from the storage proteins of its endosperm.

Giacomo Beccari was the first one to describe and call “gluten”, that is a 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. The solubility properties of seed proteins are at the basis of different purification procedures, such as that reported in Fig.1.4 (Hurkman et al., 2004).

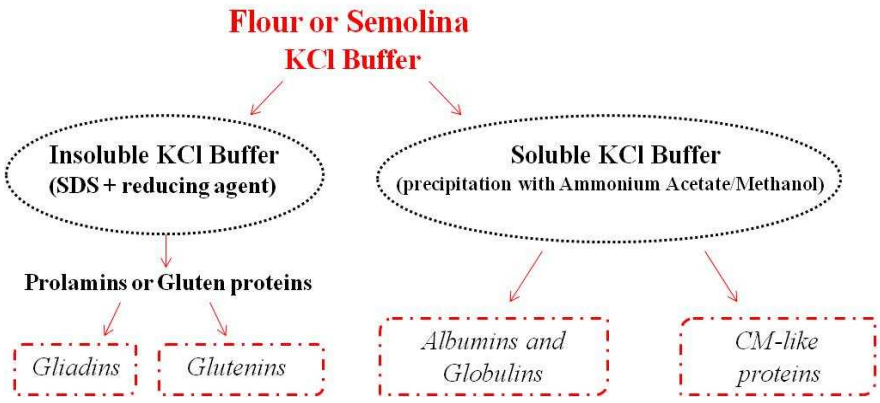


Fig. 1.4: Separation of wheat flour proteins into gluten, metabolic, and CM-like protein fractions (Hurkman et al., 2004).

1.3.5.1 Metabolic proteins (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 (Fig 1.4). 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 and they include metabolic and structural proteins involved in different pathways and functions (Dupont et al., 2011). Albumins are usually prevalent than globulins. 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, in these protein groups there are several proteins well known as human allergens, that trigger wheat allergies in sensitive individual.

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 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 bread-making quality has been established.

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 (Pogna et al., 1991).

Protocols developed to obtain protein fractions enriched in albumins and globulins (KCl-soluble) (see Fig. 1.4, Hurkman et al., 2004) have allowed the analysis of the largely unexplored albumins (Vensel *et al.*, 2005; Vensel et al., 2011).

1.3.5.2 Gluten proteins

Gluten proteins may amount to about 80% of the total proteins in the wheat caryopsis. Gluten is typically divided into alcohol-soluble (gliadin) and insoluble (glutenin) fractions, which are further separated by electrophoretic analysis.

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). Because the former two groups are structurally identically, they are now collectively known as α/β gliadins (Kasarda et al., 1983).

The glutenins consist of polymeric proteins stabilized by inter- and intra-chain 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) glutenin subunits, with the latter being further divided into B-, C-, and D-type subunits according to size, isoelectric points, and composition (rev. in 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 “prolamin superfamily” (Kreis et al., 1985) 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) (Shewry and Halford, 2002; Shewry et al., 1995).

Furthermore, it is possible to define three groups of prolamins, which contain gliadin and/or glutenin proteins. These are: a) the high molecular weight prolamins, which comprise only the HMW subunits of glutenin polymers; b) the sulfur-poor (S-poor) prolamins, which consist of ω-gliadins and D-type LMW subunits of glutenin polymers, and c) the S-rich prolamins, which include α/β gliadins, γ-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 gliadins, and are considered to be derived from these components by mutations resulting in the presence of unusual cysteine residues, which are able to form interchain disulfide bonds. In contrast, the B-type LMW subunits form a discrete group (D'Ovidio and Masci, 2004).

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

1.3.5.2.1 Gliadins

The three groups, α/β-, γ- and ω-gliadins can also be classified according to their N-terminal amino acid sequence (Dupont et al., 2004); that is for the α/β-gliadins are represented by a very small sequence of five amino acid residues (VRVPV) (Bietz et al 1977), for the N-terminal region of the γ-gliadins is formed by 12 amino acid residues (NMQVDPSGQVQW) (Autran et al 1979; Kasarda et al 1983), instead for ω-gliadins 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).

Their 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) (Fig. 1.5). Two-dimensional electrophoresis (2D-PAGE)

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/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 more 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.

Fig. 1.5: Acid-poliacrylammide electrophoretic gel(A-PAGE) of wheat gliadins.

1.3.5.2.1.1 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 bread-making properties, and also established a ranking order of the different blocks of gliadin components.

The contribution of different gliadin components to variation in bread-making 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 bread-making properties compared with the *Gli-2* loci. In most of these studies correlations between gluten components and bread-making properties of flour have been assessed by the SDS

sedimentation test, an indirect test in which good bread-making 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).

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 present 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 (2D-PAGE) after reduction of disulfide bonds (the A-, B-, C- and D-regions of electrophoretic mobility) (Fig 1.6). 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/β-gliadins (Payne and Corfield 1979; Payne et al 1985; Thompson et al 1994, D'Ovidio and Masci, 2004). 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).

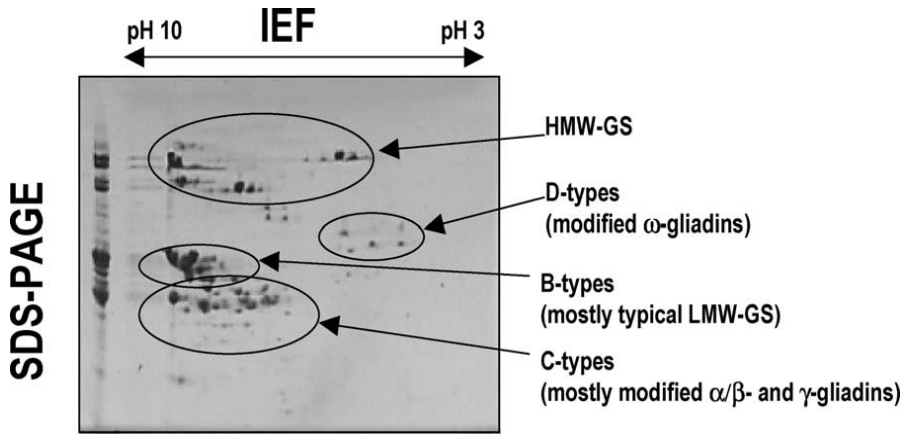


Fig. 1.6: Two-dimensional electrophoresis (IEF X SDS-PAGE) of glutenin subunits of the bread wheat cultivar Chinese Spring. The HMW-GS and the B-, C- and D-type groups of LMW-GS are indicated.

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 bread-making because they are major determinants of gluten elasticity (Tatham et al 1985a; rev. by Shewry et al., 2003) 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 (Fig 1.6). 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).

1.3.5.2.2.1.1 Relationship of HMW-GS to wheat quality

The establishment of allelic variation in HMW glutenin subunits as a major contributor to genetic variation in bread-making 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 may actually be different. 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).

The effect of individual proteins (HMW-GS, LMW-GS, hordeins, gliadins) on dough properties can also be evaluated by studying the mixing performance of a base flour, modified either by incorporation or addition of the specific proteins (Bekes et al 1994a, b). Glutenin subunits 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, these proteins 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 consideration 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; Vensel et al., 2011), 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 discovery was the first evidence that gliadin-like subunits were present and incorporated in the glutenin polymers (Masci et al., 1993; Masci et al., 1999). LMW-GS with α/β- and γ-type gliadin-like 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/β- 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). The 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/β-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 polypeptide: serine, methionine, or isoleucine, respectively. LMW-s type subunits are the most abundant in all genotypes analyzed 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) (Tab 1.2 and 1.3). However, both LMW-s and LMW-m type subunits contain eight cysteine residues, two of which are involved in intermolecular disulphide bonds.

Types of LMW-GS	N-terminal sequences
LMW-s	SHIPGL-
LMW-m	**MET**SHIPGL- **MET**SCIPGL- **MET**SRIPGL-
LMW-i	ISQQQQ-

Tab. 1.2: Comparison of LMW-GS N-terminal sequences. Between the different groups (LMW-GS m-, s- and i-type) the mainly difference is the presence/absence of the three first amino acids (MET, in bold).

Among typical LMW-GS, the LMW-i type (Cloutier et al., 2001, Ikeda et al., 2006), 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), Ikeda et al. (2002, 2006) and more recently, Masci et al (2012) 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.

A summary of the different classifications of the LMW-GS, based on electrophoretic mobility and N-terminal amino acid sequence is reported in Table 1.3.

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

1.3.5.2.2.2.1 The 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. Information on the structure of genes encoding LMW-GS derives from the characterisation of more than 70 DNA clones reported in databanks. 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 sometimes contains the first cysteine residue, a repetitive domain rich in glutamine codons and a C-terminal region, that contains most of the cysteine residues. 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 (Fig. 1.7). 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 Da.

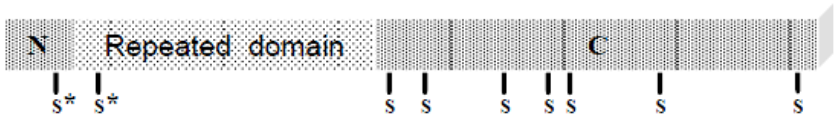


Fig. 1.7: Schematic illustration of a B-type LMW-GS. Black bars with letter s represent cysteine residues available for intermolecular or intramolecular disulphide bonds. s* indicates the first cysteine residue alternatively located in the N-terminal region or at the start of repetitive domain.

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: CCA1–2 TTT (T/C)C(A/G) CA(G/A) CAA1–4. 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: (1) those with one cysteine in the short N-terminal domain; (2) those with a cysteine residue in the repetitive domain replacing that in the N-terminus; and (3) those with eight cysteines in the C-terminal part of the protein. This different cysteine distribution could lead to functional and practical 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. 1.3). 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 (D'Ovidio and Masci, 2004).

The analysis and the characterization of LMW-GS is at partial stage, due to the large number of subunits expressed in the endosperm of wheat, and also for their similarity in chemical and physical characteristics that makes them difficult to purify (Tosi et al., 2009).

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 (Lambourne et al.,2010; Tatham et al., 1985; Tatham and Shewry, 1985), 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 Cb* according to Köhler et al. (1993)) and the seventh (Cys-295 or Cx). 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 intramolecular disulphide bonds, as suggested by direct amino acid sequencing of cysteine 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 (Cb*) and the seventh (Cx) 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, Vitale and Boston, 2008).

The dynamics of formation of both intra- and intermolecular disulphide bonds nowadays is still 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, Dupont et al, 2011). 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 stabilize 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 played a part in in the glutenin polymeric structure (Gianibelli et al., 2001, 2002a).

1.3.5.2.2.2.2 Relationship of LMW-GS to wheat quality

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, Gianibelli et al., 2001). 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 bread-making 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 36 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 assumed 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 quality 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 excellent technological properties (Brites and Carrillo, 2001; Ruiz and Carrillo, 1995, 1996; Vàzquez et al., 1996). The 42K 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 significant 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).

1.3.5.2.2.3 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 organization 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 support a 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 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. 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 bread-making quality (Southan and MacRitchie 1999).

1.4 What is the origin of gluten?

1.4.1 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, Shewry, 2009). 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 latter 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 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 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). 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). The prolamin box in its entirety is also not present in HMW prolamin gene promoters (Shewry et al., 1999).

1.4.2 Seed storage proteins synthesis and deposition

Cereal seed storage proteins are produced by the secretory pathway and deposited in discrete and separate protein bodies (Rubin et al., 1992). However, the mechanisms that determine the pathway of storage protein trafficking and deposition are still partly 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 (Rubin et al., 1992, Shewry et al., 1995; Vitale and Bollini, 1995, Gupta et al., 1996). Seed storage proteins are synthesized by mRNA associated with polyribosomes on the rough ER (Chrispeels and Herman, 2000). 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.

Rubin et al., 1992 also provided the evidence for the existence of two different types of protein body in wheat (Herman and Larkins, 1999) (Fig. 1.8). These bodies differed in density and accumulated simultaneously and independently in wheat endosperm cells. The denser protein bodies appeared to be formed by the aggregation of storage proteins within the ER while the lighter bodies appeared to result from aggregation at a post-ER location. The authors also showed that, in young grains, most of the gliadins were present in the light protein bodies, whereas at more mature stages they were found in both protein both types (Levanony et al., 1992). By contrast, the HMW subunits were highly enriched in the dense protein bodies during the entire period of grain development. No clear results were obtained for the LMW subunits.

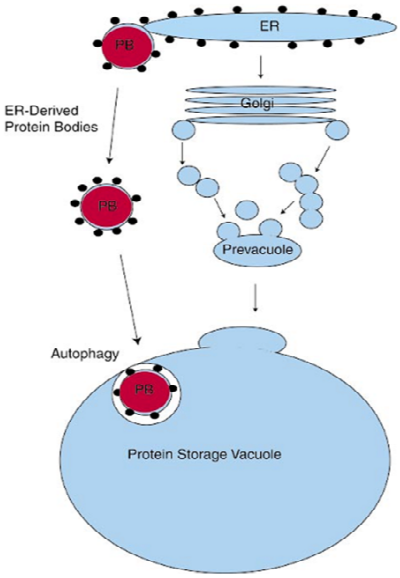


Fig 1.8: A representation of the existence of two different trafficking way in wheat endosperm Taken from Herman and Larkins, 1999.

It's well know that the folding and assembly of the gluten proteins is assisted by ER lumenal proteins, such as the enzyme protein disulphide isomerase (PDI) and the molecular chaperone binding protein (BiP) although this is still not conclusively established (Grimwade et al. 1996; DuPont et al. 1998). It has been reported that some prolamins accumulate in the ER before being deposited into protein bodies (PBs) (Galili et al. 1993). These PBs are derived from the ER and it has been suggested that they are transported directly to the storage vacuole bypassing the Golgi (Levanony et al. 1992; Galili et al. 1993; Vitale and Galili 2001) (Fig 1.7). However, other reports show the trafficking of some prolamins via the Golgi particularly during the early stages of seed development (Parker and Hawes 1982; Shy et al. 2001; Loussert et al. 2008; Tosi et al. 2009). In mature seeds, the prolamins form a matrix resulting from the disappearance of the PB structure (Rubin et al. 1992).

The glutenin proteins are elastomeric, similar to silk protein, abductin or elastin protein. Despite their different amino acid sequences, all of these proteins present common characteristics such as short repeated sequence conferring elasticity and the ability to form stable intermolecular links (Tatham and Shewry 2000). There are currently few reports of prolamin fusions with fluorescent protein (Foresti et al. 2008; Saito et al. 2009), although GFP has been widely employed as a marker protein to demonstrate passage of protein through the plant secretory pathway. It has been suggested that

accumulation in the ER lumen could be a consequence of the ability of glutenins to form insoluble aggregates (Shewry, 1999). The ER-resident chaperone BiP has been shown to be associated with prolamin PBs in rice (Muench et al. 1997) and wheat (Levanony et al. 1992). In addition, BiP has been suggested to be involved in PB biogenesis because of its role in retention of prolamins in the ER lumen by facilitating their folding and assembly into insoluble PBs in developing seeds (Zhang and Boston 1992; Li et al. 1993) and leaves of transgenic plants (Bagga et al. 1997; Mainieri et al. 2004).

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, Vitale and Denecke 1999). BiP may bind permanently to misfolded proteins (Vitale et al. 1993, Vitale and Denecke 1999) 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, Pedrazzini and Vitale, 1996). 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.

The synthesis, folding and trafficking of the gluten proteins take place within the endomembrane system of the plant cell; determination of the locations and trafficking of individual gluten proteins in developing wheat grain has been limited in the past by the complexity of the protein mixture with a high degree of sequence similarity between some components, making it difficult to develop monospecific antibodies (Tosi et al., 2009).

1.5 Proteomic analysis

The large-scale investigation of proteins in organisms (proteome-protein complement expressed by a ‘genome’ is equally important, as it provides information on the actual ‘factories’ (enzymes) of the metabolic process. However, unlike other disciplines, such as genomics and transcriptomics, proteomics and its current state-of-the-art techniques and strategies are still largely in development. Proteomics seeks to study the total proteins expressed in any given system, whether by abundance, activity, structure, state of posttranslational or other modification, or how these proteins interact with each other in networks or complexes. Proteomics investigations can span either a top-down or bottom-up strategy. While the scope of the analysis is strictly dependent on the goals of the research, proteomics in systems biology generally refers to the study of proteins at the organism level, emphasizing both qualitative and quantitative strategies. While strategies enabling proteome elucidation are continually being developed and improved,

current methodologies vary significantly in precision, applicability, and scale. Important remaining proteomics challenges include the limited detection range of expressed proteins- dynamic range (for example, proteomes can range from at least 7 to over 9 orders of magnitude) Other notable challenges include the efficient high throughput measurement and analysis of post-translational modifications (PTMs), precise large-scale quantification, and understanding complex protein-protein and protein-component interactomes. 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 (Dupont et al., 2011; Vensel et al., 2011). Proteomics can be divided into three main areas: (i) identification of proteins and their posttranslational 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. Comparative proteomics studies have been largely used to investigate the processes that take place in plant response to biotic, abiotic stresses and nutritional quality (Hurkman et al., 2004; Laino et al., 2010).

The advent of high-throughput technologies has facilitated a more holistic approach to the study of gene expression, allowing the coordinated characterization of many genes simultaneously, compared with previous studies that looked at genes or proteins individually (Anderson et al., 2000; Dutt and Lee, 2000; Lopez, 2000). Proteomics is

the systematic analysis of “all” the individual proteins within a cell or a tissue populations at a given time.

1.6 Future projection for genetic engineering of wheat

Transformation of specific wheat genotypes is a powerful tool to study the function of genes and non-coding regulatory sequences, especially for disease resistance and end use quality traits that cannot be easily studied in model plant species. However, currently there are major difficulties in the direct commercialisation of genetically modified staple foods. These stem partly from the technical challenges of moving promoters, marker genes and coding sequences across different kingdoms to create genetically-stable, metabolically-balanced, environmentally- benign agronomic improvements and partly from the requirements of regulatory authorities and consumer resistance. There is clear potential to develop new areas of research for the improvement of agronomic and quality traits that utilise our understanding of cell biology and molecular genetics but without the use of foreign DNA. Modifying the plant’s own genome could provide a route to consumer acceptance and commercialization (Shewry and Jones, 2005).

Because of this wide range of applications, the processing quality of wheat has been the subject of an immense volume of research and was one of the first targets for genetic modification (GM) in the late 1990s. Studies on wheat have so far focussed on two aspects of quality that are determined principally by the grain protein composition: dough strength and grain texture (hardness).

AIM OF THE WORK

2. AIM OF THE WORK

We are interested in the clarification of synthesis, maturation processes and deposition of wheat gluten proteins, and the aim of this PhD thesis has been to shed light on two of these aspects, that are: 1) the maturation processing of LMW-GS 2) the determination of the precise trafficking of gluten proteins, in particular of LMW-GS.

With regard to the first aspect, among the various classes of LMW-GS, the most abundant are LMW-m and LMW-s, so called according to the first amino acid of their mature sequence (methionine for LMW-m and serine for LMW-s). The two classes of LMW-GS differ mainly for the presence of three additional N-terminal amino acids in the LMW-m types. The comparison between the amino acids sequences of the N-terminal region of the two types of LMW-GS has suggested the possibility of a different maturation process. We have thus hypothesized that the presence of an Asn residue at position 23 in LMW-s types determines the cleavage of the peptide MEN by an asparaginyl endoprotease (Masci et al, 1998). For this reason, the genes coding for these two target proteins (LMW-m and –s) were mutated in order to have a threonine instead of asparagine and *viceversa* in position 23. The mutated gene versions, along with the wild type version of the presumed LMW-m type, have been used to transform, using the biolistic method, plants of durum wheat (cv. Svevo) and two different tags (His- and FLAG-tags) have been added at the C-terminus, in order to facilitate the recognition and purification of the encoded proteins.

In regard to the second issue addressed in this work, two trafficking pathways have been proposed to occur in wheat, with proteins either being transported via the Golgi apparatus into the vacuole or accumulating directly within the lumen of the ER.

It has been suggested that the same individual protein could be trafficked by either pathway, possibly depending on the stage of development, and that segregation of gluten proteins both between and within protein bodies may occur. In literature it has been proposed the existence of two different protein bodies types: the denser protein bodies formed by the aggregation of storage proteins within the ER, and the lighter bodies resulting from aggregation at a post-ER location. These authors (Rubin et al., 1982; Levanony et al., 1992) also showed that, in young grains, most of the gliadins

were present in the light protein bodies, whereas at more mature stages they were found in both protein body types. By contrast, the HMW-GS were highly enriched in the dense protein bodies during the entire period of grain development. No clear results were obtained for the LMW subunits. In fact, the determination of the locations and trafficking of individual gluten proteins in developing wheat grain has been limited in the past by the complexity of the protein mixture with a high degree of sequence similarity between some components, making it difficult to develop monospecific antibodies. For this reason, we have made different gene constructs containing specific markers for cellular compartments (for vacuole, Golgi and ER) in which GFP, YFP or Cherry and HA-tag, have been added. These constructs have been used to transform genetically wheat plants in order to localize, through immunofluorescence tests, these specific compartments and eventually get information about the modality of deposition of gluten subunits, by performing crosses between these transgenic plants and other transgenic wheat plants previously transformed with tagged gluten proteins.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 MATURATION PROCESSING OF LMW-GS

Before entering into technical details, I would like to give a rough outline of the work performed, in order to facilitate the comprehension of the approach used to reach the proposed goal.

Because our initial hypothesis was that the responsible of the presumed differential processing of LMW-s and LMW-m types was the presence of either an Asparagine or a Threonine at the beginning (position 23) of the coding sequence (Fig. 3.1), we thought to use two genes, presumed to code for the two LMW-GS types, and to mutagenize them in the codons corresponding to amino acids in position 23. In a previous PhD thesis (Ferrante, 2006), an attempt to reach the same goal was performed by using transient transformation, but methodologies available at that time, and likely the use of a different biological system (*Nicotiana benthamiana*), did not allow to get reliable results, although indications that our hypothesis was correct were obtained, at least in regard to the LMW-m type (Ferrante et al, 2006).

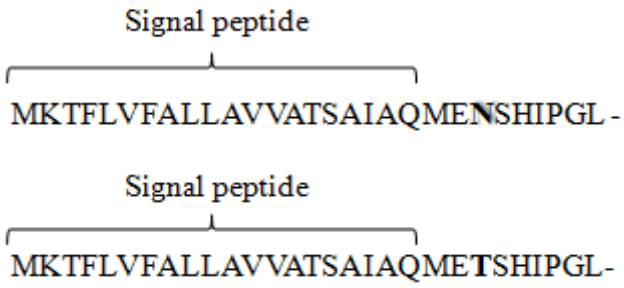


Fig. 3.1: The two immature polypeptide sequence of LMW-GS (-s and –m types). Amino acids (N and T) at position 23 are bolded.

In order to elucidate the process of maturation of LMW-GS, we thus expressed these constructs in the native system, namely we used stable transformation of wheat plants with these genes, and eventually isolated the corresponding polypeptides. We used both the mutated versions of the LMW-m and LMW-s type genes, along with the wild type version of the LMW-m type gene, because, in this latter case, the processing as m-type

was only hypothesized, whereas, in the case of the LMW-s type gene chosen, the processing had been previously confirmed (Masci et al, 1998).

3.1.1 Plant material

Since the durum wheat cultivar Svevo is commonly used in our laboratory as a background for performing genetic transformation, this was used also in our case.

3.1.2 Preparation of constructs and genetic transformation

The three genes that have been used for genetic transformation were isolated from the PVX vectors used by Ferrante (2006) for transient expression in *Nicotiana benthamiana*. One gene corresponds to a native gene (named HB1133WT) that was presumed to code for a LMW-m polypeptide, although it is reported in GenBank as a gamma-gliadin (accession number M11077, Okita et al., 1985); another one corresponds to the same gene (named HB1133T23N) that Ferrante (2006) mutated in position 23; and the third one (named HLMW42KN23T) corresponding to a LMW-s type (accession number Y17845) previously mutated by Ferrante (2006) at the same position 23. In order to use these genes for genetic transformation, we cloned them into pLRPT vector (Tosi et al, 2004). Transgenes were put under the control of the HMW-GS Dx5 promoter (Tosi et al., 2004). Two different tags were added at the C-terminus (His- and FLAG-tags) of each target gene, in order to facilitate their identification and characterization. Vector pUBI::BAR was used to co-transform embryos of wheat, in order to screen and select transgenic plants. The biolistic method was used for producing transgenic plants (Blechl and Anderson, 1996), in collaboration with Prof. Renato D'Ovidio and Dr. Michela Janni (DAFNE, Tuscia University, Viterbo).

3.1.3 DNA analysis

3.1.3.1 Cloning of the HLMW42KN23T gene

The reaction of amplification was prepared in a volume of 50 µl containing 5 µl of 10X buffer by addition of MgCl2, 100 ng of genomic DNA, 400 uM of each nucleotide, 200 ng of each oligonucleotide, 2.5 units of Fast Start High Fidelity DNA polymerase (Roche). The PCR program was as follows: 95 ° C 2 min, 1 cycle, 95 ° C 1 min, 62 ° C 2 min, 72 ° C 2 min, 30 cycles; 72 ° C, 5 min, 4 ° C ∞. The amplification product of 1144 bp was recovered from 1.2 % agarose gel and re-amplified with the pair of oligonucleotides LMW42KSalF and LMW42KXbaR, that allowed the insertion of the restriction sites SalI and XbaI, useful for cloning in pLRPT vector (Tosi et al., 2004). Following the sequence of oligonucleotides used is reported. The SalI sites (forward) and XbaI (reverse) are highlighted in gray.

SalHLMWF

5’ ACAGTCGACATGAAGACCTTCCTCATCTTT 3’

XbaHisFlagR

5’TCTAGATCACTTGTCATCGTCATCCTTGTAGTCGTGATGGTGATGGTGATG GT 3’

3.1.3.2 Cloning of the HB1133T23N and HB1133WT genes

The gene coding for the presumed LMW-m type (LMW-GS B1133, accession M11077) was initially isolated from the bread wheat cv. Cheyenne (Okita et al. 1985), kindly provided by Dr. Ceriotti (IBBA, CNR, Milano) and cloned in PVX201 carrier by Ferrante (2006), either in the mutated or wild-type gene versions.

In order to use these genes for the transformation constructs, PVX vectors were amplified with a couple of oligonucleotides SalHB1133F (forward primer) and

XbaHisFlagR (reverse primer). In this way, the restriction sites SalI and XbaI were inserted (highlighted in the sequence of the primers reported below).

SalHB1133F

5'ACAGTCGACATGAAGACCTTCCTCGTCTTT 3'

XbaHisFlagR

5'TCTAGATCACTTGTCATCGTCATCCTTGTAGTCGTGATGGTGATGGTGATG GT 3'

3.1.3.3 The PCR analysis

Transformant cells containing our specific constructs, were identified by PCR using specific primers, as shown above. The PCR program was as follows: 94°C for 2 minutes 1 cycle, 94°C 1 min, 62°C for 1 min, 72°C for 1 min and 30 seconds, 35 cycles; 72 ° C, 5 min, 4 ° C ∞, using GoTaq Green-MasterMix (Promega).

3.1.3.4 The ligation reaction

The ligation reactions were carried out so that the ratio between the molar concentration of the insert and that of the carrier is 3:1.

The reactions were performed in a solution that contained amounts of carrier and control insert DNA, depending on the size of the vector and the insert, to which 5X buffer of T4 DNA ligase, 0.1 U of T4 DNA ligase (Promega) and water to a final volume of 10 µl were added. The reaction mixture was incubated overnight at 4 °C.

To calculate the appropriate amount of PCR product (insert) to include in the ligation reaction, the following equation has been used:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

3.1.3.5. Transformation of E. coli competent cells

The bacterial strains employed are:

- ***E. coli* XL1-Blue Supercompetent cells (Agilent Technologies).** This strain has been used for the transformation of LMW-GS-m type genes (HB1133T23N and HB1133WT).
- ***E. coli* SURE 2 Supercompetent cells (Agilent Technologies).** It has been employed for the transformation and propagation of gene coding for HLMW42KN23T.

The transformation of *E. coli* competent cells was carried out as described by Sambrook et al. (1989) with some modifications. An aliquot of competent cells, kept at -80 C, is left to thaw on ice for 10-15 min. Half the volume of the ligation reaction mixture or 20-50 ng of plasmid DNA were added to competent cells and left on ice for 30 min.

The cells were submitted to heat shock at 42 °C for 1 minutes and 50 seconds and then placed on ice for 2 min. After this, 1 ml of Luria Bertani (LB) medium was added and incubated about one hour by gentle agitation at 37 °C (*E. coli* XL1-Blue Supercompetent cells), or at 30 °C (*E. coli* Sure 2 Supercompetent cells). Finally, bacteria were plated on solid medium containing the appropriate antibiotic and incubated at 37 °C or 30°C (as above specified) for 16-18 hours. Colonies are tested by extraction of plasmid DNA, and digestion with restriction enzymes and PCR.

3.1.3.6 Isolation of plasmid DNA

The bacterial strain were transformed with a plasmid or a ligation mix and the DNA was extracted from each colony. Single bacterial colonies were picked to inoculate 1,5 ml of LB containing the appropriate antibiotic and shaken at 250 rpm, 37°C. overnight. The day after, 1,5 ml culture were centrifuged in eppendorf tubes at 14000 rpm for 1 min and the pellet resuspended in 1ml TE (1M Tris-HCl pH 8, 1mM EDTA) and centrifuged as above. The pellet obtained was resuspended in 150 µl of SET (50 mM Tris-HCl pH 8, 50 mM EDTA pH 8, 20% Sucrose) using a vortex. 350 µl of lysis buffer (0,2 M NaOH, 1% SDS) were added and, after mixing gently, tubes were left on ice for 10 min.

250 μl of 3M sodium acetate pH 5.2 were added and the tubes left again on ice for 20 min to allow the SDS and chromosome DNA to precipitate. After centrifuging at 14000 rpm for 10 min, the supernatant was collected and the plasmid DNA precipitated adding an equal volume of cold isopropanol and centrifuged for 10 min at 14000 rpm. Pellet was then rinsed with 70% ethanol and dried under vacuum for 10-15 min. Plasmid DNA was resuspended in 20 μl of sterile water and treated with RNase (2 μl of 1 mg/ml) at 37°C for 20 min. The average yield using this method was about 2 μg per 1,5 ml of bacterial culture.

3.1.3.7 Maxi preparation of plasmid DNA

Plasmid DNA was extracted using the "PureLink™ HiPure Plasmid Filter Purification" kit (Invitrogen), following the manufacturer's instructions.

3.1.3.8 Gel extraction and purification of DNA fragments

For the purification of DNA fragments from agarose gels a "Nucleospin Extract II" kit (Macherey-Nagel) was used according to the procedures specified from the manufacturer.

3.1.3.9 Digestion with restriction enzymes

All the restriction enzymes were purchased from Promega and used with the supplied buffers. Different quantities (from 500 ng to 5-20 μg) of plasmid or genomic DNA were used whether they were for analytical or preparative digestion. Incubation was performed for 1-2 hours at 37°C with specific restriction enzymes in appropriate reaction conditions. Usually 1U of enzyme were added per μg of DNA. Digestion of genomic DNA was performed in a appropriate reaction buffer using 5-10 μg of DNA in a final volume of 40 μl.

3.1.3.10 DNA sequencing

DNA sequence analyses were conducted by a commercial sequencing service (Eurofins MWG Operon, Ebersberg, Germany).

3.1.4 Nucleic acids extraction from plant material and relative analyses

3.1.4.1 Total DNA extraction

Half wheat kernels were ground with a mortar and pestle, and 80 µl of extraction buffer (100 mM Tris-HCl pH 8, 50 mM EDTA pH 8, 500 mM NaCl, 1,25% SDS), pre-heated at 65°C, were added to each sample before the incubation at 65°C in a water bath for 10 min. After this step, 26.7 µl of 5M potassium acetate were added and the samples were kept for 40 min on ice, centrifuged for 10 min at 13000 rpm at 4°C and the supernatant stored. For the DNA precipitation, 80 µl of isopropanol were added; samples were centrifuged again at 4°C for 10 min, the supernatant was poured out and the pellet rinsed twice with 150 µl of 70% ethanol. The pellet was then rinsed twice with 70% ethanol, dried under vacuum and resuspended overnight at 4°C with 20 µl of TE or water. The DNA was stored at 4°C (-20°C for longer periods).

3.1.4.2 PCR analysis of wheat lines transformed with LMW-GS

Transformed T_1 plants were identified by PCR by using primers specific for the HMW-GS DX5 promoter and the terminator (shown below). The PCR program was as follows: 94°C for 2 minutes 1 cycle, 94°C 1 min, 62°C for 1 min, 72°C for 1 min and 30 seconds, 35 cycles; 72 ° C, 5 min, 4 ° C ∞.

PRDX5F (forward oligonucleotide specific for promoter)

5’ CATGCAGGCTACCTTCCAC 3’

PRDX5R (reverse oligonucleotide specific for promoter)

5’ CGGTGGACTATCAGTGAATTG 3’

Dx5TermR (reverse oligonucleotide specific for terminator)

5’ TTCAGTGGCTGACAATGAGC 3 ’

3.1.4.3 DNA electrophoresis

To identify and separate DNA fragments, 1,2-1,5% agarose gels were prepared in TBE 1x (Tris, Boric acid and EDTA), with 0,5µg/ml Ethidium Bromide. Amplified DNA samples were prepared by adding a suitable volume of 6x loading Dye (Fermentas). As molecular weight markers, either GeneRuler 100 bp DNA Ladder or GeneRuler 1kb DNA Ladder (“Fermentas”) were used.

3.1.5 Proteomic analysis

3.1.5.1 Isolation of glutenin subunits

3.1.5.1.1 Total glutenin subunits extraction

50 mg of flour from the durum wheat cultivar Svevo as well from the obtained transgenic lines were washed three times with 1 mL of 50% (v/v) propanol in order to remove the soluble fraction. The pellet was treated with a solution (1 mg: 10 mL) of 50% propanol containing, 50 mM Tris-HCl pH 8.8, 1 mM EDTA, 10 mM iodoacetamide or 1,4% of 4VPP, 1% (w/v) DTT for 1 h at 70 °C. After centrifugation at 13000 rpm for15 min, the recovered supernatant was added with four volumes of cold acetone and kept overnight at -20 °C to precipitate the glutenins. After centrifugation at 13000 rpm for 15 min the precipitated proteins were recovered and dried in a Savant centrifuge.

3.1.5.1.2 Enrichment of B-type low-molecular-weight glutenin subunits

In order to obtain a fraction enriched in B-type low-molecular weight glutenin subunits, to which LMW-m and LMW-s mostly belong, we used the procedure reported by Masci et al. (1999).

3.1.5.1.3 2-D quant Kit Assay

In order to load comparable amounts of proteins for 2D analyses, quantification was performed with the 2-D quant Kit Assay (GE Healthcare). This is based on the specific binding of copper ions to protein. Precipitated proteins are resuspended in a copper-containing solution and unbound copper is measured with a colorimetric agent. The color density is inversely related to the protein concentration. The assay has a linear response to protein in the range of 0-50 μg. This Kit was also used to have an accurate estimation of the protein extractions prior the IEF. Six dilutions (0-50 μg) of BSA provided in the kit (2 mg/mL) were used as standards. Five and ten μL of samples (containing the protein extractions dissolved in Rehydration Buffer) have been assayed in each test.

3.1.5.1.4 2D electrophoretic analysis (IEF vs SDS-PAGE) of LMW-GS

Total glutenin subunits or B subunits of LMW-GS were suspended in 250 μL of a solution composed of 7 M Urea, 2 M Thiourea, 4% (w/v) CHAPS, 1.2% (v/v) Destreak Reagent, 0.5% (v/v) IPG buffer pH 3-10 and 6–11 at least for 2 hour. Isoelectric focusing (IEF) was performed with *IPGphor*^{TM} *Isoelectric Focusing System* (Amersham Pharmacia Biotech) and carried out on immobilized pH gradient (IPG) strips (18 cm, 1mm) pH 3-10 and pH 6-11 (for LMW-m, B1133 and for LMW-s type, 42K, respectively). The strips were hydrated with samples overnight (12.30 h) at room temperature. IEF was performed at 90.000 volts-hours at 20 °C. After focusing, the strips were equilibrated for 30 min at room temperature in a solution of Urea 6 M, 2% (w/v) SDS, 30%(v/v) Glycerol, 50 mM Tris-HCl pH 6.8, 1% (w/v) DTT and loaded on the top of a 1 mm thick SDS polyacrylamide gel 18 cm, T 11% C 1.28%. Second

dimension is performed in vertical 12% polyacrylamide gels using *Protean* II *xi multi-cell* and *Protean Plus Dodeca cell* (Bio-Rad). Electrophoretic separation was carried out at 40 mA/gel, with cooling at 10 °C. Gels were stained with Coomassie Blue G250 (Neuhoff et al, 1988) and destained in water before image acquisition.

The gel analyses were performed using the softwares: SameSpots Progenesis (vers. 4.5.4293.47197, Nonlinear Dynamics, UK). Briefly, after automatic pixel level geometric alignment, a software automatic management of spot measurement, background subtraction and normalisation were generated. Then, the biological gel grouping process drove the generation of *p* value from the application of analysis of variance (ANOVA). In addition the software includes determination of False Discovery Rate (FDR, $q \leq 0.05$), and Principle Component Analysis (PCA; according to O‟Gorman *et al.* (2007).

3.1.5.2 Western Blotting and amino acid sequencing

The Western blotting of the 2-D patterns was performed using a *Mini Trans Blot Cell* module (Biorad). 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, the region containing the polypeptides of interest was cut out of the unstained gels and gel pieces 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 sequencing was performed according to the procedure reported by Tao and Kasarda (1989).

3.1.5.3 Immunoblotting

For immunoblotting experiments, the resolving gel (or gel pieces containing the region of interest, in case of big gels) was incubated in transfer buffer (25 mM Tris- HCl, pH8, 192 Mm glycine and 0,04% SDS) for 15 minutes after SDS-PAGE. The blotting was performed in a *Mini Trans-blot* apparatus (Bio-Rad) using a PVDF membrane (Bio-Rad) according to the manufacturer's protocol. After transferring, the membrane was saturated in 100ml of Blocking solution (10 mM Tris-HCl pH 8, 150 mM NaCl, 0,1% Tween20 and 5% not fat dry milk) at room temperature, on an orbital shaker, for 2h. The membrane was then washed twice in Washing buffer (10 mM Tris-HCl pH 8, 150 mM NaCl and 0,2% Tween20) and incubated overnight with specific primary antibodies from rabbit. In particular, a polyclonal antibody that recognizes the eight amino acids of Flag-tag (Sigma-Aldrich) was used. Removed from the incubation buffer, the membrane was washed extensively and incubated with the secondary antibody (goat anti rabbit horseradish peroxidase coniugated) at room temperature, for 1 hour. The antigen-antibody complex was detected using the "Western blotting Luminol reagent" kit ("Santa Cruz Technologies") and following the manufacturer's procedure.

3.1.5.4 Mass Spectrometry Analysis

These analyses were performed by Dr. William H. Vensel (USDA, WRRC, ARS, Albany, CA).

3.1.5.4.1 Protein digestion

In preparation for mass spectrometry Coomassie brilliant blue (Sigma-Aldrich Corp. St. Louis, MO, USA) stained bands were removed from the polyacrylamide gels and stored in 1.5 mL Eppendorf tubes. Immediately prior to enzymatic digestion the gel pieces (1-3) were placed into a 96-well reaction plate that was positioned in an automated xyz robot (DigestPro, Intavis, Langenfeld, Germany). The robot automatically destained, reduced, alkylated and digested the proteins in the gel plugs with Sequencing Grade Modified Trypsin (Promega Corporation, Madison, WI, USA). Twenty μg of trypsin was used for each 96-well sample plate and digestion was performed at 35°C. At the

end of the digestion period the instrument eluted the tryptic peptides into a 96-well receiving plate that was then inserted into the autosampler interfaced with the QSTAR pulsar *I* hybrid quadrpole-TOF mass spectrometer (Applied Biosystems/MDX Sciex, Toronto, Canada) configured with an electrospray ionization (ESI) source (Protana, Odessa Denmark).

3.1.5.4.2 Electrospray Ionization

The samples from the DigestPro 96 well receiving tray were loaded into an autosampler (FAMOS, LC Packings/Dionex) that then loaded the sample into a sample loop. A loading pump (Switchos, LC Packings/Dionex) then pumped the sample (typically 10uL) through the loop onto a reverse phase sample trap. Typically 100-200 ul of loading solvent was pumped onto the sample-containing trap before it was switched in-line with the analytical column and gradient elution started. (Note: To aid in preventing hydrophilic peptides from not sticking to the trap the loading pump solvent contained 0.01% HFBA. Additionally the sample from the DigestPro was in 0.03%TFA) The analytical column was a C-18 monomeric, Vydac EVEREST column (238EV5.07515; Grace/Vydac & Co). The column effluent passed through an emitter (spray tip) that had a 8 micron orifice (catalogue no. FS360-75-8-CE-20, New Objective, Woburn, MA, USA). The column exit was plumbed into one end of a stainless steel union and the spray A liquid junction at the SS union provided contact for the spray voltage (2200) A co-axial counter-flow of ultra high purity (UHP) nitrogen (curtain gas at a setting of 20) was used to aid spray desolvation and to shield the mass spectrometer. Mobile phase A was 0.5% glacial acetic acid (Fisher Scientific, reagent grade) diluted in HPLC-grade water (Burdick and Jackson, Muskegon, MI, USA). Mobile phase B was 80% acetonitrile that was 0.5% in acetic acid. Samples were eluted with the a gradient profile of: 8% B at 0 min to 80% B by 12 min through 13 min to 8% B by 14 min continuing at 8% B to 28 min using a low rate of 200-250 nl per minute.

3.1.5.4.3 Mass spectrometry particulars

Calibration of the TOF mass analyzer was with the 187.0713 and 1245.5444 mass to charge (m/Z) m/z fragment ions from the collision induced dissociation (CID) of the +2 charge state (m/z 785.8) of glufibrogen.(Note here that the parent mass of the glufibrinogen fragment is doubly charged, but that the fragment ions each carry a single

charge. Thus the parent m/z is less than the fragment at m/z of 1245.5. is Data acquisition was performed using the information dependent acquisition (IDA) mode of Analyst QS software. (An aside here: the operator can set certain characteristics for whether an ion is fragmented or not and the instrument will follow those rules. See this unfold below.) The instrument was configured to carry out a survey scan (Q1 passing m/z of 300-1500 into the Time of Flight -TOF) followed by two product ion scans. Different scan ranges were used but in general, from an initial survey scan of mass range m/z 300–1500, the most abundant single, double or triple charged ion above a threshold of 5 counts was selected for fragmentation. The quadrupole mass filter (Q1) was adjusted so that only ions of the selected m/z entered the collision cell (Q2). CID of the mass-selected ion in Q2 was carried out using UHP nitrogen. Fragment ion range for the TOF mass analyzer was set to 70–2000 m/z. The precursor ion was precluded from further MS/MS experiments for 45 seconds. An IDA script was used to determine the optimum collision energy for each precursor ion. Following the 3 s MS/MS fragmentation period(S), the MS survey scan was repeated until another MS/MS period was triggered.

3.1.5.4.4 Database analysis and identification of proteins

Data were extracted from the Wiff files generated by Analyst QS with the use of Mascot version 2.3.01. Mascot not only searches a user specified database it also generates a set of files that contain the information used to query that database. Those files allows the use of a second program to search the same database. Using two search engines increases the confidence that one places in the data and can lead to an increase statistically significant protein hits. The data was searched, using Mascot and X!Tandem against a database containing 11,589 wheat protein sequences from NCBI *Triticum aestivum* plus a list of common laboratory contaminants (The Global Proteome Machine) as well as expected sequences from the mutant and wild type expressed proteins. The results of the searches were combined, analyzed and visualized using Scaffold version 3.04.

3.2 TRAFFIKING OF GLUTEN PROTEINS

3.2.1 Preparation of constructs for plant transformation

The base vector of each gene construct was the pSPORT-GUS *(*Invitrogen). A scheme of this vector is reported in Fig. 3.2 (Wiley et al 2007).

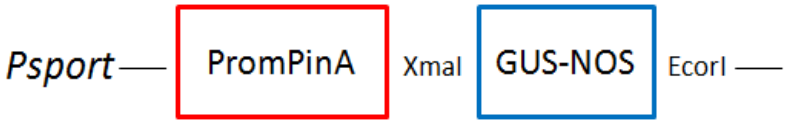


Fig.3.2: Schematic representation of PSPORT construct (Wiley et al., 2007).

Each construct contains a specific marker for the recognition of the different subcellular compartments: for the vacuole we used the α-Tip (Arabidopsis alpha-tonoplast intrinsic protein, Hunter et al., 2007); for the endoplasmic reticulum we chose the Sec61β (the single membrane-spanning subunit of rice, Onda et al., 2009); and for the Golgi compartment the α-1,2-Mannossidase I (ManI) (soybean key enzyme involved in N-linked Glycan processing, Nelson et al., 2007); all the marker gene sequences are shown in the following section. All the constructs include the promoter PinA (sequence showed below), that was used because it allows an even expression throughout the endosperm (Wiley et al., 2007). Moreover, in each construct we added the HA tag, for facilitating the identification of the protein products.

The description of the different steps performed to obtain the constructs is reported in the Results section.

PinA promoter sequence

5′CATTTCCTGTTTATCCTATGATCTGCATGACTGTGTGCAACTGCAAATGAGCCTCCACCCTA
GGCTTATTTCCATATTGATTATTACTCACCTCGATTTTTGTTTGTAAGTGTGTTTATTTATTGG
TCAAAGATAATTGTTTCTGGGGAAAAATGAAGGATTAGAAGAAGCTTGTCATGTGCCGAATCTC
AATCTTCGATAAACCAAGGGAGAATAATTAGAAAAGATGGCTATCTAATATGACTCATTGCACT
TTCTAGGTCAGAATCAAAACCCTTTTATGGCATTGTACATGGGGAGGTGAGCTCTGTTCTAAGG
TTAATCCTAACCCGTGTTGTTTGAAATACCTATTTACCCCCTCCGATGCATCTAGATCCTCGGA
CACCTTGTTAAAAAAAATTACTTCCTTGGAGACAAATACTATTTTGGAGAATAATGAAAATCAT
TTAGAAACATTTGACATGAACGACATGATTCCAGAGACAATAAAAAACTTAAAAACAAACGATG
GATAAGAAGATGATGTCTTGATAAATCATTACTTGTCGAGTTGTCGTGTACTACTAGTCTGTAA
AATACAGTCTCTAACAAATTTGGTACAATCTGAGATCCATCTTGAACAACCTGCACAATCCTAC

AAGTTTAGTTTCGCAAAAGAATAACAATATGAACCATGTGACTTTCTTGGTACGTACAAAACCA
CAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAAGGGTCACACATCATTAGATAAGGTCTA
TCTTCACAAAGTTGCACATACATGTACAGTACAGGAAGCGACATGTATCTCAATACCACATGGT
TCTAGATACTGGACGAAAAAGCAGTGGCTAGAAAGATGACGATATATAGATGCATTACTTATCA
TATACTACTACCTAGAAAAATACAATATCTAATTTCCTCTTGATCCTTCTTGAACAACCTGCAC
AACACTACAAGTTCAGTTTCACAAAAGCGTAAGTCTAAAGCTTTGGTACAACAACAACTTATGG
TTTATTTTGAGAAAAGGTCAGATTCAGTACACGGAACATCACATATCTCAACAACTTCCACCAG
TTTTGTGTGCTTTCAAAGTAACTTTGATTGGTATCCAGCTATACAACACACAACCGCACACAGA
AATCGTGCCACCTCAATTATAAATAAAGGTGTGGCCTCATCTCATCTATTCATCTCCACCTGCA
CCAAAACACACTGACAACATG 3′

3.2.1.1 PCR analysis and specific oligonucleotide design

The next table (Tab 3.1) shows all the oligonucleotides (forward and reverse) that were used for cloning (with specific enzyme restriction sites) the specific constructs. For all the analysis about manipulation of DNA see from par. 3.1.3.4 to 3.1.3.10.

Specific primers were designed for using the function primer design of the DNAMAN software (Lynnon Biosoft, Quebec, Canada) (Tab. 3.1).

Primer Name	**Oligonucleotide Forward Sequences 5'-3'**	**Utilization**
PinA	AGGTCAGATTCAGTACACGGAACATC	Promoter
XmaI.Alpha-TIP	ATACCCGGGATGGCCGCAACATCAGCTCGTAGAGCAT	PSPORT-α-Tip
BamhI.NOS	ATAGGATCCGCTACCGAGCTCGAATTTC	Terminator
XmaI.cherry	ATACCCGGGATGGTGAGCAAGG	PSPORT-Sec1β
BamhImut.F*	AGCTGTACAAGGGAGGTGGTTCC	SOE PCR
BamhI.sec61-mut*	GGGAGGTGGTTCCATGGTGG	SOE PCR
XmaI.BamhI.mut*	CGGTCCAGGGGGTTCCATG	SOE PCR
XmaI.Alpha-mann	ATACCCGGGATGGCGAGAGGGAGCAGAT	PSPORT-α-MannI
Alpha-mann	ATGGCGAGAGGGAGCAGATCAG	PSPORT-α-MannI
Primer Name	**Oligonucleotide Reverse Sequences 5'-3'**	**Utilization**
BamhI.HA.YFP	TCAAGCGTAATCTGGAACATCGTATGGGTACATGATCACCTTGTACAGC	PSPORT-α-Tip
EcorI.NOS	CCGGAATTCCCGATCTAGTAAC	Terminator
BamhI.sec61.HA	ATAGGATCCTCAAGCGTAA TCTGGAACAT CGTATGGGTA CAT	PSPORT-Sec1β
XmaI.BamhI.mut*	CATGGAACCCCCTGGACCG	SOE PCR
BamhI.sec61-mut*	CCACCATGGAACCACCTCCC	SOE PCR
SEC61	GGCGGAGGTGCGGTAAAG	PSPORT-Sec1β
GFP2	CTTGTACAGCTCGTCCATGCCGT	PSPORT-α-MannI
BamhI.HA.GFP2	CGCGGATCCTCAAGCGTAA TCTGGAACAT CGTATGGGTA CAT	PSPORT-α-MannI

Table 3.1: Oligonucleotides used for the cloning of PSPORT-Alpha-Tip, PSPORT-Alpha-Mannosidase I and PSPORT-Sec1β.

3.2.1.2 PCR-splicing by overlap extension (SOE PCR)

The cloning of Cherry-Sec61β into the vector pSPORT required the use of SOE-PCR to remove the enzyme restriction sites (XmaI and BamhI) contained into the middle of gene coding regions, because these sites were not compatible with the cloning in the vector.

SOE-PCR involves three separate PCRs: the two DNA fragments produced in the first stage reactions are mixed to form the template for the second stage (Warren *et al*,1997). Four primers are required for each construct: two flanking primers, and two hybrid primers, one of each for each of the two first stage reactions. The hybrid oligonucleotides are designed on the basis of the known nucleotide sequences to generate fragments that will have overlapping sequence. One of the first PCRs produces a DNA fragment with the sequence 5' to splice point, and the other a DNA fragment with the sequence 3' to the splice point (Fig. 3.3). However, since the hybrid oligonucleotide spans the splice point, each of the first stage products is tipped with a short sequence derived from the other. For this reason, when the two products are mixed, they can partially anneal, and, using the original flanking primers, participate in the second-stage PCR to produce the final product.

PCR reactions were performed in a reaction volume of 25 μl with 10 ng of plasmid DNA (pSPORT), 2,5 units of FastStart High Fidelity PCR system (Roche Diagnostics, Monza, Italy), 1x Taq PCR buffer, 0,5 μM of each of the two primers, and 100 μM each deoxyribonucleotide. Amplification conditions were: 1 cycle at 94°C for 2 min; 30 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec; and a final step at 72°C for 5 min. The two DNA fragments produced, corresponding to the genes of both *Cherry* and *Sec61β*, were recovered, purified and then used as templates in a final PCR reaction with the original flanking primers. Since the two DNA fragments have overlapping sequence, they can partially anneal in this last PCR step and produce the final product in which the two genes are spiced together. For SOE-PCR we followed the same condition described above. SOE-PCR products recovered were digested with XmaI and BamhI and inserted into the pSPORT vector. For the ligation reaction, T4-DNA Ligase (Promega) was used, according to the manufacturer's instructions and then, 10μl of the ligation mix were used to transform BL21 Competent cells. The

correct coding sequence of Cherry-Sec61β and the insertion sites were confirmed by nucleic acid sequencing.

Primers indicated in the Table 2.1 with an asterisk (*) were used for PCR-splicing by overlap extension for cloning ER specific marker construct.

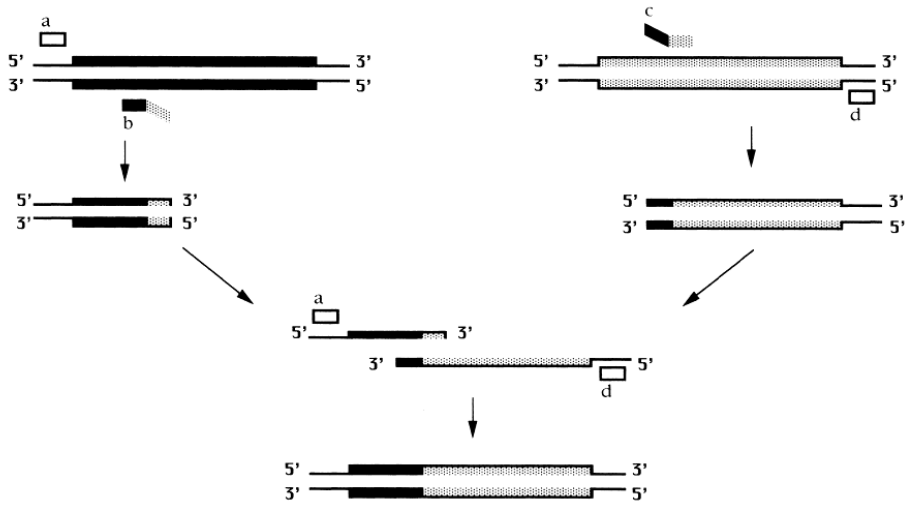


Fig 3.3: Schematic representation of SOE-PCR (taken from Warren et al, 1997).

3.2.2 Production of transgenic wheat plants

The bread wheat cv. Cadenza was transformed with constructs containing markers for specific cellular compartments at Rothamsted Research, Harpenden, UK.
T_1 plants were screened by PCR analysis by using primers specific for the PinA promoter and NOS terminator regions (Tab. 3.1). T_2 plants were obtained by embryo rescue (Tosi et al., 2004) and developing seeds analysed by confocal microscopy.

3.2.3 Confocal microscopy analysis

I performed this analysis at Rothamsted Research (Harpenden, UK), in collaboration with Dr. Paola Tosi

Developing wheat kernels collected at 16 dpa (days post anthesis) were sectioned with a Vibratome (Leica) and 150 µm thick sections collected on polylysine coated microscopy slides. Sections were analysed for transgenic fluorescent proteins (FP) signal on a Zeiss LSM 780 confocal microscope.

Some of the main features of this microscope include optical sectioning and the ability to acquire Z stacks to form a 3-dimensional image. This means that it is possible to image a thin optical slice (less than 500 nm) out of a thick specimen. A large number of optical slices can then be acquired in the Z-axis and used to create a 3D data set of the sample. The LSM 780 also makes it possible to overlay transmission electron images with confocal fluorescence images of the same area in a specimen. Another feature of this microscope is that it has multiphoton capabilities, which enables greater penetration depth and reduces photodamage and photobleaching.

3.2.4 Immunofluorescence microscopy

Slides with grain sections fixed by High pressure Freezing and embedded in LR White were pre-incubated (50 µl drop/well) in 3% (w/v) bovine serum albumin (BSA) (Fraction V, A 2153, Sigma) in PBS at pH 7.4 for 30 min, then incubated for 2 h in primary antibody. The primary antibody rabbit polyclonal anti-HA (Sigma H6908) was used at 1:200 dilution in PBS containing 1% BSA 0.05% Tween20. Slides were rinsed three times for 5 min with PBS Tween, then incubated for 2 h, in the dark, with secondary antibody (anti-rabbit Alexa 488 conjugated, Invitrogen) diluted 1:200 in PBS, 1% BSA, 0.5% Tween. Slides were then rinsed twice with PBS Tween, two times with PBS and three times with water. Samples were analysed on a Zeiss confocal microscope LSM 780 fluorescence microscope.

Samples were analysed on a Zeiss confocal microscope LSM 780 fluorescence microscope.

RESULTS AND DISCUSSIONS

4. RESULTS AND DISCUSSIONS

4.1 MATURATION PROCESSING OF LMW-GS

In order to shed light on LMW-GS maturation process, we have taken into consideration two protein genes mutated in such a way to have either an Asn or a Thr in the coding sequence, as explained in par. 3.1: a presumed LMW-m (LMW-B1133), showing a Thr at the beginning of the mature sequence in the wild type version, and thus having a Asn at the same position in the mutated version (Fig. 3.1), and a LMW-s (LMW-42K) type, instead showing a Asn in place of the Thr in the wild type version, and thus having a Thr at the same position in the mutated version (Fig. 3.1).

We have inserted the mutated versions of these two genes in the durum wheat cultivar Svevo by particle bombardment, by using the wild-type version of the LMW-B1133 as control. The wild-type version of the LMW-42K has not been used to transform plants, because its actual N-terminal sequence was already known (Masci et al, 1998).

4.1.1 Preparation of constructs for genetic transformation

Although this part would be placed more appropriately in the methods section, I decided to report it in the results section, in order to describe the details of the realization of constructs, since it was a significative part of my PhD work.

4.1.1.1 Cloning of the HLMW42KN23T gene

The mutated version of the LMW-42K gene will be named HLMW42KN23T from now on.

The gene sequence encoding for HLMW42KN23T was isolated from PVX201 vector (Ferrante, 2006), and cloned into the pLRPT vector. We used SalHLMWF and XbaHisFlagR primers (see par. 3.1.3.1) to amplify the region corresponding to HLMW42N23T gene, that was recovered from 1, 2% agarose gel. The purified PCR product, was digested with SalI and XbaI enzymes and ligated into the vector pLRPT (Fig 4.3). An aliquot of the ligation reaction was used to transform *E. coli* Sure2

Supercompetent cells, with a low efficiency of transformation. Although we have not explored this aspect further, this could be due to a non-canonical conformation of the DNA sequences encoding the LMW-GS for the presence of the particularly long repeated domain (Ferrante, 2006).

Although we expected the excision of one insert, and namely the presence of a single PCR band, we found a deleted band instead of 42K gene (accession number Y17845). The presence of the latter band might be due to deletions that may have occurred in the repetitive region (Masci et al. 1998). The use of the bacterial strain SURE 2, that lacks some components of the metabolic pathways that catalyze rearrangements and deletions of not canonical secondary and tertiary structures of polypeptides, along with the growth of bacteria at 30° C, instead of 37 ° C, significantly reduced this phenomenon, even if most of the clones analyzed contained deletions and minor substitutions, that did not affect the N-terminal region (Fig. 4.2a, b).

```
42KA  ATGAAGACCTTCCTCATCTTTGCCCTCCTCGCCGTTGCGGCGACAAGTGCAATTGCACAA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
42KB  ATGAAGACCTTCCTCATCTTTGCCCTCCTCGCCGTTGCGGCGACAAGTGCAATTGCACAA

42KA  ATGGAGACTAGCCACATCCCTAGCTTGGAGAAACCATTGCAACAACAACCATTACCACTA
      |||||||||||||||||||| | ||||||| ||||| ||| |||||||||||||||| |
42KB  ATGGAGACTAGCCACATCCCTGGTTTGGAGAGACCATCGCAGCAACAACCATTACCACCA

42KA  CAACAAATATTATGGTACCA...ACAACAACAACCCATCCAACAACAACCACAACCATTT
      ||||||| ||||  | ||||   ||||||||||||||||||||||||||||| | ||||
42KB  CAACAAACATTAACGCACCACCAACAACAACAACCCATCCAACAACAACCACACCAATTT

42KA  CCACAACAGCCACCAGGTTCACAGCAACAACAAC...CACCATTATCGCAACAACAACA.
      |||||||||| |||| ||||||||||||||||||   ||||||||||||||||||||||
42KB  CCACAACAGCAACCATGTTCACAGCAACAACAACAACCACCATTATCGCAACAACAACAA

42KA  CCACCATTTTCACA...ACAACAACCACCATTTTCGCAGCAACAACAACCCGTTCTACCG
      |||||||||| ||   |||||||||||||||||| |||||||||||| |||||||||
42KB  CCACCATTTTCGCAGCAACAACAACCACCATTTTCACAGCAACAACAACCAGTTCTACCG

42KA  CAACAACCACCATTTTCGCAGCAACAACAACAA.........................
      ||||||||| |||||||||||||||||| || |
42KB  CAACAACCATCATTTTCGCAGCAACAACTACCACCATTTTCGCAGCAACAACAACCACCA

42KA  TTTCCGCAGCAACAACAACCACTTTTACCGCAACAACCACCATTTTCGCAGCAACAACCA
      ||| | |||||||||||||| || |||||||||||| |||||||||||||||||| |
42KB  TTTTCACAGCAACAACAACCAGTTCTACCGCAACAACCATCATTTTCGCAGCAACAACTA

42KA  CCATTTTCTCAGCAGCAGCAACAACCACCATTTTCGCAGCAACAACAAC...........
      |||   | |  |||||| |||||||||||||||||||||||||||||||
42KB  CCACCATTTTCGCAGCAACAACAACCACCATTTTCGCAGCAACAACAACCAGTACTACCG

42KA  ..................................................

42KB  CAACAACCACCATTTTCGCAGCAACAACTACCACCATTTTCACAGCAACTACCACCATTT

42KA  ............................AACCACTTTTACCGCAACAACCACCATTT
                                  ||||||  ||  |||||||||||||||||
42KB  TCGCAGCAACAACAACCAGTACTACCGCAGCAACCACCATTTTCGCAACAACCACCATTT
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42KA  TCACAACACCAACAACCAGTTCTACCGCAACAACAAATACCATCTGTTCAGCCATCTATC
      || ||||| ||||| |||||||||| |||||||||||||||| |||||| |||||||||
42KB  TCGCAACAACAACAGCCAGTTCTACTGCAACAACAAATACCATTTGTTCATCCATCTATC

42KA  TTGCAGCAGCTAAACCCATGCAAGGTATTCCTCCAGCAGCAATGCAGCCCTGTGGCAATG
      |||||||| |||||||||||||||||||||||||||||||||||||||||||||||| |||
42KB  TTGCAGCAACTAAACCCATGCAAGGTATTCCTCCAGCAGCAATGCAGCCCTGTGGCTATG

42KA  CCACAAAGTCTTGCTAGGTCGCAAATGTTGTGGCAAAGTAGTTGCCATGTGATGCAGCAA
      |||||||||||||||||||||||||||||  ||| || ||||||||||||||||| |||
42KB  CCACAAAGTCTTGCTAGGTCGCAAATGTTGCAGCAGAGCAGTTGCCATGTGATGCAACAA

42KA  CAATGTTGCCGGCAGCTGCCGCAGATCCCCGAACAATCACGCTACGATGCAATCCGTGCC
      |||||||||| |||| ||||||| |||||| | ||||| ||||| || ||||||||||
42KB  CAATGTTGCCAGCAGTTGCCGCAAATCCCCCAGCAATCCCGCTATGAGGCAATCCGTGCT

42KA  ATCATCTACTCGATCGTCCTACAAGAACAACAACATGGTCAGGGTTTGAACCAACCTCAG
      ||| ||||||| ||| |||| |||||||||||||| | ||||||||  | |||| |||||
42KB  ATCGTCTACTCCATCATCCTGCAAGAACAACAACAGGTTCAGGGTTCCATCCAAACTCAG

42KA  CAGCAACAACCCCAACAGTCGGTCCAAGGTGTCTCACAACCCCAACAACAACAGAAGCAG
      ||||| |||||||||||||| || |||| | || || ||||||||||||||
42KB  CAGCAGCAACCCCAACAGTTGGGCCAATGCGTTTCCCAACCCCAACAACAGTC.......

42KA  CTCGAACAGTGTTCTTTCCAACAACCTCAACAACAACAACTGGGTCAATGGCCTCAACAA
                                  |||||| ||||| || |||   |||||||||
42KB  ............................ACAACAGCAACTCGGGCAACAACCTCAACAA

42KA  CAACAGGTACCCCAGGGTACCTTGTTGCAGCCACACCAAATAGCTCAACTTGAGGTGATG
      |||||  |  | |||||||||| ||||||||||||||||||||| ||||||||||||
42KB  CAACAATTGGCACAGGGTACCTTTTTGCAGCCACACCAAATAGCTCAGCTTGAGGTGATG

42KA  ACTTCCATTGCACTTCGTACCCTGCCAACGATGTGCAGTGTCAACGTGCCGGTGTACGGC
      |||||||||| || |||||||||||||| ||||||| ||| || ||| || ||||  |
42KB  ACTTCCATTGCGCTCCGTACCCTGCCAACAATGTGCAATGTTAATGTGTCGTTGTATAGA

42KA  ACCATCACTATTGTGCCATTCGGCGTTGGCAC.GGAATTGGTGACTACTGA
      |||| | |||  |||||||||||||||||||| ||| |||||| |||||||
42KB  ACCACCCCTAGGGTGCCATTCGGCGTTGGCACCGGAGTTGGTGCCTACTGA
```
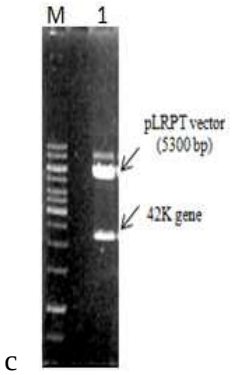

Fig. 4.2a: Alignment of the sequence of the pLRPTHLMW42KN23T (42KA) with those obtained from amplification of the clone PVX-LMW-42K (42KB, Ferrante, 2006).

```
42kA    MKTFLIFALLAVAATSAIAQMETSHIPGLERPSQQQPLPPQQTLTHHQQQQPIQQQPHQF 60
42kB    MKTFLIFALLAVAATSAIAQMETSHIPSLEKPLQQQPLPLQQIL-WYQQQQPIQQQPQPF 59
        ****************************.**:* ****** ** * :**********:  *

42kA    PQQQPCSQQQQQPPLSQQQQPPFSQQQQPPFSQQQQPVLPQQPSFSQQQLPPFSQQQQPP 120
42kB    PQQPPGSQQQQ-PPLSQQQQPPFSQQQ-PPFSQQQQPVLPQQP--------PFSQQQQQ- 108
             *** * ***** *************** ***************    *******

42kA    FSQQQQPVLPQQPSFSQQQLPPFSQQQQPPFSQQQQPVLPQQPPFSQQQLPPFSQQLPPF 180
42kB    FPQQQQPLLPQQPPFSQQ---------QPPFSQQQQ-----QPPFSQQ------------ 142
                    *.*****:*****.****     *********   *******

42kA    SQQQQPVLPQQPPFSQQPPFSQQQQPVLLQQQIPFVHPSILQQLNPCKVFLQQQCSPVAM 240
42kB    --QQQPLLPQQP------PFSQHQQPVLPQQQIPSVQPSILQQLNPCKVFLQQQCSPVAM 194
             ****:*****   ****:***** ***** *:**********************

42kA    PQSLARSQMLQQSSCHVMQQQCCQQLPQIPQQSRYEAIRAIVYSIILQEQQQVQGSIQTQ 300
42kB    PQSLARSQMLWQSSCHVMQQQCCRQLPQIPEQSRYDAIRAIIYSIVLQEQQHGQGLNQPQ 254
         ********** ************:******:****:*****:***:*****: ** *.*


42kA    QQQPQQLGQCVSQPQQQS-----------QQQLGQQPQQQQLAQGTFLQPHQIAQLEVM 348
```

```
42kB    QQQPQQSVQGVSQPQQQQKQLEQCSFQQPQQQQLGQWPQQQQVPQGTLLQPHQIAQLEVM 314
          ****** * *******.      ****** *****:.***:************

42kA    TSIALRTLPTMCNVNVSLYRTTPRVPFGVGTGVGAY 398
42kB    TSIALRTLPTMCSVNVPVYGTITIVPFGVGTELVTT 350
        ************.***.:* * . ******* : :
```

Fig. 4.2b: Alignment of the proteins sequence on the LMW-42K (PVXLMW42K, 42KA) with those obtained from amplification of the clone pLRPTHLMW42KN23T (42KB).



c

Fig. 4.3: Electrophoretic analysis on 1.2% agarose gel of clone of the digestion of pLRPTHLMW42KN23T using SalI and XbaI restriction enzymes. M, marker Gene Ruler 1KB. Lane 1, the arrow on the top indicates the pLRPT vector and the lower arrow indicates the HLMW42KN23T gene.

4.1.1.2 Cloning of the HB1133WT and HB1133T23N genes

The mutated version of the B1133 gene mutated will be named HB113T23N and the B1133 wild-type will be named HB1133WT from now on.

In order to subclone the HB1133WT and HB1133T23N genes in the expression vector pLRPT, the two genes were amplified from pPVX201HB1133WT and pPVX201HB1133T23N with the pairs of oligonucleotides previously described (par. 3.1.3.2). The PCR inserts were ligated into the vector pLRPT and analysis of transformed clones allowed to identify several recombinant plasmids. Two of the positive clones, as tested by PCR, one for each type, were chosen for further analysis and sequencing, and eventually used for biolistic transformation of wheat plants.

In Fig 4.3, the restriction analysis performed with the endonuclease SalI and XbaI on clones pLRPTHB1133WT and pLRPTHB1133T23N is shown.

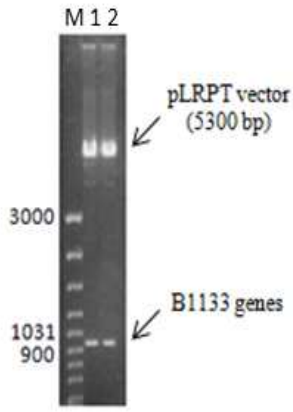


Fig. 4.3: Electrophoretic analysis on 1.2% agarose gel of clones on pLRPTHB1133WT (lane 1) and pLRPTHB1133T23N (lane 2) using SalI and XbaI restriction enzymes. M, marker Gene Ruler 100bp. Lane 1, the arrow on the top indicate the pLRPT vector and the other arrow the HB1133WT and lane 2, the arrow on the top indicate the pLRPT vector and the other arrow the HB1133T23N genes.

4.1.1.3 Complete maps of LMW-GS constructs

The complete coding region of the three LMW-GS genes (two mutated and one wild type) were inserted into the pLRPT vector (Tosi et al., 2004) under the control of the DX5 promoter, that is a strong endosperm-specific promoter. To facilitate the identification of the transgenic product, the His and FLAG-tags were fused to the C-terminus of the sequence. The resulting constructs of LMW-GS (Fig.4.1a, b), and the pUBI::BAR construct (Fig 4.1 c), carrying the *bar* gene that confers resistance to the bialaphos herbicide, were co-transformed into immature wheat embryos (*Triticum durum* cv. Svevo) by particle bombardment following the procedure reported by Weeks *et al*, 1993 with some modifications as described by Janni *et al*, 2008.

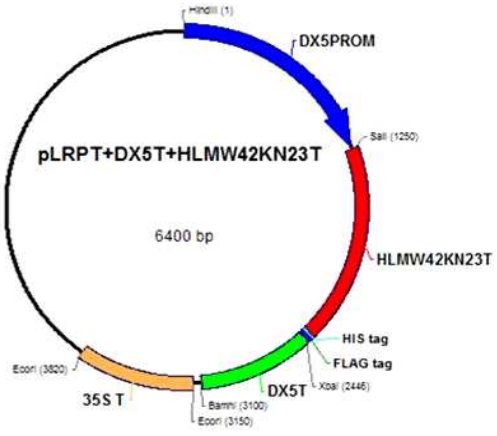


Fig. 4.1b: Plasmid used for biolistic transformation of wheat, pLRPT, containing the *Dx5* promoter and 42K gene.

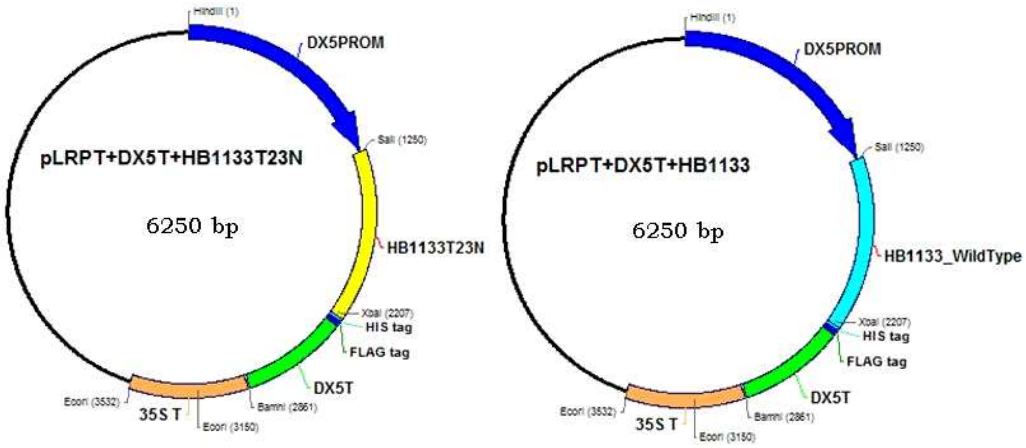


Fig.4.1a: Plasmids used for biolistic transformation of wheat. pLRPT, containing the *Dx5* promoter and B1133 mutated (on the left) and wild-type (on the right) genes.

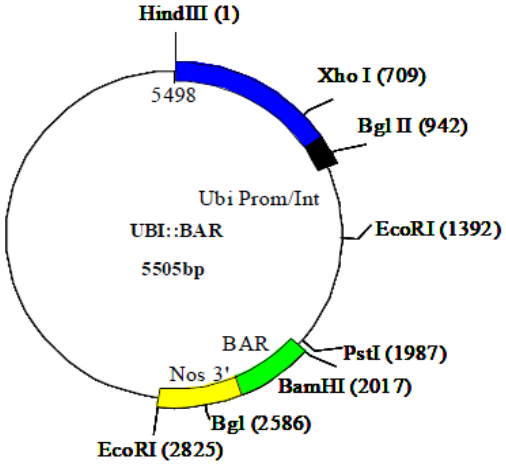


Fig. 4.1c: pUBI::BAR containing the Ubi1 promoter and the bar gene, used for the biolistic bombardment.

4.1.2 Screening of transgenic plants by PCR analysis

We have obtained different transgenic lines for each of the three constructs (pLRPT-HLMW42KN23T, pLRPT-HB1133T23N and pLRPT-HB1133WT) (Tab 4.1). PCR analysis have been used to screen T_0 wheat lines, with primers specific for HMW-GS DX5 promoter region and for Dx5 terminator (for primers, see par. 3.1.3), as shown in Fig. 4.4 a, b, c.

Cv	Bombardment	Construct	Shoots regenerated	Positive To plants
Svevo	MJ 19	HLMW42KN23T	65	32
Svevo	MJ 36-40	HB1133T23N	82	27
Svevo	MJ 43-49	HB1133WT	27	11

Table 4.1: Bombardment experiments with LMW-GS constructs. Transgenic lines obtained and analyzed by PCR.

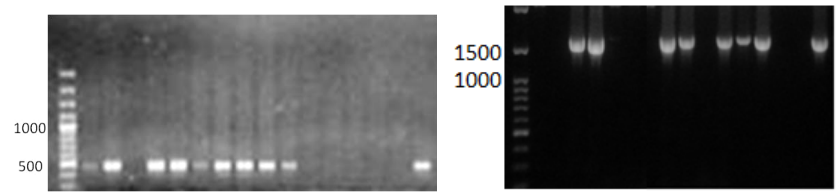


Fig.4.4a: Screening by PCR of plants transformed with LMW-s mutated (HLMW42KN23T). Primers used are Dx5F and DX5R, specific for the Dx5 promoter (on the right) and SalHLMWF and DX5TermR (on the left). Results do not correspond because they are relative to different transgenic lines.

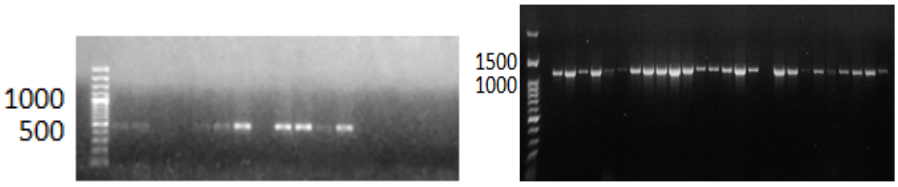


Fig.4.4b: Screening by PCR of plants transformed with LMW-s mutated (HB1133T23N). Primers used are Dx5F and DX5R, specific for the Dx5 promoter (on the right) and SalHB113F and DX5TermR (on the left). Results do not correspond because they are relative to different transgenic lines.

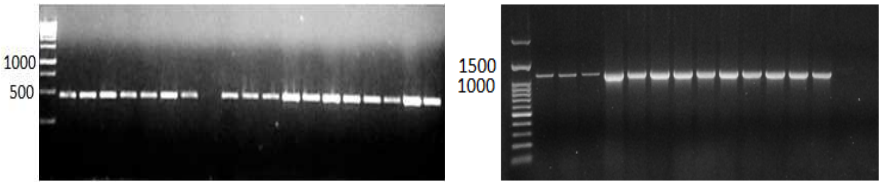


Fig.4.4c: Screening by PCR of plants transformed with LMW-s mutated (HB1133WT). Primers used are Dx5F and DX5R, specific for the Dx5 promoter (on the right) and SalHB113F and DX5TermR (on the left). Results do not correspond because they are relative to different transgenic lines.

4.1.3 Proteomic analysis

4.1.3.1 2D electrophoretic analysis (IEF vs SDS-PAGE) of LMW-GS and relative immunoblottings

T_1 plants that resulted positive to the presence of the transgenes were grown up to maturation, and T_2 seeds were used for proteomic analysis, in order to detect the transgenic polypeptides in comparison to untransformed genotypes.

2D electrophoresis and relative Western Blottings (Fig. 4.6a, b, c) were performed on the glutenin proteins extracted from transformed and untransformed lines. We used different immobilized pH gradient (IPG) strips: pH 3-10 for transgenic plants containing LMW-GS B1133, either wild type or mutated, and pH 6-11 for the mutated 42K protein), in order to get a better separation on 2D gel, since the pI of the mutated 42K protein is ~9.2, whereas that of B1133 is ~7.2.

In case of the transgenic plants containing the mutated LMW-42K protein, Western Blotting was not performed, since we found that the FLAG-tag was lost during the cloning of HLMW42KN23T gene, due to multiple rearrangements and deletions of this gene (Masci et al., 1998).

We analyzed different 2D gels (technical replicas), in order to individuate and characterize the HLMW42KN23T corresponding polypeptide (Fig. 4.6a). The protein spot on the proteomic map was not separated easily from the other LMW-GS B-types because these proteins are very abundant and close each other in the 2D gel. But thanks to the Progenesis SameSpot software was more simple to discriminate our polypeptide, as shown in the figure 4.6a.

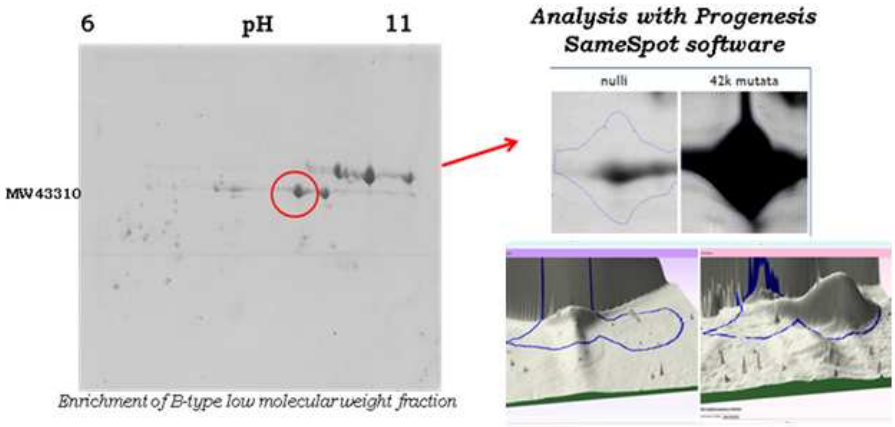


Fig. 4.6a: The 2D electrophoretic analysis of 42K mutated polypeptide and relative analysis with Progenesis SameSpot. The red circle indicates the polypeptide on 2D map. To isolate the 42K polypeptide a protocol for enrichment of LMW-GS B-type was used.

For HB1133T23N and HB1133WT proteins was easier to isolate them on 2D map because the polypeptides are overexpressed and are evident, respect the other LMW-GSs (Fig.4.6b, c). The presence of the transgenic proteins was also verified by western blotting..

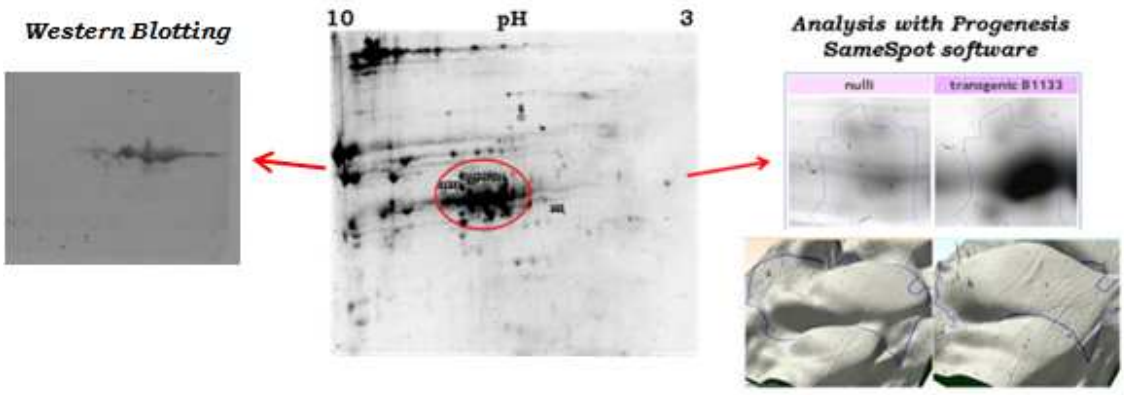


Fig. 4.6b: The 2D electrophoretic analysis of B1133 mutated polypeptide and relative analysis with Progenesis SameSpot (on the right) and Western Blotting (on the left). The red circle indicate the polypeptide on 2D map.

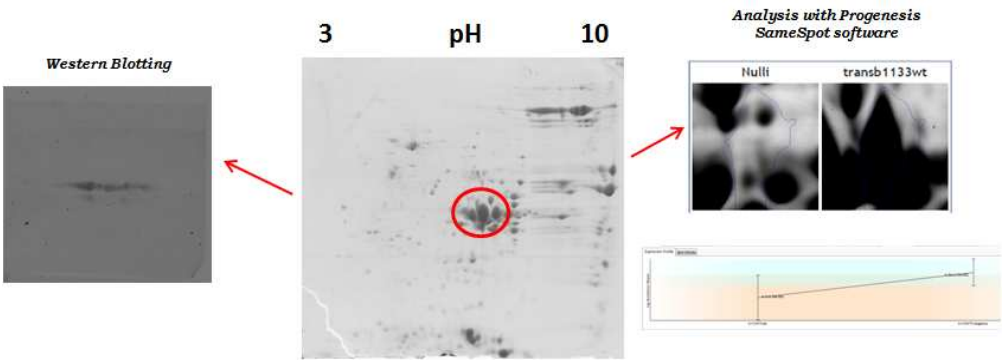
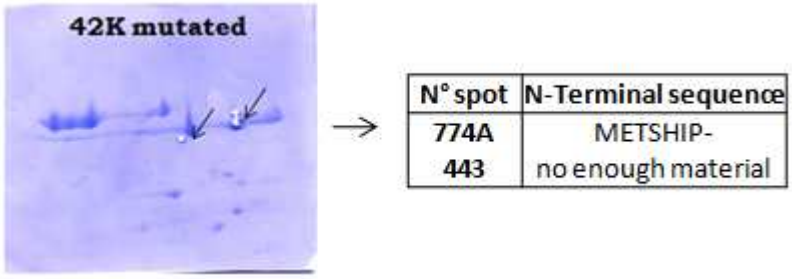


Fig. 4.6c: The 2D electrophoretic analysis of B1133 wild-type polypeptide and relative analysis with Progenesis SameSpot (on the right) and Western Blotting (on the left). The red circle indicates the polypeptide on 2D map, pH 3-10.

4.1.3.3 The N-terminal sequencing

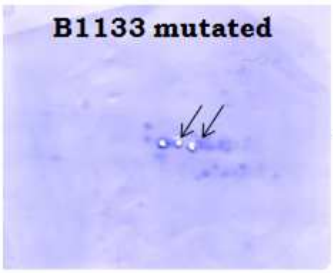
We first performed N-terminal sequencing in order to check if the mutations introduced affected LMW-GS processing.

The figures below showed the N-terminal blotting membrane used to excised the spots corresponding to the polypeptides (42K mutated, B1133 mutated and wild-type) and their analysis (Fig. 4.7a, b, c). The 4-vinyl pyridine (4VP) was used to alkylate the polypeptides, to solve the ambiguity problem of E and C in N-terminal sequencing.



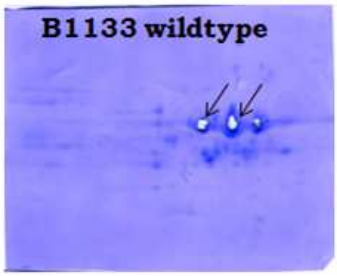
N° spot	N-Terminal sequence
774A	METSHIP-
443	no enough material

Fig. 4.7a: N-terminal blotting membrane of 42K polypeptide. The arrows indicate the spots excised from the membrane to perform the N-terminal sequence analysis and the table on the right indicate the sequence obtained.



N° spot	N-Terminal sequence
220B	SCIPGLERP-
224B	SCIPGLERP-
22A6	SCIPGLERPW-
219A	SCIPGLERP-

Fig. 4.7b: N-terminal blotting membrane of B1133 mutated polypeptide. The arrows indicate the spots excised from the membrane to carry out the N-terminal sequence analysis and the table shows the sequence.



N° spot	N-Terminal sequence
88B	METSCIFGLERPWQQ-
187	METSCIFGLERPWQ-

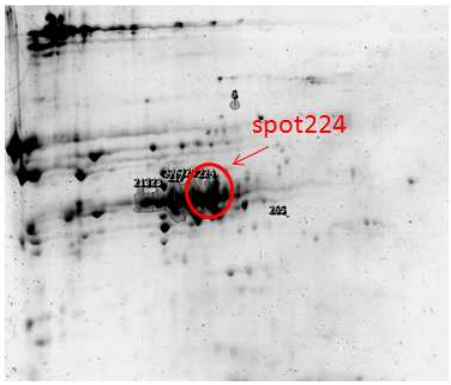
Fig. 4.7c: N-terminal blotting membrane of B1133 wild-type polypeptide. The arrows indicate the spots excised from the membrane and the table illustrates the N-terminal sequence.

The analysis of the N-terminal sequencing carried out on the LMW-GS has confirmed our hypothesis that one of the substantial differences between the LMW-GS -s and m-type, is the presence of the first three amino acid residues MET in the mature polypeptide sequence. It has been hypothesized that this difference is due to a different processing N-terminal of LMW-m and LMW-s type (Masci et al., 1998). In particular, the presence of an Asn in position 23 in the LMW-s, would lead to the cutting sequence performed by an asparagynil endo-peptidase and then to the production of sequences as a LMW-s type (DuPont et al., 2004). Conversely, the presence of a threonine at position 23 in the sequences of m-type, would lead to their canonical processing, characterized only by the cleavage of signal peptide (for details about the classification and the N-terminal sequences, see Introduction Tab. 1.2).

4.1.3.4 Mass spectrometry analysis

Mass Spectrometry analysis of B1133 mutated protein spots allowed to identify a polypeptide showing the expected N-terminal sequence, that is SCISGLE- (Fig. 4.8),

thus confirming the hypothesis that the presence of an Asn in position 23 likely causes a proteolytic cleavage by an Asparagynil endo-peptidase, similarly to what occurs in the LMW-s type. We identified different polypeptides on electrophoretic gel, likely due to protein deamidation because of the high amount of Gln residues (Righetti, 2006). The polypeptide sequence showed a 63% of coverage (Fig. 4.8). The analysis of the LMW-s (42k mutated) (Fig. 4.9) verifies our theory that with T instead of N, the mature polypeptide is METSHIP- as LMW-m type. The last proteins, B1133 wild-type is the positive control for our thesis (Fig. 4.10). The Mass spectrometry analysis of this protein permitted to identy the METSCIF sequence in N terminal domain, validating our work.



HB1133T23N_After (100%), 33.628,8 Da
ASN cleavage
22 unique peptides, 22 unique spectra, 32 total spectra, 187/295 amino acids (63% coverage)

SCISGLERPW	QQQPLPPQQS	FSQQPPFSQQ	QQQPLPQQPS	FSQQQPPFSQ
QQPILSQQPP	FSQQQQPVLP	QQSPFSQQQQ	LVLPPQQQQQ	QLVQQQIPIV
QPSVLQQLNP	CKVFLQQQCS	PVAMPQRLAR	SQMWQQSSCH	VMQQQCCQQL
QQIPEQSRYE	AIRAIIYSII	LQEQQQGFVQ	PQQQQPQQSG	QGVSQSQQQS
QQQLGQQCSFQ	QPQQQLGQQP	QQQQQQQQVLQ	GTFLQPHQIA	HLEAVTSIAL
RTLPTMCSVN	VPLYSATTSV	PFGVGTGVGA	YHHHHHHDYK	DDDDK

Fig. 4.8: Mass spectrometry analysis of B1133 mutated polypeptide. The spot 224 was cut off from the 2D gel and sequenced. Matched and modified amino acids are highlighted in yellow and green, respectively.

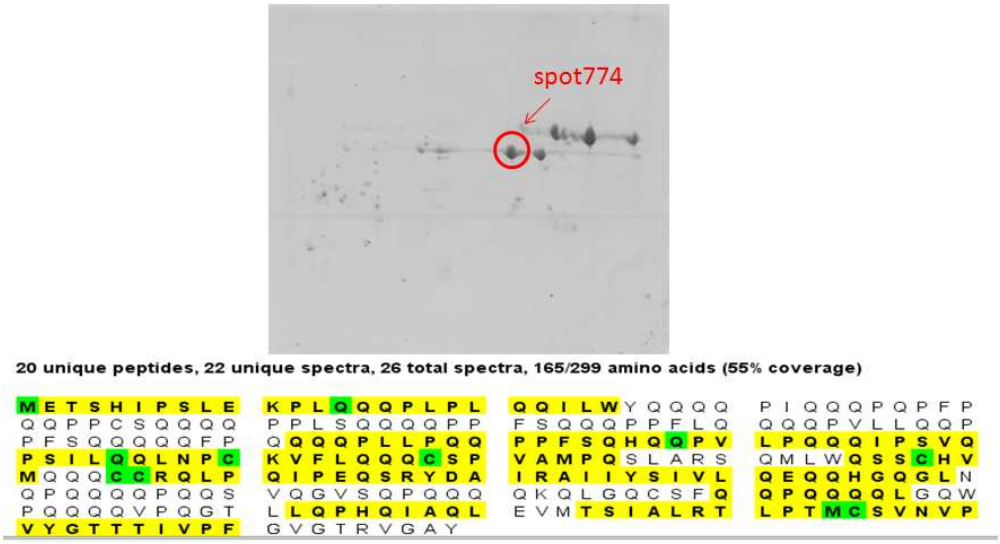


Fig. 4.9: Mass spectrometry analysis of 42K mutated polypeptide. The spot 774 was cut off from the 2D gel and sequencing. Matched and modified amino acids are highlighted in yellow and green, respectively.

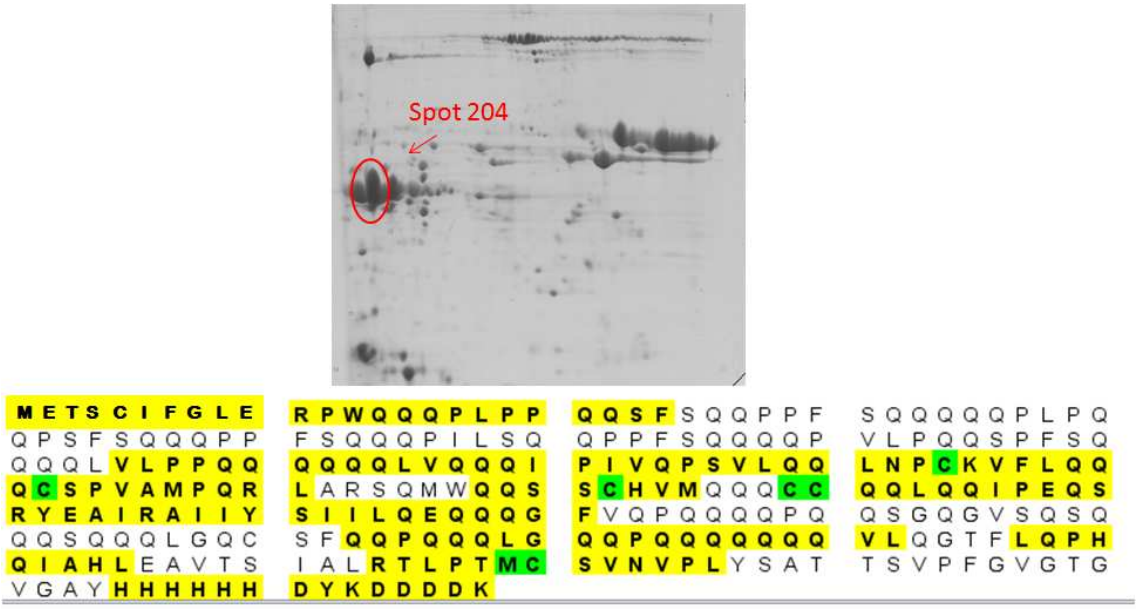


Fig. 4.8: Mass spectrometry analysis of B1133 wild-type polypeptide. The spot 204 was cut off from the 2D gel and sequencing. Matched and modified amino acids are highlighted in yellow and green, respectively.

4.2 TRAFFIKING OF GLUTEN PROTEINS

The determination of the precise route of trafficking of individual glutenin subunits is still a matter of debate. It has been suggested that the same protein could be trafficked

by different pathways, depending on the stage of development, and that segregation of gluten proteins both between and within protein bodies may occur. The existence of two different protein body types has been hypothesized: the denser protein bodies formed by the aggregation of storage proteins within the ER, and the lighter bodies resulting from aggregation at a post-ER location. No clear results were obtained for the LMW subunits. In fact the determination of the locations and trafficking of individual gluten proteins in developing wheat grain has been limited in the past by the complexity of the protein mixture with a high degree of sequence similarity between some components (such as LMW-GS and gliadins), making it difficult to develop monospecific antibodies (Tosi et al., 2009).

In order to gain more precise information at this regard, the first approach was to transform wheat plants with LMW-GS genes containing specific tags, such as c-myc (Tosi et al, 2009). This approach gave indication of the route followed by LMW-GS, but, because only the polypeptides were marked and not membranes of the cellular compartments, the interpretation of microscopy images was rather complicated.

For this reason, we produced transgenic wheat plants transformed with constructs containing specific markers for cellular compartments (for Vacuole, Golgi and ER) in which YFP, GFP or Cherry and HA-tag, have been added, in order to localize, through immunofluorescence tests, these specific compartments. As a second step, crosses or co-transformation will be performed between these latter and transgenic wheat plants previously transformed with LMW-GS containing different tags, in order to get unequivocal information about the modality of deposition of glutenin subunits.

4.2.1 Preparation of constructs for plant transformation

In this case also, I decided to report details about constructs preparation in the results section, since it was an important part of my thesis.

4.2.1.1 Cloning of specific marker for cellular compartments

4.2.1.1.1 Preparation of the pSPORT-Alpha-TIP construct as a marker of the vacuolar compartment

The Arabidopsis Alpha-TIP (Tonoplast Intrisec Protein) gene was chosen first, because the TIP family is well characterized in different plant species and tissues, and the encoded polypeptides can be used as vacuole markers. Second because in Arabidopsis seeds, α-TIP was very clearly localized to the rim of the PSV (Plant Storage Vacuols) by immunofluorescence and immuno-electron microscopy (Hunter et al., 2007).

Initially, we used a construct (kindly provided by prof. L. Frigerio, Warwick, UK) that contained the Arabidopsis Alpha-TIP gene (At1g73190, TAIR databank) and the YFP Hunter et al., 2007). The two genes were isolated by using the primers Xma.Alpha-TIPF and BamhI.HA.YFPR (see Materials and Methods, Tab. 3.1), and cloned into pSPORT vector, already containing the PinA promoter and the NOS terminator (Wiley et al, 2007).

The HA-tag was added at the C-terminus of the YFP, that is functional to identify the marker gene by immunolocalization. *E. coli* XL1-Blue Supercompetent cells was chosen for the transformation; screening was done by PCR analysis (Fig. 4.11) and the complete construct containing the vacuolar marker gene (7120 bp) has been used for biolistic transformation (Fig. 4.12).

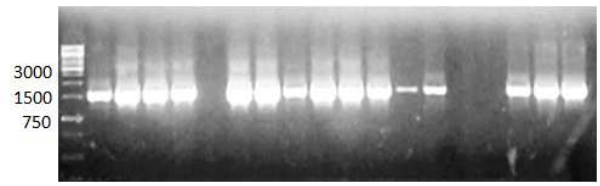


Fig. 4.11: PCR colony to screen positive colonies containing pSPORT-Alpha-TIP construct, using primers Xma.Alpha-TIPF and BamhI.HA.YFPR.

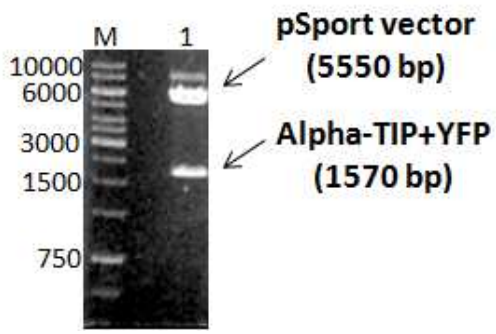


Fig. 4.12: Electrophoretic analysis on 1% agarose gel of clones excised from pSPORT-Alpha-TIP (lane 1, 2) using XmaI and BamhI restriction enzymes. M, marker GeneRuler™ 1 kb DNA Ladder. Lane 1,the arrow on the top indicates the pSPORT vector and the other arrow indicates the Alpha-TIP plus YFP genes.

4.2.1.1.2. Preparation of the pSPORT-Sec61β construct as a marker of the ER compartment

We chose Sec61β because is a rER membrane marker. The Sec61αβγ complex is in fact the essential core of the protein translocation machinery in the ER membrane and is tightly associated with membrane-bound ribosome in rice (Onda et al., 2009). For this construct, we took the ER marker gene from the binary vector BV1, accession number C20385 (see Supporting information, Onda et al., 2009).

Since the restriction sites XmaI and BamhI present in the binary vector BV1 between the Cherry marker and the Sec61β gene were not in an appropriate position for our cloning vector (pSPORT), it was necessary to remove these sites by SOE-PCR (see Materials and Methods, par. 3.2.1.2), and place them in flanking positions to the Cherry marker and the Sec61β gene. The steps performed are described in the detail in the next paragraph.

4.2.1.1.2.1 SOE-PCR of Cherry-Sec61β genes

We have used the technique of Splicing by Overlap Extension by the Polymerase Chain Reaction (SOE by PCR).

The different steps performed are illustrated below (Fig. 4.13).

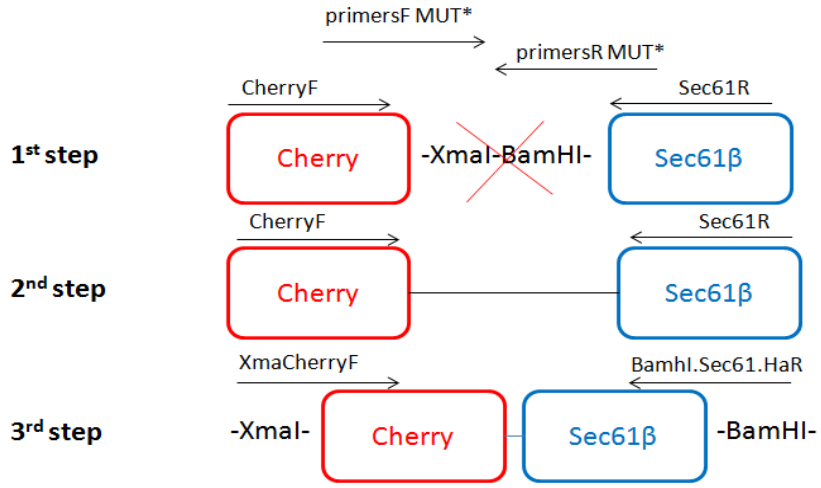


Fig. 4.13: SOE-PCR schematic representation.

1st step:

To remove the XmaI and BamhI sites in between of the cassette containing Cherry-Sec61β, mutated primers were used (see Materials and Methods, Tab. 3.1). Two fragments were obtained (Fig. 4.14a).

2nd step:

The second step involved a PCR strategy performed with primers CherryF (forward primer) and Sec61 (reverse primer) that allowed to ligate the two fragments to obtain a single PCR product (Cherry-Sec61β) (Fig 4.14b).

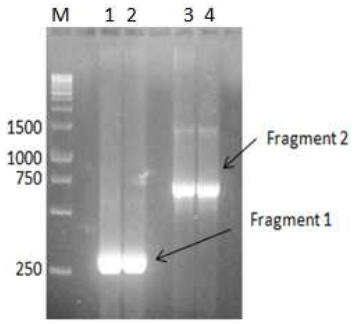


Fig.4.14a: Electrophoretic analysis on 1.2% agarose gel of the first step of SOE-PCR. Two fragments were obtained. M, marker Gene Ruler 1Kb. Lane1, 2 correspond to the Cherry gene (fragment 1) and lane 3, 4 correspond to the Sec61β gene (fragment 2).

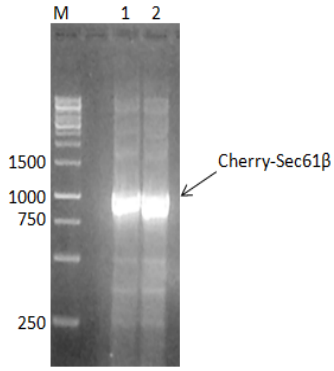


Fig.4.14b: Electrophoretic analysis on 1.2% agarose gel of second step of SOE-PCR. M, marker Gene Ruler 1Kb. Lane1 and 2 are the ligated Cherry-Sec61β genes.

3rd step:

In the last step of SOE-PCR, we used XmaI.cherryF (forward primer) and BamhI.sec61.HAR (reverse primer) to insert the restriction sites in flanking positions of the Cherry-Sec61β gene construct (Fig. 4.14c) that allowed cloning into the pSPORT vector.

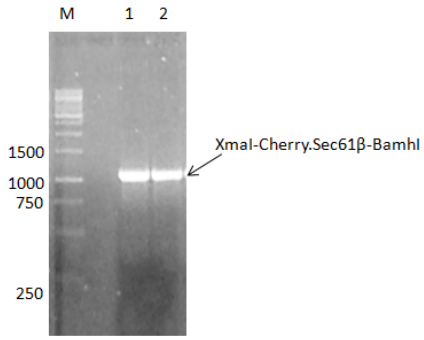


Fig.4.14c: Electrophoretic analysis on 1,2% agarose gel of the third step of SOE-PCR. M, marker Gene Ruler 1Kb. Lane1 and 2 indicate the gene construct.

4.2.1.1.2.2.. Cloning of Cherry-Sec61β in pSPORT vector

Before inserting the Cherry-Sec61β gene construct into pSPORT, the HA-tag was added at the C-terminus, in order to facilitate recognition of the polypeptide products. The transformation was performed by using *E. coli* cells and the screening of positive colonies was performed by PCR colony (Fig. 4.15).

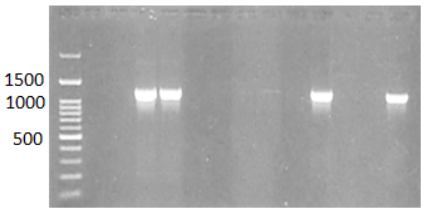


Fig. 4.15: PCR colony to screen positive colonies containing pSPORT-Sec61β construct.

4.2.1.1.3. Preparation of the pSPORT-Alpha-Mannosidase I construct as a marker of the Golgi compartment

Golgi localization was based on the cytoplasmic tail and the transmembrane domain (first 49 aa) of GmMan1, a soybean α-1,2-mannosidase I (Saint-Jore-Dupas et al., 2006). The first Golgi enzymes to be involved in N-glycan maturation are the glycosidases, more precisely *a*-mannosidase I and II and the soybean GmMan I is the only Golgi glycosidase cloned so far in plants.

We used the construct α-1,2-mannosidase I-GFP (G-gk CD3-963) from the binary plasmids available through the Arabidopsis stock centers (http:// www.arabidopsis.org)., This construct was then amplified and inserted in our vector (pSPORT), according to the procedure reported in Par. 3.2.1.1 (Fig. 4.16, 4.17), in order to use this construct for the production of wheat transgenic plants.

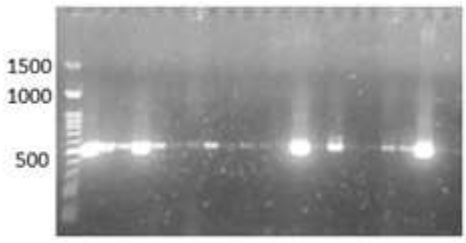


Fig. 4.16: PCR colony to screen positive colonies containing the pSPORT-Alpha-Mannosidase I construct. Primers used are specifics for GFP gene region.

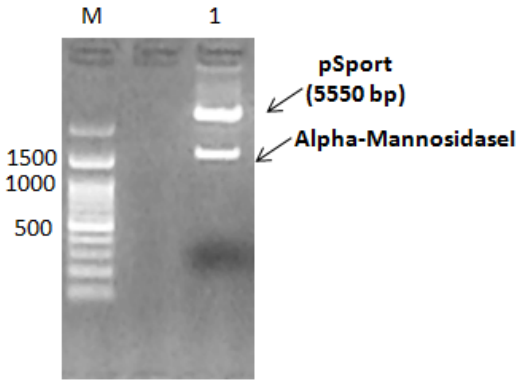


Fig. 4.17: Electrophoretic analysis on 1% agarose gel of clones excided from pSPORT-Alpha-Mannosidase I (lane 1). M, marker GeneRuler 100bp. Lane 1, the arrow on the top indicates the pSPORT vector and the other arrow indicates the Alpha-Mannosidase I and GFP genes.

4.2.1.1.4 Maps of specific markers constructs

For each of the specific marker constructs described above, we report the specific map and sequence. The **target gene** for each cellular compartment is **in bold**, the YFP (yellow fluorescent protein) gene is highlighted in yellow, the GFP (green fluorescent protein) gene is in green and the Cherry gene is highlighted in red, the NOS sequence is light blue, the HA tag is in pink. Figures 4.8, 4.9 and 4.10 give a schematic representation of constructs.

4.2.1.1.4.1 Vacuolar marker (α-TIP) plasmid sequence

PINA-XmaI-
ATGGCCGCAACATCAGCTCGTAGAGCATACGGTTTCGGTAGAGCCGATGAGGCTACACA
CCCTGACTCCATTAGAGCAACTTTAGCTGAGTTTCTCTCCACTTTTGTCTTCGTCTTTG
CAGCTGAAGGCTCTATCCTCTCTCTCGATAAGTTGTATTGGGAACATGCGGCTCATGCG
GGGACAAACACACCAGGAGGGCTGATTTTAGTAGCGTTGGCTCATGCGTTTGCTCTGTT
TGCTGCTGTTTCAGCAGCCATTAATGTCTCCGGCGGACACGTTAACCCGGCAGTCACTT
TTGGTGCTCTTGTTGGAGGCAGAGTTACAGCGATCCGCGCCATCTACTACTGGATCGCT
CAGCTTCTTGGAGCCATCCTCGCTTGTCTCTTGTTAAGGCTCACAACAAACGGCATGAG
ACCAGTTGGTTTCCGTCTAGCATCAGGTGTTGGAGCGGTTAATGGACTTGTATTAGAGA
TCATTTTAACATTTGGCTTAGTCTACGTAGTGTATTCCACTTTGATTGATCCAAAACGT
GGAAGCCTCGGGATCATAGCACCGCTTGCAATCGGACTCATAGTTGGGGCAAACATCTT
AGTAGGTGGACCATTTTCTGGTGCTTCGATGAATCCAGCTAGAGCTTTTGGTCCAGCGT
TGGTGGGATGGAGATGGCATGACCACTGGATCTATTGGGTCGGACCATTCATCGGTAGT
GCTTTAGCCGCCCTTATATATGAGTACATGGTCATACCCACCGAACCACCTACCCACCA
CGCACATGGTGTACACCAGCCCTTGGCCCCTGAAGATTACGCCATGGGCAGCAAGGGCG
AGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGC
CACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCT
GAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCT

TCGGCTACGGCCTGCAGTGCTTCGCCCGCTACCCCGACCACATGAAGCAGCACGACTTC
TTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGA
CGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCA
TCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAG
TACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAA
GGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACT
ACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTG
AGCTACCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCT
GGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCAAGGACGAGCTGTACAAGGTGATC-
HA-BAmhI-NOS-EcoRI

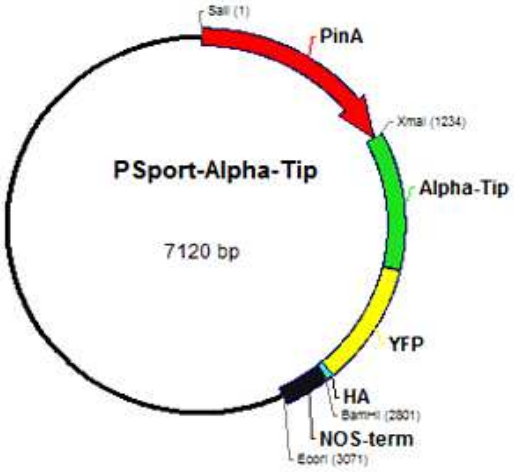


Fig.4.8:The *pSPORT-Alpha-TIP* construct.

4.2.1.1.4.2 ER marker (Sec61β) plasmid sequence

PINA-XmaI-
ATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATGCGCTTCAA
GGTGCACATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGG
GCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTG
CCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCCTACGTGAA
GCACCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGG
AGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTCCTCCCTG
CAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCGACGG
CCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCCCG
AGGACGGCGCCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACGGCGGCCAC
TACGACGCTGAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCGGCGC
CTACAACGTCAACATCAAGTTGGACATCACCTCCCACAACGAGGACTACACCATCGTGG
AACAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAAG
GGAGGTGGTTCC**ATGGTGGCCAATGGAGATGCCCCGGCTAGAGGGAGTGCAGCAGCTGC**
TGCAAGCTTGCGCAGGCGTAGAACCACCAGCAGTGGCACTGGTGGAGGCGGTGCCAGCA
CAATGCTCCAGTTCTACACCGATGAGGCTGCTGGGCGCAAGATGTCCCCGAACTCAGTC
CTCATCATGAGCATTGGGTTCATTGCCGTCGTTGCTCTTCTGCATGTTTTCGGCAAGCTT
TACCGCACCTCCGCC-HA-BAmhI-NOS-EcorI

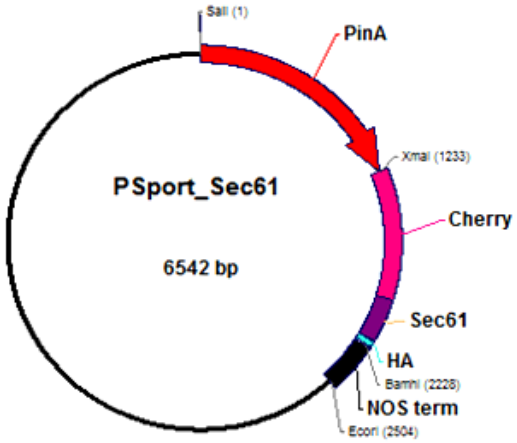


Fig.4.9:The *PSPORT-Sec61β* construct.

4.2.1.1.4.3 Golgi (α-mannosidase I) marker plasmid sequence

PINA-XmaI-
ATGGCGAGAGGGAGCAGATCAGTGGGTAGCAGCAGCAGCAAATGGAGGTACTGCAACCC
TTCCTATTACTTGAAGCGCCCAAAGCGTCTTGCTCTGCTCTTCATCGTTTTCGTTTGTG
TCTCTTTCGTTTTCTGGGACCGTCAAACTCTCGTCAGAGAGCACCAGGTTGAAATTTCT
GAGCTGCAGAAAGAAGTGACTGATTTGAAAAATTTGGTGGATGATTTAAATAACAAACA
AGGTGGTACCTCTGGGAAGACTGACTTGGGGAGAAAAGCTACCAAGTCCAGTAAAGACG
TCTCGAGGACCGGTCCAGGGGGTTCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGG
GTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTC
CGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCA
CCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCTTCACCTACGGCGTGCAG
TGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCC
CGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCC
GCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATC
GACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCA
CAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCC
GCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCC
ATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCT
GAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCG
CCGGGATCACTCACGGCATGGACGAGCTGTACAAG-HA-BAmhI-NOS-EcorI

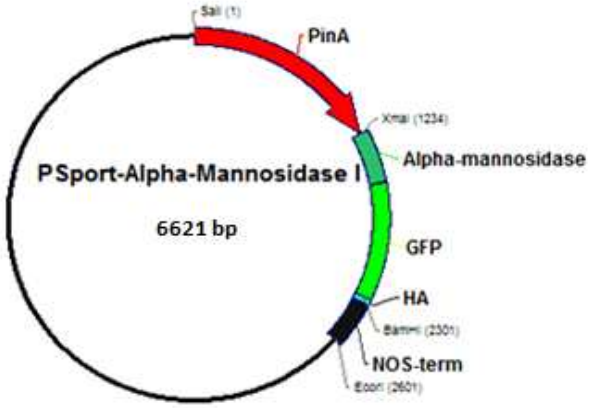


Fig.4.10:The *pSPORT-Alpha-Mannosidase I* construct.

4.2.2 PCR analysis and screening of transgenic plant

PCR analysis was used to screen T_0 wheat lines (plants regenerated by *in vitro* culture), with primers specific for PinA promoter and NOS terminator regions. T_1 seed were germinated and embryo rescue techniques were used on developing grains to shorten time for production of T_2 plants. Seeds from transgenic T_2 plants were screened by confocal microscopy, in order to confirm expression of fluorescence tags (Fig. 4.18).

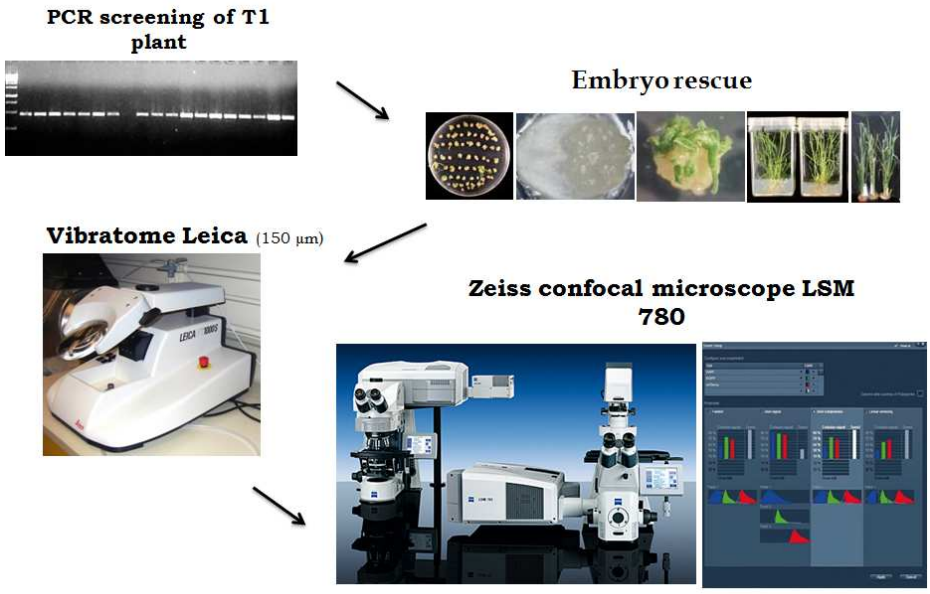


Fig. 4.18: Schematic illustration of screening of trangenic plants, embryo rescue, vibratome sectioning of positive seeds and confocal microscopy.

4.2.2.1 PCR analysis with specific oligonucleotide design

In the figure 4.19 the primers used for PCR analysis of transgenic lines containing specific cellular marker (see Materials and Methods, par. 3.2.1.1) are illustrated. Each set of transgenic lines were grown under controlled conditions.

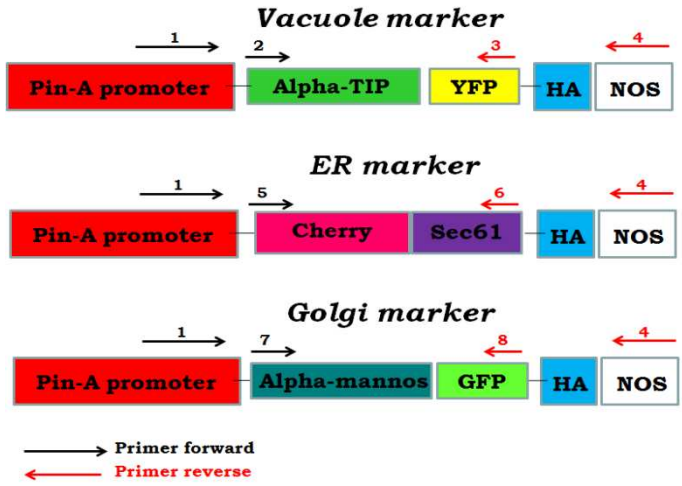


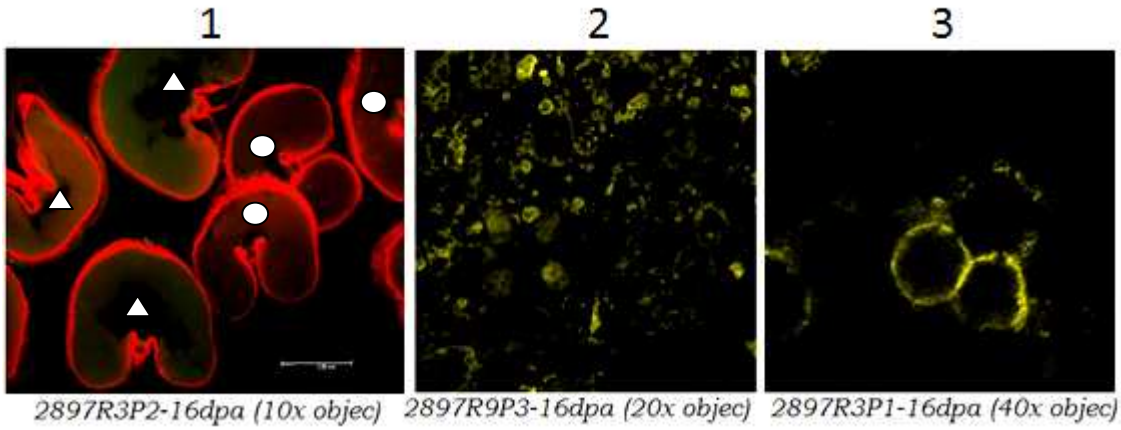
Fig. 4.19: Specific oligonucleotides used for PCR screening of transgenic lines.

4.2.3 Confocal microscope analysis

Analysis of transgenic plants containing the fluorescent markers for Vacuole, Golgi and ER was carried out on a LSM 780 confocal microscope (Zeiss), using a “smart setup” option which allows automatic adjustment of imaging parameters for best acquisition speed/ signal quality (Fig 4.20, 4.21, 4.22). We used different sets of wheat transgenic lines but collected at the same stage of development, in order to compare the expression of the markers for the different compartment.

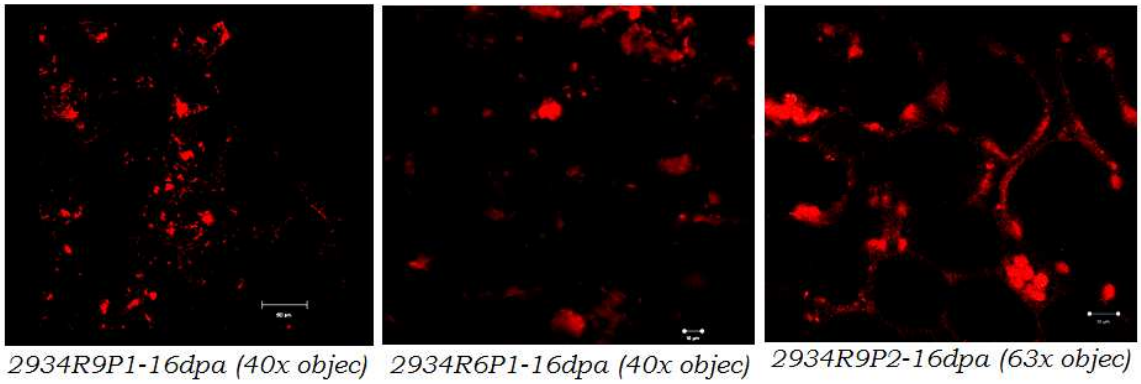
The tonoplast is the delimiting membrane around the vacuole, and in fully expanded cells it stretches throughout the cell revealing the large size of the central vacuole (Figure 4.20). Some cells, particularly in rapidly expanding tissues of seedlings, contain circular structures of varying size (1–10 µm) inside the lumen of the vacuole (Figure 4.20-2).

Our results indicate that the α-TIP marker was localized in the tonoplasts of vacuoles of different sizes. This would be in accordance with the results previously reported by Park et al. (2004), but different to what reported by Hunter et al. (2007), who localized exogenously expressed hemagglutinin (HA)-tagged α-TIP in the central vacuole only.



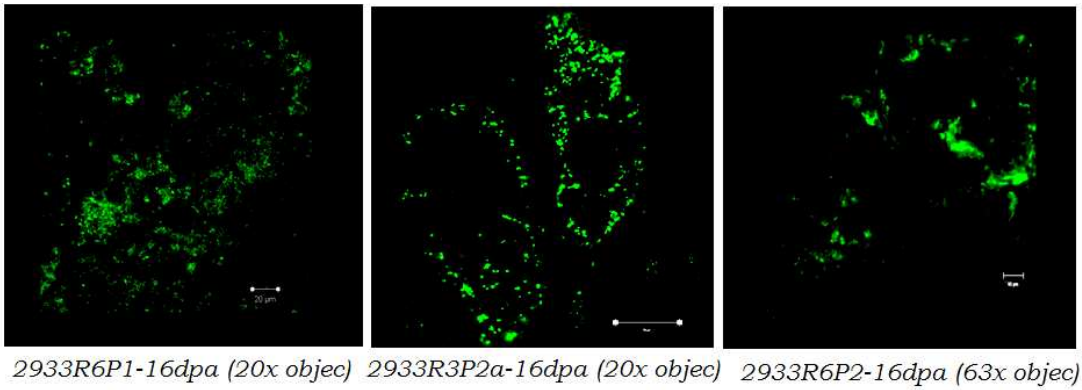
4.20: Confocal analysis of 16 dpa seed sections from T_2 emizygous line expressing the transgenic α–TIP-YFP. At the bottom of each picture the transgenic lines used and the magnification are specified. 1 Transgenic (△) and null segregant grains (◯). 2 and 3 YFP labelling of vacuolar membranes.

The ER forms an extensive network throughout the cytoplasm. This network is better observed by focusing on the cortical cytoplasm just under the plasma membrane (Figure 4.21). In this case, a fine network of clearly delineated tubular and sheet-like ER elements can be discerned. The signal difference between the dark areas in the lacunae and the ER elements is substantial and distinct. Moreover, we observed fluorescent structures attributable to ER derived protein bodies, but further analysis using a storage protein marker will be necessary to confirm this conclusion.



4.21: Confocal analysis of 16dpa seed sections expressing the ER marker, Sec61β. At the bottom of each picture the transgenic lines used and the magnification are reported.

The Golgi apparatus consists of a large number of small independent stacks (Fig. 4.22). Our observations are in perfect agreement with Nebenführ et al., (1999) who found that the individual Golgi stacks appeared as perfectly round discs, as short lines or in the shape of small rings, depending on orientation and status.



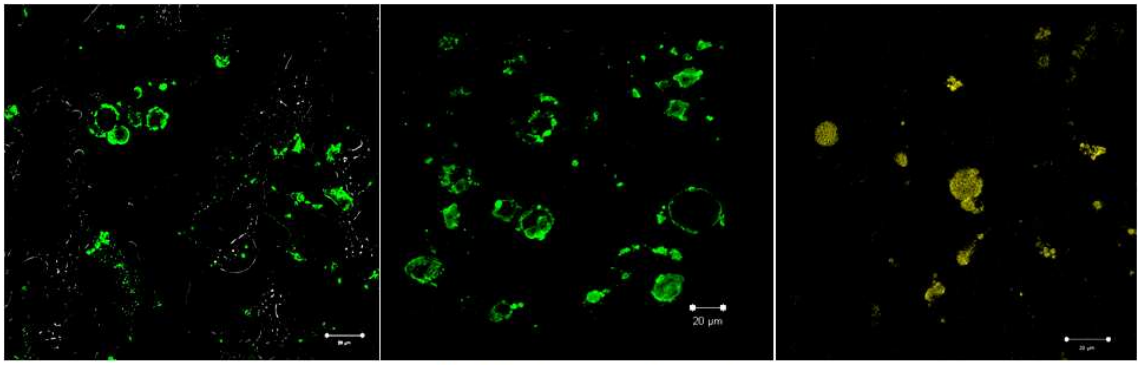
4.22: Confocal analysis of 16dpa seed sections expressing the Golgi marker, Alpha-Mannosidase I. On the bottom of each picture the transgenic lines used and the magnification are reported.

So each fluorescent protein (GFP, YFP and Cherry) was detected with a high definition by confocal microscopy, as reported in the figures above. The specificity of the signal confirms the efficacy of this organelle tagging approach to study the trafficking routes in wheat.

4.2.4 Immunofluorescence test of pSPORT-Alpha-TIP construct

At the moment, immunofluorescence microscopy was carried out on transgenic seeds expressing the α-TIP marker. The polyclonal anti-HA antibody was used for immunofluorescence tests, in order to screen and visualize the α-TIP polypeptide in wheat kernel sections (28 dpa).

These preliminary results show that the α-TIP marker was recognized in all the vacuoles of different sizes, confirming the results obtained monitoring GFP fluorescence (fig. 4.23).



4.23: Immunofluorescence analysis of 28 dpa seed sections from obtained from T_2 emizygous line expressing the transgenic α–TIP-YFP. At the bottom the transgenic line used and the magnification are reported. The polyclonal anti-HA antibody was used to recognize the α–-TIP polypeptide. In the rigth most panel, the YFP signal is in yellow. In the other panels, it is in green.

CONCLUSIONS

5. CONCLUSIONS

Wheat gluten proteins are typical secretory proteins in that their synthesis, folding, maturation and deposition take place within the endomembrane system of the plant cell. As for the glutenins, they are divided into high (HMW-GS) and low (LMW-GS) molecular weight glutenin subunits. The first have been extensively studied and characterized, whereas study of the second is still at incomplete stage, due to the large number of subunits expressed in the endosperm of wheat, and also for their similarity in chemical and physical characteristics that makes them difficult to purify.

The LMW-GS are divided into three groups according to their electrophoretic mobility: B, C and D. In the B group the typical LMW-GS are mostly present, which are subdivided, according to the first amino acid present in the mature polypeptide, in LMW-s (serine), LMW-m (methionine) and LMW-i (isoleucine) type. LMW-s and LMW-m seem practically identical, on the basis of the complete nucleotide sequence. This is because the main difference between these two sequence types is the absence of the expected first three N-terminal amino acids (MET-, or MEN- in some minor cases,) in the mature sequence of LMW-s type, although the nucleotide sequence corresponding to these three amino acids is present in the sequences encoding both -s and -m type LMW-GS precursors.

According to signal sequence cleavage site prediction algorithms, cleavage by signal peptidase would generate a QMET- (or QMEN-) N-terminal sequence. Removal of the N-terminal Gln residue would be therefore required to generate m-type LMW-GS. On the other hand, the identification of the gene coding for a specific LMW-s type showed the presence of an Asn instead of a Thr before the start of the mature sequence (MENS- instead of METS-). Thus we have hypothesized that the presence of the Thr of the immature polypeptide instead of the Asn residue could determine a preferential processing at the N-terminal end of LMW-s type sequences, that might generate the cleavage of the peptide MEN by an asparaginyl endoprotease, similarly to what likely occurs in ω-gliadins (Dupont et al., 2004). For this reason, the genes for these two target proteins (LMW-m and –s) were mutated in position 23 of the coding sequence in order to have a threonine instead of asparagine and *viceversa*. The durum wheat cultivar Svevo was used for genetic transformation with mutated and wild-type LMW-GS.

The genes coding for the mutated versions of LMW-GS s-type and LMW-GS m-type and for the wild-type LMW-GS m-type were cloned into pLRPT vector for the expression in plants. Both were put under the control of the HMW-GS Dx5 promoter. Two different tags were added at the C-terminus (His- and FLAG-tags) of each target gene. Vector pUBI::BAR was used to co-transform wheat embryos , in order to screen and select transgenic plants. SDS-PAGE, 2D electrophoresis and relative Western Blottings were performed on the glutenin proteins extracted from transformed and untransformed lines, in order to identify the mutated LMW-GS. The N-terminal sequencing and/or mass spectrometry characterization of the identified spots was performed in collaboration with Dr. W.H. Vensel, USDA, Albany, CA. These analyses allowed to identify polypeptides showing the expected sequence of both mutated LMW-m type (that was SCISGLE-), and LMW-s type (that was METSHIPGLE-), thus confirming our hypothesis that the presence of an Asn or a Thr in position 23 causes a proteolytic cleavage (by an Asparagynil endo-peptidase). This was verified also by results obtained on the transgenic wild type LMW-m, that showed an unchanged N-terminal sequence.

We produced also the constructs containing markers for specific cellular compartments, to be used to transform wheat plants in order to follow the deposition pathway of wheat storage proteins, and, in particular, of LMW-GS. Each construct contained a specific marker for the recognition of the different subcellular compartments: for the vacuole, we used the Arabidopsis α-Tip (α-tonoplast intrinsic protein) (Hunter et al., 2007); for the endoplasmic reticulum, we chose rice Sec61β (membrane protein translocator,) (Onda et al., 2009); and for the Golgi compartment, we chose a soybeanα-Mannossidase I (a key enzyme involved in N-linked Glycan processing) (Nelson et al., 2007).

All the constructs included the promoter, PinA (from the wheat puroindoline-a gene), because it allows an even expression throughout the endosperm (Wiley et al., 2007). As fluorescent markers, we used either the yellow or the green fluorescent protein (YFP or GFP), or the Cherry protein.

In collaboration with Rothamsted Research, the bread wheat cultivar Cadenza was transformed with *PSport-Alpha-Tip*, *PSport-Alpha-Mannosidase I* and *PSport-Sec1β*

constructs and the corresponding transgenic lines were analysed by confocal microscopy.

PCR analysis was used to screen T_1 wheat lines, and embryo rescue was performed to obtain T_2 plants. Our results confirmed that this approach is a valid tool to study trafficking routes in wheat kernel. To reach this goal, these transgenic plants will be either crossed with other transgenic wheat plants transformed with LMW-GS containing specific tags, or co-transformed with LMW-GS with specific tags.

The information obtained so far and those that will be obtained by the continuation of this research, besides shedding light on these unknown mechanisms, will help in the understanding of gluten proteins organization, that is at the basis of qualitative properties of wheat.

6. REFERENCES

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AKNOWLEDGEMENTS

First of all I would like to thank my professor Stefania Masci from whom I learnt all that I needed for my PhD thesis, but most important for her professional behaviour as well as for her availability whenever I needed her. Thanks to Prof. Domenico Lafiandra for giving me hospitality in their laboratories. I would like to extend my gratitude also to Prof. Renato D'Ovidio and his collaborators of Plant Physiology laboratory for the transgenic transformation that they have made, especially to Dr. Michela Janni. A particular thanks is to Dr. Paola Tosi for teaching me some experimental methodologies of confocal microscopy and to Dr. Aldo Ceriotti, Dr. Lorenzo Frigerio and Dr. Yayoi Onda for providing me the compartment marker genes. Thanks also to Dr. William Vensel for Mass spectrometry analysis and for the advice that he gave me about proteomic matter. Thanks to AgroGen Grant that supported my research and for CIB which gave me the grant to spend a period at Rothamsted Research Centre, UK. My immense gratitude is of course to the people of my laboratory Francesco Sestili, Linda Botticella, Samuela Palombieri, Lina Maria Rivera Ortiz, , Daniela Panichi, Roberta Lupi, Gaetan Gombaud and Federica Paoletti. They were always available to answer to all my question, give me useful advice and support me during my research. Thanks to Tiziana Bozzini and Valentina Rocchi, that I met at university course and we became true friends, thanks also for funny moments in the laboratory.

My final thought is to Sergio and my family; thanks for always believing in me and for helping me whenever I needed them most, while at the same time letting live my life without any interference.