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THE EFFECTS OF THE ABIOTIC AND BIOTIC STRESSES ON THE WHEAT PROTEOME

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A mio nonno e al cucciolo Luca:

"Lentamente muore chi
abbandona un progetto prima
di iniziarlo, chi non
fa domande sugli argomenti
che non conosce, chi non
risponde quando gli
chiedono qualcosa che
conosce".
(P. Neruda)

THE EFFECTS OF THE ABIOTIC AND BIOTIC STRESSES ON THE WHEAT PROTEOME

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ABSTRACT

The wheat is one of the world's most popular and cultivated crop and the abiotic and biotic stresses are damaging factors affecting the yield and the quality traits of the wheat grain's derived products. The protein and starch content of the durum and bread wheat grain is the prime measure of wheat quality. The kernel proteins are typically classified according to their solubility properties into albumins (water soluble), globulins (salt soluble) and prolamins (gluten proteins). The non-prolamin fractions include proteins with metabolic activity or structural function, that could be involved in the plant stress response. High temperature during grain filling was recognized to alter quality characteristics of bread wheat doughs, as consequence of modified accumulation of gluten proteins that are responsible for their technological properties. Also low temperature has a great impact on plant productivity, mostly because it alters the wheat metabolism and physiology. Among the biotic stress Fusarium head blight (FHB), caused by *Fusarium* spp., is the most destructive disease of small grained cereals, and leads to significant reduction of the grain yield and quality.

In order to investigate on the effects of both these stressors (heat, low temperature, and *Fusarium graminearum* infection) on the wheat grain and leaf proteome, different gel-based comparative proteomic analyses (IEF/SDS, DIGE and MS identification) were used. To study the heat stress response on the accumulation of non-prolamin protein compound, the durum wheat cultivar Svevo, was subjected to two thermal regimes (heat stress *vs.* control) during grain filling. The comparative proteomic analysis revealed 132 differentially expressed polypeptides (both up- and down-regulated), 47 of which were identified by MS and found to include heat shock proteins, proteins involved in the glycolysis and carbohydrate metabolism as well as stress related proteins. Many of the heat induced polypeptides are considered to be allergenic for sensitive individuals.

The results from the low temperature study provided an optimized method to discriminate on 2DE gels the total leaf proteins, although for technical problems we did not reach the optimal temperature conditions for the cold stress investigation.

Finally, to evaluate the biotic stress effects on the seed proteome, transgenic bread wheat genotypes encoding a PoliGalactoronase Inhibitor Protein (PGIP) were used. Previous analyses showed that the transgenic genotypes resulted more tolerant to *Fusarium graminearum* infection. None of the grain proteins expressed at maturity resulted differentially regulated (by DIGE analysis) comparing the transgenic plant seeds to their relative controls after the fungal infection. Also the total starch accumulation did not change.

This study provides a global picture of the effects of both the heat stress and the *Fusarium graminearum* infection (on susceptible and resistant genotypes) on the wheat seed proteome.

1.INTRODUCTION

1.1 The wheat

Wheat is one of the world's most popular crop. It is grown over a large area and under a wide range of conditions and, with about 620 million tons produced annually worldwide, it provides about one-fifth of the calories consumed by humans. It is to note that in 2007, the total world harvest was about 605 m tonnes compared with 652 m tonnes of rice and 785 m tonnes of maize (http://faostat.fao.org/). Additionally to the adaptability of the wheat to grow over a wide range of climatic conditions and soil fertility, there are other several advantages of the wheat consumption: it is easily transported and safely stored over long periods of time and it can be consumed by humans with minimal processing.

The wheat seeds (caryopsis or kernel) can be easily ground into flour (from bread wheat) or semolina (from durum wheat) which form the basic ingredients to prepare leavened and unleavened bread, noodles, cakes, biscuits and pasta. Wheat, especially the endosperm, also contributes carbohydrates, essential minerals, vitamins, beneficial phytochemicals, dietary fibres and proteins to the human diet.

The main components of the grain are the starch (about 80%) and the proteins, that are mostly responsible for the derived products quality.

The protein content of wheat grains can vary from 10 to 22% 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 (Schofield 1994). Consequently, the gluten proteins have been widely studied in order to determine their structures and properties and to provide a basis for manipulating and improving end use quality.

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 the wheat genomes

The first cultivation of wheat occurred about 10,000 years ago, as part of the 'Neolithic Revolution', which saw a transition from hunting and gathering of food to settled agriculture. These earliest cultivated forms were diploid (genome AA) (einkorn) and tetraploid (genome AABB) (emmer) wheats and their genetic relationships indicate that they originated from the South-Eastern part of Turkey (Heun *et al*., 1997; Nesbitt, 1998; Dubcovsky and Dvorak, 2007).

Taxonomically, wheat belongs to the genus *Triticum* of the family *Gramineae*; it includes several species forming a polyploid series, with a basic chromosome number (x) equal to 7, comprising diploid (2n=2x=14), tetraploid (2n=4x=28) and hexaploid (2n=6x=42) wheats. Today's commercial wheat, tetraploid and hexaploid wheats, contain two and three homeologous genomes, respectively. These genomes are named A, B, D and G, according to the donor species, each of which consists of seven pairs of chromosomes numbered 1 to 7. They are products of natural hybridisation of ancestral types, none of which nowadays is still of any commercial importance. In the hybridisation process, spontaneous crosses between wild species with different chromosomes have been followed by spontaneous doubling of chromosomes to originate a fertile allopolyploid. This hybridization probably occurred several times independently, with the novel hexaploid (genome AABBDD) being selected by farmers for its superior properties, included a wider adaptability.

The A genomes of tetraploid and hexaploid wheats are clearly related to the A genomes of wild and cultivated einkorn (*T. urartu* is the A genome donor), while the D genome of hexaploid wheat is derived from that of *T. tauschii*. In fact, the formation of hexaploid wheat occurred so recently that little divergence has occurred between the D genomes present in the hexaploid and diploid species. By contrast, the B genome of tetraploid and hexaploid wheats is probably derived from the S genome present in the Sitopsis section of *Aegilops*, with *Ae. speltoides* being the closest extant species. The S genome of *Ae. speltoides* is also close to the G genome of *T. timopheevi*, a tetraploid species with the A and G genomes (Feldman, 2001). A schematic representation of the origins of the different types of wheats is reported in Fig. 1.1.

The cultivated tetraploid wheats have evolved from two wild type groups, *T. dicoccoides* (AABB) and *T. araraticum* (AAGG) (Feldman, 2001), derived from diploid progenitors.

A second tetraploid species, *T. timopheevii*, as its wild progenitor *T. araraticum* , contains the G genome, which is closely related to the genome of *Ae. speltoides* (S genome). *T. timopheevii* is still grown to a limited extent in Armenia and Transcaucasia.

Among 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 and couscous in the Mediterranean Region, is getting more and more importance with the increase of pasta products demand.

The hexaploid wheats originated 6,000-7,000 years ago by natural hybridisation of tetraploid wheat, most likely *T. dicoccum* (AABB) with the diploid wild grass *Aegilops squarrosa* (DD), also known as *T. tauschii* (Miller, 1987). The resulting hexaploid species, *T. aestivum* (AABBDD), the common "bread wheat", is the dominant species in world agriculture. Most bread wheat is the free threshing var. *aestivum*, but other free threshing types (vars. *compactum* and *sphaerococcum*) are grown in restricted areas.

The hexaploid wheat species *T. zhukovskyi*, whose genomic composition is AAAAGG, could have been originated recently by interspecific hybridization of cultivated tetraploid *T. timopheevvii* (AAGG) with the cultivated diploid *T. monococcum* (AA).

Although bread wheat is the mostly widely cultivated all around the world, durum wheat is more adapted to the dry Mediterranean climate and is often called pasta wheat to reflect its major end-use. However, it may also be used to bake bread and is used to make regional foods, such as couscous and bulgur in North Africa.

Small amounts of other wheat species (einkorn, emmer, spelt) are still grown in some regions including Italy, Spain, Turkey, the Balkans, and the Indian subcontinent. In Italy, these hulled wheats are together called "farro", while spelt continues to be grown in Europe, particularly in Alpine areas.

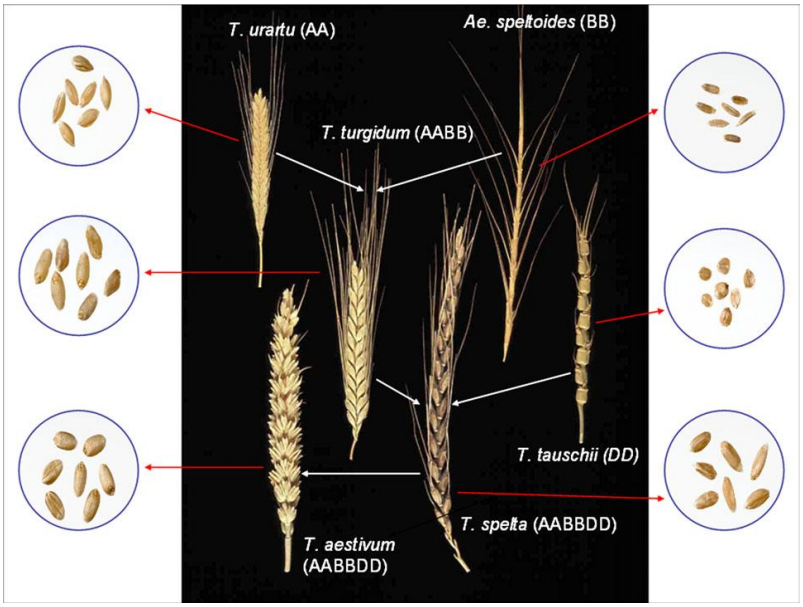


Fig 1.1. The evolutionary and genome relationships between cultivated bread and durum wheats and related wild diploid grasses, showing examples of spikes and grain (from Shewry, 2009).

1.3 The grain structure and composition.

The wheat grain is botanically a single-seeded fruit, called ‘‘caryopsis’’ or ‘‘kernel’’. 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 higher as it stands on its base), ~3.0-3.5 mm wide, ~6.0-7.0 mm in length, with an average weight of ~30-40 mg. It is constituted by three distinct parts: the bran, the starchy endosperm and the embryo or germ (Fig. 1.2). They account for 13-17%, 80-85% and 2-3% of the dry weight of the seed, respectively. A simplified representation of the wheat grain chemical composition is reported in the tab. 1.1 (Belderok, 2000).

1.3.1 The bran

The bran of the wheat grain is composed by a series of different cell layers. The ‘‘pericarp’’ (fruit coat) surrounds the entire seed and consists of two portions, the outer pericarp and the inner pericarp. The outer pericarp is composed by the epidermis (epicarp), the hypodermis, and by the innermost layer, called the remnants of thin-walled cells. The inner pericarp, adjacent to the remnants, is composed of intermediate cells (cross cells and tube cells). A further inner layer of cells is the seedcoat (also called “testa”) where the pigments confer the grain colors. 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 major compounds of the wheat mature grain are the endosperm and the embryo or germ. They derive from two different fecundation process. One of the two sperm cells, fuses with an egg cell give rise to the diploid zygote and consequentially to the scutellum and the embryonic axis. The other sperm cell fuses to two polar nuclei rises to one cell of the endosperm (triploid) that develops and differentiates in the starchy endosperm and in the aleurone.

The starchy endosperm is about 80% of the matter dry of the grain and contains about 70% of proteins f the seed. It is composed of about 80% of carbohydrate (starch), 12-17% of proteins and 1-2% of fats, and it is mainly acts as a source of nutrients for the accompanying embryo when it begins to germinate. The aleurone, the layer between endosperm and bran, is a maternal tissue that is retained as part of the seed in many small grains. The aleurone functions for both storage and digestion functions, indeed during the germination phase it secretes the amylase enzyme that breaks down endosperm starch into sugars to nourish the growing seedling.

The wheat embryo consists of two compounds: the embryonic axis, that represents the primordial root and shoot, and the scutellum. The latter, adjacent to the endosperm and contains oils and storage globulins, serves to absorb nutrients from the endosperm during germination.

Finally, the germ is rich in proteins, fats, carbohydrates and dietary fibres.

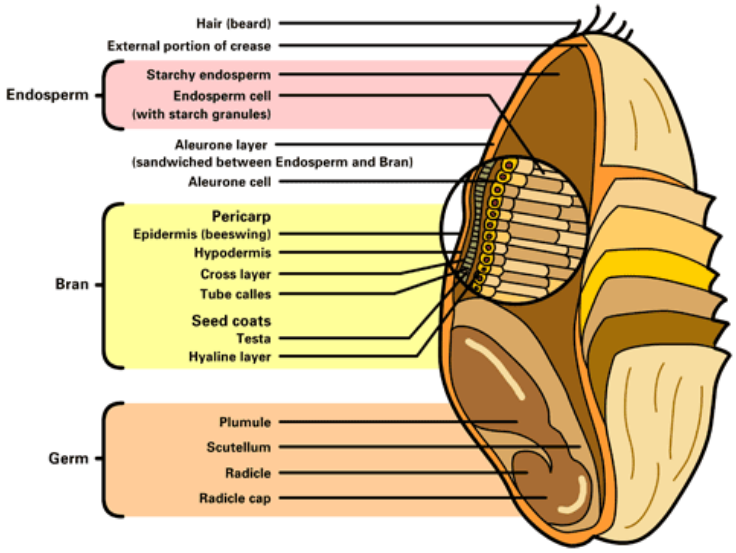


Fig. 1.2: Structure of the wheat grain.

	WHOLE GRAIN	ENDOSPERM	BRAN	GERM
Carbohydrate	16	13	16	22
Proteins	2	1.5	5	7
Fats	68	82	16	40
Dietary fibres	11	1.5	53	25
Minerals	1.8	0.5	7.2	4.5

Tab1.1 Chemical composition of the whole wheat grain with its various parts. Numbers are average percentages converted on a dry matter basis. Redrawn from Belderok (2000).

1.3.3 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 via α-1,6 linkages. Starch is generally described as containing two broad classes of molecules, amylose and amylopectin, that differ in degree of polymerisation and branch frequency. Amylopectin is a very large molecule with a degree of polymerisation from 10^5 to 10^7 and contains frequent branch points, on average approximately one branch for every 15–20 glucose units. Amylose has a lower degree of polymerisation (10^3 to 10^4) and contains from zero to a few branch points. These differences in amylose and amylopectin are functionally important and are reflected in the variety of applications these polymers find in the food and chemical industries. In bread and durum wheats, amylose content ranges from about 18 to 35%, although waxy wheats containing effectively zero amylose have now been produced.

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 2, and sometimes 3, size classes of granules, a feature shared with starches from other *Triticeae*, notably barley and rye. The larger "A" granules have a diameter from 15 to 30 μm whereas "B" granules, initiated at a different stage of development than "A: granules, have a diameter that is typically below 10 μm. A third "C" class of very small granules is seen under cooler environmental conditions allowing a long grain fill period. Additionally wheat starch granules contain a defined set of prominent proteins located within the interior of the starch granule, including Granule-Bound Starch Synthase, Starch Synthase I, Branching Enzymes IIa and IIb, and Starch Synthase IIa. 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).

1.3.4 The kernel proteins

Wheat grains contain relatively little protein compared to legume seeds, with an average of about 10–12% dry weight. Nevertheless, they provide over 200 million tones (mt) of protein for the nutrition of humans and livestock, which is about three times the amount derived from the more protein-rich (20–40%) legume seeds. In addition to their

nutritional importance, cereal seed proteins also influence the utilization of the grain in food processing. In particular, the unique property of wheat flour to form an extensible and elastic dough resides primarily in the storage proteins of its endosperm.

Beccari (1745) gave the name gluten to the water-insoluble protein fraction of the grain; two centuries later, Osborne (1924) developed a classification of the kernel proteins on the basis of their solubility in different solvents. He established that albumins were soluble in water, globulins in dilute saline solutions, prolamins in alcohol-water mixtures and glutelins, soluble in dilute acid or basic solutions. A schematic representation of the grain proteins is reported in fig. 1.3.

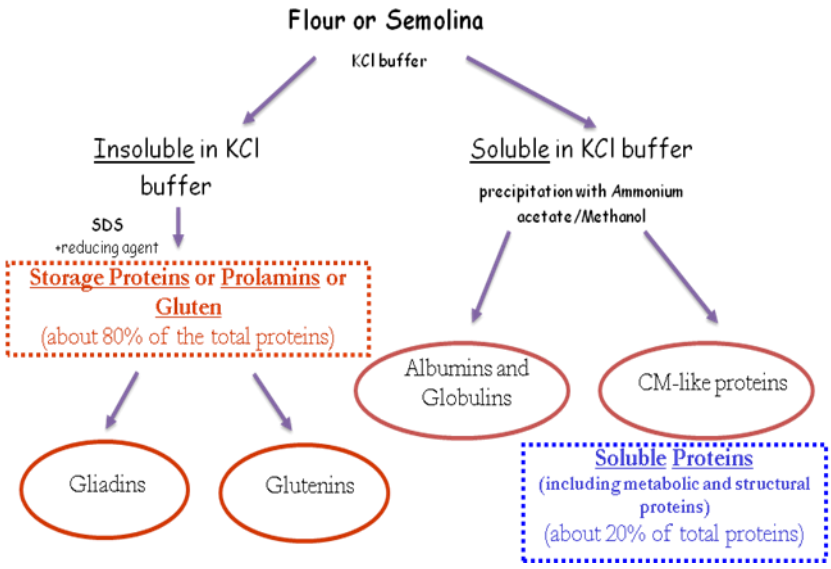


Fig.1.3 Schematic representation of the different wheat seed proteins divided in according to their solubility prprieties in a salt buffer (KCl buffer).

1.3.4.1 Gluten

Gluten proteins may amount to about 80% of the total proteins in the caryopsis. They are structurally and evolutionarily related and can all be defined as “prolamins” (for the high content of the amino acids proline and glutamine)

Gluten is classically divided into alcohol-soluble (gliadin) and insoluble (glutenin) fractions, which are further separated in different subgroups by electrophoresis.

The gliadins consist of monomeric proteins, which are separated into α, β, γ, and ω groups by polyacrylamide electrophoresis at low pH (Woychik *et al*., 1961), although actually α and β are structurally identical (Kasarda *et al* 1890, 1987).

The glutenins consist of polymeric proteins stabilized by inter- and intrachain disulfide bonds. These bonds need to be reduced before the component subunits are 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 (Payne and Corfield, 1979; Jackson *et al*., 1983).

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 additional 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).

As regards gliadins, the molecular weight range is ≈30,000 to 75,000 Da. Using one-dimensional electrophoresis, gliadins of a single wheat grain can be separated into 20–25 components (Bushuk and Zillman 1978; Autran *et al* 1979; Wrigley *et al* 1982; Metakovsky *et al* 1984). Two-dimensional electrophoresis allows better separation with a resolution of up to 50 components (Wrigley 1970; Payne *et al* 1982; Lafiandra and Kasarda 1985; Pogna *et al* 1990). Due to extensive polymorphism, these proteins have been widely used for cultivar identification in hexaploid and tetraploid wheats.

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

1.3.4.2 Metabolic proteins (albumin and globulin)

In classifications of storage proteins based on solubility, those that are soluble in water and in salt solutions are called albumins and globulins, respectively. Many water or salt-soluble wheat proteins are located in the embryo and aleurone layers; others are distributed throughout the endosperm. They may amount to about 20% of the total proteins in the caryopsis and they are considered as metabolic and structural proteins involved in different pathways and functions.

Albumins are usually more abundant 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 in sensitive individual trigger wheat allergies.

Payne and Rhodes (1982) noted that soluble proteins are complex mixtures containing: 1) metabolic enzymes that survived dehydration, 2) hydrolytic enzymes necessary for germination, 3) enzyme inhibitors. Also present are proteins related to legumins (the seed storage proteins of legumes), called “triticins”, which were extensively studied by Singh and Shepherd (1985). They account for about 5 % of the total seed proteins and are located in the protein bodies of the starchy endosperm.

Protocols developed to obtain protein fractions enriched in albumins and globulins (KCl-soluble) (see Fig. 1.4) have allowed the analysis of the largely unexplored albumins and globulins that function in metabolism and related biochemical processes (Vensel *et al.*, 2005). By using a classical proteomic approach based on 2DE, 254 proteins, grouped in 13 different biochemical processes of the endosperm, were identified in this fraction: ATP interconversion reactions, carbohydrate metabolism, cell division, cytoskeleton, lipid metabolism, nitrogen metabolism, protein synthesis/assembly, protein turnover, signal transduction, protein storage, stress/defense, transcription/translation, and transport (Vensel *et al.*, 2005). A complete list of the protein identifications by Vensel *et al* (2005) is reported in Tab 1.2.

Spot no.	Swiss-Prot no.	Protein	Spot volume		Method	E-value[a]
			10 dpa	36 dpa		
6	Q8S1A5	Carbamoyl phosphate synthetase	0.197	0.013	M[g]	9.60E-06
10	Q8L5C2	4SNc-Tudor protein, (NTPase)	0.207	0.047	QE[h]	1.90E-17
11	Q8L5C2	4SNc-Tudor protein, (NTPase)	0.465	0.029	M	2.00E-04
25	Q42669	Aconitase	0.349	0.099	F[i]	1.60E-03
26	Q9LZF6	Cell division cycle protein	0.724	<0.004[f]	M	7.10E-07
27	P49608	Aconitase	0.246	0.080	M	1.00E-05
31	O23927	Pyruvate Pi dikinase	2.029	0.539	F	1.90E-27
33	O23755	Elongation factor 2	0.225	0.15	QM[j]	2.50E-05
35	O98447	ClpC protease	0.190	0.013	F	1.10E-10
42	Q43638	Heat shock protein, 82K, precursor	0.171	<0.004	QM	4.70E-03
43	Q9LF88	Late embryogenesis abundant protein-like	0.022	0.051	QE	3.30E-04
48	Q8W0Q7	Methionine synthase	0.418	0.145	QE	5.10E-11
49	Q9XGF1	Heat shock protein 80–2	0.241	0.044	QM	2.50E-05
53	Q9M6E6	Poly(A)-binding protein	0.133	<0.004	QE	1.20E-04
55	Q9AT32	Poly(A)-binding protein	0.162	0.009	QM	9.40E-05
57	Q9M6E6	Poly(A)-binding protein	0.137	<0.004	QE	2.90E-07
60	P93616	Poly(A)-binding protein	0.145	0.015	QE	5.10E-14
61	P93616	Poly(A)-binding protein	0.076	<0.004	QE	2.70E-02
63	P93616	Poly(A)-binding protein	0.126	0.012	QE	1.10E-20
65	Q39641	Heat shock protein 70	0.360	0.079	M	2.30E-05
66	P93616	Poly(A)-binding protein	0.157	0.01	QE	8.70E-13
69	P93616	Poly(A)-binding protein	0.277	0.072	QE	1.70E-13
70	Q01899	Heat shock protein 70	0.317	0.052	QE	5.50E-31
72	Q9SPK5	10-Formyltetrahydrofolate synthetase	0.420	0.091	QE	1.40E-10
74	Q40058	DNAK-type molecular chaperone HSP70	1.973	0.568	QE	4.60E-56
77[b]	Q95I76	F23N19.10, putative stress-induced protein	0.204	0.056	QE	5.70E-10
77[b]	P93616	Poly(A)-binding protein	0.204	0.056	QE	2.70E-06
82	Q8L724	Stress-induced protein, sti1-like	0.250	0.050	QE	7.00E-22
93	Q9FME2	RNA-binding protein, similarity	0.152	0.018	QE	1.10E-14
94	Q9FME2	RNA-binding protein, similarity	0.237	<0.004	QM	2.20E-09
96	Q95NX2	Phosphoglucomutase	0.149	0.040	F	2.30E-18
97	Q944F5	Fructokinase	0.175	0.037	M	4.40E-03
98	O65305	Acetohydroxyacid synthase	0.220	0.016	M	2.60E-05
102	Q9ZR86	Protein disulfide isomerase-like protein	0.166	0.040	QE	6.80E-08
103	Q94DV7	Phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent	0.426	0.075	QE	2.60E-49
104	Q94DV7	Phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent	0.312	0.059	M	6.10E-10
110	Q8GWX8	2-Isopropylmalate synthase	0.249	0.108	F	4.20E-05
111	Q8W4M5	PPi-fructose-6-P 1-phosphotransferase	0.085	0.182	M	3.40E-09
112	Q41141	PPi-fructose 6-P 1-phosphotransferase beta subunit	0.565	0.125	F	1.30E-09
117	Q9LWT6	Chaperonin 60 kDa beta subunit	0.532	0.079	M	7.60E-04
118	P52589	Protein disulfide isomerase	8.808	3.348	M	2.90E-14
119	O49485	Phosphoglycerate dehydrogenase-like protein	0.286	<0.004	QE	3.30E-12
121	O82783	Importin α-2 subunit	0.703	1.974	F	2.90E-28
125	P16098	β-Amylase	0.009	0.641	F	1.80E-14
128	P12299	ADP-glucose PPase, LS	0.245	0.049	M	1.50E-05
130	P12299	ADP-glucose PPase, LS	0.216	0.058	F	1.60E-31
132	P12299	ADP-glucose PPase, LS	0.661	0.159	M	1.20E-11
135	P30184	Leucine amino peptidase	0.592	0.157	QE	7.50E-33
137	P30184	Leucine amino peptidase	<0.004	0.185	QE	9.20E-13
138	Q8RZF3	Ketol-reductoisomerase	0.599	0.446	M	9.50E-09
141	Q9M7E0	Elongation factor 1-α	0.170	0.295	F	1.50E-07
145	Q8RZW7	Selenium binding protein	0.286	0.034	QE	2.00E-30
147	Q8GU01	Globulin-2	0.019	0.286	QE	1.20E-25

Spot no.	Swiss-Prot no.	Protein	Spot volume		Method	E-value[a]
			10 dpa	36 dpa		
148	P55307	Catalase isozyme 1	0.260	0.116	M	1.60E-05
149	Q93YR3	Heat shock associated protein	0.161	0.142	QE	5.00E-22
151	Q8RZW7	Selenium binding protein	0.204	0.165	QE	1.90E-05
153	Q8LST6	Aldehyde dehydrogenase	0.246	0.050	QE	1.10E-41
154	Q9FPK6	Aldehyde dehydrogenase	0.158	0.189	QE	4.20E-16
155	Q8GU01	Globulin-2	<0.004	0.392	M	4.80E-07
156[b]	Q9M4Z1	ADP-glucose PPase, SS	<0.004	0.224	QE	2.10E-21
156[b]	O49218	Methylmalonate-semialdehyde dehydrogenase	<0.004	0.224	QE	7.30E-08
157	Q43772	UDP-glucose PPase	0.412	0.138	M	1.10E-08
158	Q43772	UDP-glucose PPase	0.608	0.216	QE	4.70E-53
159	Q9M4Z1	ADP glucose PPase, SS	0.343	0.145	M	6.10E-05
161	Q9ASP4	Dihydrolipoamide dehydrogenase	0.178	0.111	QE	2.30E-55
163	Q93YR3	Heat shock associated protein	0.132	0.190	QE	1.60E-23
165	Q8W3W6	LMW glutenin subunit group 3 type II	0.315	0.420	QM	9.60E-06
166	Q8GU18	LMW glutenin subunit	0.659	1.365	QE	1.10E-10
168	Q9ASP4	Dihydrolipoamide dehydrogenase	nd[e]	0.314	QE	7.30E-18
171	Q8GU18	LMW glutenin subunit group 3 type II	0.101	0.302	QE	7.20E-04
173	P52894	Alanine amino transferase 2	0.351	0.093	M	2.70E-06
175	Q8GU01	Globulin-2	0.038	0.201	QE	7.50E-04
176	P52894	Alanine amino transferase 2	1.533	0.694	F	2.20E-16
178	Q42971	Enolase	0.230	0.123	M	7.60E-08
179	Q42971	Enolase	0.877	0.322	F	1.80E-33
182	Q9ZRR5	Tubulin α-3 chain	0.369	0.032	M	1.90E-08
184	Q9ZRB0	Tubulin β-3 chain	0.467	0.008	M	2.30E-06
185	Q42971	Enolase	2.053	0.917	F	3.60E-08
193	O81237	6-Phosphogluconate dehydrogenase	0.325	nd[e]	QE	3.00E-02
195[b]	Q8W3N9	26S Proteasome regulatory particle triple-A ATPase subunit 3	0.287	0.027	QE	7.80E-12
195[b]	Q8VZ47	Argininosuccinate synthase-like protein	0.287	0.027	QE	2.70E-13
195[b]	Q9AUV6	UDP-glucose dehydrogenase	0.287	0.027	QE	6.70E-19
199	Q08837	Triticin	0.089	0.209	M	4.60E-04
201	Q8W516	SGT1	0.265	0.078	M	2.70E-06
203	Q8W3W4	LMW glutenin subunit group 4 type II	0.186	0.675	QE	4.30E-02
205	Q9FXT8	26S proteasome regulatory particle triple-A ATPase subunit 4	0.085	0.251	QE	4.00E-27
206	P41378	Eukaryotic initiation factor 4A	0.301	0.129	M	2.00E-05
212	Q40058	DNAK-type molecular chaperone HSP70	<0.004	0.567	QE	6.00E-21
220[b]	Q9FXT8	26S Proteasome regulatory particle triple-A ATPase subunit 4	0.351	0.369	QE	7.60E-04
220[b]	P37833	Aspartate amino transferase	0.351	0.369	QE	1.70E-49
225	Q9SAU8	Heat shock protein 70	0.042	0.318	QE	6.90E-20
228[c]	Q9FXT8	26S Proteasome regulatory particle triple-A ATPase subunit 4b (10 dpa)	0.182	0.280	QE	4.20E-08
228[c]	Q9XGU8	Isocitrate dehydrogenase (NAD) (36 dpa)	0.182	0.280	QE	4.30E-26
229	Q8GU01	Globulin-2	0.068	0.196	M	1.60E-04
230[b]	Q9ZRI8	Formate dehydrogenase	1.059	0.148	M	8.40E-07
230[b]	P37833	Aspartate amino transferase	1.059	0.148	QE	2.10E-31
231	P93693	Serpin WZS2	0.490	1.960	M	3.00E-08
232	P37833	Aspartate amino transferase	0.197	0.166	QE	7.70E-28
233	Q9ST58	Serpin	<0.004	0.366	QE	1.50E-04
234	Q43492	Serpin homolog WZS3	0.085	0.022	QE	2.90E-05
236	P37833	Aspartate amino transferase	0.222	0.682	QE	9.70E-48
237	P93692	Serpin homolog WZS3	0.111	0.826	QE	1.20E-40
238	P04727	α/β-Gliadin clone PW8142	0.129	0.126	QE	5.40E-04
240[b]	Q9FUS4	Actin	0.430	0.283	F	1.50E-35

Spot no.	Swiss-Prot no.	Protein	Spot volume		Method	E-value[a]
			10 dpa	36 dpa		
240[b]	P93692	Serpin homolog WZS3	0.430	0.283	QE	9.70E-10
244	Q40676	Aldolase	0.570	0.259	QE	2.30E-36
245	P93692	Serpin homolog WZS3	<0.004	0.632	QE	5.90E-25
246	Q41593	Serpin	0.157	1.115	M	7.50E-06
247[c]	Q9ZR33	Reversibly glycosylated polypeptide (10 dpa)	0.671	0.148	M	1.30E-07
247[c]	P93692	Serpin homolog WZS3 (36 dpa)	0.671	0.148	QM	7.30E-05
249	Q9ZRI8	Formate dehydrogenase	1.159	0.472	QE	1.20E-13
250	Q9ZR33	Reversibly glycosylated polypeptide	0.162	0.168	M	6.90E-08
251	Q9ZRI8	Formate dehydrogenase	0.379	0.105	QM	2.90E-05
252	Q40676	Aldolase	1.418	0.293	QE	1.30E-19
255	Q8LK23	Peroxidase 1	0.091	1.330	M	1.80E-07
256[b]	O81221	Actin	0.354	0.045	F	1.20E-21
256[b]	Q41319	Acyl-acyl-carrier protein desaturase	0.354	0.045	F	9.80E-04
256[b]	Q93Y71	Protein disulfide-isomerase precursor	0.354	0.045	QE	1.10E-09
257	Q40676	Aldolase	<0.004	0.572	QE	9.40E-21
259	Q8LK23	Peroxidase 1	0.110	1.494	QM	1.30E-09
262	Q9M4V4	Glyceraldehyde-3-P dehydrogenase (NAD)	1.816	1.907	QE	8.10E-43
263	Q40069	Peroxidase BP1	0.200	2.242	F	2.20E-16
264	P25861	Glyceraldehyde 3-P dehydrogenase (NAD)	4.518	3.130	QM	1.50E-09
265[b]	Q8LK23	Peroxidase 1	0.045	0.605	QE	1.40E-09
265[b]	Q9ST58	Serpin	0.045	0.605	QE	5.10E-07
272	Q94CS6	Legumin-like protein	0.025	0.247	QE	4.90E-13
273[c]	Q942N5	Auxin-induced protein (10 dpa)	0.275	2.569	QE	8.60E-15
273[c]	Q9M4V4	Glyceraldehyde-3-P dehydrogenase (NAD) (36 dpa)	0.275	2.569	QE	1.10E-10
274	Q9M4V4	Glyceraldehyde-3-P dehydrogenase (NAD)	0.421	0.490	QE	1.10E-33
275	P26517	Glyceraldehyde-3-P dehydrogenase (NAD)	0.023	0.282	F	2.80E-03
278	Q94KS2	TGF-β receptor-interacting protein 1	0.233	0.213	QE	5.60E-25
279	Q9XGC6	Adenosine kinase	0.291	0.082	F	1.20E-22
281	Q03678	Globulin Beg 1	0.027	0.240	QM	3.40E-03
282	Q9FRV1	Chitinase-a	0.078	0.199	QM	2.40E-12
283	Q94KS2	TGF-β receptor-interacting protein 1	0.064	0.078	QE	7.60E-06
284[b]	P41095	60S Acidic ribosomal protein P0	0.209	0.055	QE	7.40E-09
284[b]	Q94CS6	Legumin-like protein	0.209	0.055	QE	3.40E-08
285	Q94CS6	Legumin-like protein	0.175	0.144	M	7.00E-06
289[b]	Q9C774	26S Proteasome regulatory subunit S12	0.617	0.535	F	1.50E-05
289[b]	Q09114	Avenin N9	0.617	0.535	QE	3.20E-14
294	Q9FT00	Malate dehydrogenase (NAD)	2.266	1.348	QM	4.50E-17
295	Q94JA2	Malate dehydrogenase (NAD)	0.587	0.223	QE	2.80E-12
301	P49027	Guanine nucleotide-binding protein β subunit-like protein	0.807	0.174	QE	7.10E-27
303[b]	Q945R5	Ascorbate peroxidase	0.293	<0.004	M	6.90E-06
303[b]	Q42988	PPi-fructose-6-P 1-phosphotransferase	0.293	<0.004	F	6.90E-05
305	Q9ZWJ2	Glyoxalase I	0.790	0.769	M	5.30E-07
306	Q40676	Aldolase	0.299	0.209	QE	5.30E-14
308	Q07810	Tritin	0.165	0.773	QE	1.60E-08
310	T06212[d]	Glucose and ribitol dehydrogenase	nd[e]	0.226	QE	8.80E-05
311	Q9C5Y9	Initiation factor 3g	0.367	0.205	QE	4.70E-04
312	Q8W5L9	Purple acid phosphatase	0.068	0.202	QE	1.20E-05
314	Q7X653	OSJNBb0118P14.5	<0.004	0.165	QE	9.10E-08
318	Q8L5C6	Xylanase inhibitor protein I	nd[e]	0.880	QE	1.60E-24
324	Q7X653	OSJNBb0118P14.5	0.035	0.084	QE	3.30E-08
325	Q8L5C6	Xylanase inhibitor protein I	0.133	0.587	QE	2.30E-11
327	Q8LKV8	Seed globulin	0.085	0.482	QE	1.70E-03
330	P29305	14–3-3 Protein homolog	0.301	0.047	QE	1.20E-14
335	Q8LKV8	Seed globulin	0.177	0.565	QM	2.10E-26
336	Q8LKV8	Seed globulin	0.083	0.466	M	1.90E-03

Spot no.	Swiss-Prot no.	Protein	Spot volume		Method	E-value[a]
			10 dpa	36 dpa		
432[b]	Q43723	Trypsin inhibitor CMx precursor (clones pCMx1 and pCMx3)	0.177	0.601	QE	6.90E-08
433	Q41518	Glycine-rich RNA-binding protein	0.191	nd[e]	QE	3.00E-06
434	P01084	α-Amylase inhibitor 0.53	0.050	0.138	QE	3.10E-28
436	P01084	α-Amylase inhibitor 0.53	<0.004	0.191	M	6.80E-06
440	O49956	α-Amylase inhibitor Ima 1, monomeric	nd[e]	0.224	QE	1.40E-10
441	Q09114	Avenin N9	<0.004	0.202	QE	1.10E-03
444	P35687	40S Ribosomal protein S21	0.230	0.036	QE	6.00E-06
447	Q40641	Polyubiquitin 6	0.406	<0.004	M	2.90E-04
471	Q40058	DNAK-type molecular chaperone HSP70	0.718	<0.004	F	1.80E-34
493	Q944R8	UDP-glucose dehydrogenase	0.296	0.035	F	1.00E-15
529	P40978	40S Ribosomal protein S19	0.696	nd[e]	QE	6.50E-12
530	Q93W25	Cyclophilin A-1	0.497	nd[e]	M	3.90E-03
550	Q43223	Sucrose synthase type 2	<0.004	0.047	QE	8.20E-42
551	Q9LF88	Late embryogenesis abundant protein-like	nd[e]	0.280	QE	1.90E-07
557	Q8S7U3	Embryo-specific protein	nd[e]	0.099	QE	5.40E-10
559	Q8GU01	Globulin-2	nd[e]	0.440	QE	1.10E-31
561	Q8GU01	Globulin-2	nd[e]	0.148	QE	5.20E-16
577	Q41551	LMW glutenin (fragment)	nd[e]	0.125	QE	1.90E-03
579	P26517	Glyceraldehyde 3-P dehydrogenase (NAD)	nd[e]	0.302	F	9.70E-13
582	P26517	Glyceraldehyde 3-P dehydrogenase (NAD)	nd[e]	0.646	QM	1.90E-05
583[b]	Q8LK23	Peroxidase 1	<0.004	0.112	QE	1.70E-03
583[b]	Q947H4	Plasmodesmal receptor	<0.004	0.112	QE	6.20E-05
591	T06212[d]	Glucose and ribitol dehydrogenase	nd[e]	0.176	QE	1.90E-18
620	Q03678	Globulin Beg 1	nd[e]	0.803	QE	5.00E-03
631	Q8L8I0	Globulin-like protein	nd[e]	0.199	QM	3.70E-05
633	P24296	Nonspecific lipid-transfer protein precursor	nd[e]	0.182	QM	6.10E-04
639	Q9ST58	Serpin	nd[e]	0.927	M	9.00E-05
640	P12783	Phosphoglycerate kinase	0.445	<0.004	F	2.30E-46
655	P93438	*S*-Adenosylmethionine synthetase 2	0.282	nd[e]	M	5.60E-04
656	Q9FXT8	26S Proteasome regulatory A subunit	0.136	<0.004	QE	5.00E-04
657	P50299	*S*-Adenosylmethionine synthetase 1	0.219	<0.004	QE	5.60E-19
666	Q945R5	Ascorbate peroxidase	0.405	<0.004	M	2.90E-05
667	Q945R5	Ascorbate peroxidase	0.178	nd[e]	QE	5.50E-12
672	Q9ZR33	Reversibly glycosylated polypeptide	0.339	nd[e]	M	9.10E-08
842	Q9LF88	Late embryogenesis abundant protein-like	nd[e]	0.262	QE	5.00E-05
851	Q8GU01	Globulin-2	nd[e]	0.360	QM	8.80E-04
852	Q8GU01	Globulin-2	nd[e]	0.163	QM	2.60E-03
860	P26517	Glyceraldehyde 3-P dehydrogenase (NAD)	<0.004	0.353	QE	4.00E-10
861	Q43247	Glyceraldehyde 3-P dehydrogenase (NAD)	<0.004	0.582	QM	2.30E-09
869	Q9FF52	60S Ribosomal protein L12	nd[e]	1.659	QE	1.30E-14
871	P28814	Barwin	nd[e]	0.274	QE	1.80E-02

Tab. 1.2. Proteins of wheat endosperm identified by MS.(Vensel *et al* 2005)

1.4 The abiotic and biotic stresses in plants

Abiotic stress is defined as all the external (environmental) factors which can cause harmful effects to the plants, such as soil conditions, drought or extreme temperature,

whereas biotic stresses include such living disturbances as fungi or microorganisms, or harmful insects.

Both of them cause important crop damages and physiological modification in the plant metabolism.

Generally plants undergo continuous exposure to various biotic and abiotic stresses in their natural environment. To survive under such conditions, plants have evolved intricate mechanisms to perceive external signals, allowing optimal response to environmental conditions. Phytohormones such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA) are endogenous, low-molecular-weight molecules that primarily regulate the protective responses of plants against both biotic and abiotic stresses via synergistic and antagonistic actions, which are referred to as signaling crosstalk (Fujita *et al.*, 2006).

Moreover, the generation of Reactive Oxygen Species (ROS) has been proposed as a key process that is shared between biotic and abiotic stress responses (Apel *et al.*, 2004, Torres *et al.*, 2005). Rapidly accumulating data, resulting from large-scale transcriptome analyses with DNA microarray technology, strongly support the existence of such crosstalk between signaling networks in *Arabidopsis* (Cheong *et al*., 2002).

Biotic and abiotic stresses regulate the expression of different but overlapping sets of genes. For example, the correlation of transcriptional regulation with environmental challenges, such as heavy metal (CuSO4) stress, and with incompatible necrotrophic pathogen infection reveals significant overlap between responses to biotic and abiotic stresses. These data suggest that ROS are a common signal that trigger downstream stress responses (Narusaka *et al.*, 2004).

Moreover there are evidences that some environmental stresses, such as freezing, salt and water stress, result in cellular dehydration, leading to similar changes in plant gene expression and metabolism. This means that cross-talk exists in their signalling pathways, although it is still not clear yet if similarly over- or under expressed proteins are also detectable (Knigh *et al.*, 2001).

It is not always possible to correlate the data obtained by transcriptome analysis with those of proteomics, because mRNA is not completely translated into protein. Moreover, post translational modification are not detectable by transcriptome analysis, making proteomics an important complementary tool for functional genomics studies.

For these reasons, proteomics has been widely used to investigate the effects of the abiotic and biotic stresses in the latter 10 years. For example, in 2006, several papers reporting proteomic studies on plant responses to symbionts (4), biotic (10) and abiotic stresses (17), including responses to stress-related hormones (8) have been published. Most of the studies have been carried out on *Arabidopsis*, rice, *Medicago truncatula* and wheat, studying the stress effects on different tissues, such as leaf, roof, seed, calli (reviewed in Jorrin *et al*., 2007) .

1.4.1 Heat Stress on the wheat seed proteins accumulation

The potential for heat-shock conditions to change the processing quality of the resulting grain is particularly important, as there are increasing demands to supply markets with a uniform product. This is the main reason because of the interest of many researchers is focused on the study of this phenomena, especially on *Triticum aestivum* and on the compounds responsible for the quality traits.

Generally, high temperatures (they are variable from 25 to 40°C depending on the wheat genotype) affect all stages of grain development, shortening the duration of water uptake and kernel expansion, dry weight accumulation and kernel desiccation. Apoptosis occurs earlier under high temperature regimens and coincides with physiological maturity (Altenbach *et al*. 2003).

Moreover, other studies have established that the heat stress causes a deviation of the ratio between the different compounds of the gluten: high temperatures have been shown to be favorable to synthesis of gliadins, which remains stable or increases, whereas glutenin synthesis decreases. DuPont *et al* (2006), have found that, consistently with the occurring of apoptosis, transcripts for α/β-, γ-, and ω-gliadins, high molecular weight glutenin subunits (HMW-GS), and low molecular weight glutenin subunits (LMW-GS) accumulate and disappear earlier under heat stress conditions. The relative amounts of certain α-gliadins and HMW-GS were higher and those for certain LMW-GS were lower in grain produced under a 37 °C/28 °C day/night regimen. In addition, accumulation rates increased more for the α-gliadins and HMW-GS than the LMW-GS in grain produced under this regimen.

High temperature during grain filling also affects the accumulation levels of stress/defense proteins. Skylas *et al*. (2002) reported that a shift from a 24°C/18°C to a

40°C/25°C day/night regimen from 15–17 DPA, increased the number of small heat shock protein (HSP) isoforms in total protein extracts from endosperm.

The decrease in the ratio of glutenins to gliadins in conditions of heat stress results in weakening of dough (Gibson *et al*., 1998), despite the concomitant increase in protein content in the grain (Blumenthal *et al.,* 1998). In contrast to the protein content increase, other studies in wheat and barley grains have shown that the accumulation of starch under heat stress decreased, due to the lower activity of the enzymes involved in the starch pathway (Wallwork *et al.,* 1998).

Another effect of the heat shock, especially during the grain filling, associated to the grain quality is the size of the Glutenin MacroPolymer (GMP) (Don *et al*. 2003). A high GMP content indicates a high quality of the flour and reflects not only the amount of glutenin protein, but also the ability to form large aggregated networks. The amount of GMP increased significantly after an heat shock treatment, thus opening a controversy of opinions on the heat effects on the grain traits of the flours, that it still open.

The gliadins up-regulation has been explained by Blumenthal *et al.* (1990) with the presence of heat stress elements (HSE) in the upstream regions of some gliadin genes.

In 2004 Majoul and coworkers, using a proteomic approach based on 2DE and mass-spectrometry, found out that in *T. aestivum* a thermal regime of 37°C lead to the down-regulation of some enzymes involved in the starch synthase pathway, such as glucose-1-phosphate adenyltransferase and the Granule Bound Starch Synthase, thus giving an explanation to the shifting in the starch accumulation of the stressed individuals. The same group investigated about the heat stress effect on hexaploid wheat in both the total endosperm and the non-prolamin protein fraction. They reported several protein families involved in the heat stress response. They mainly belong to Heat Shock Protein (HSP), enzymes involved in the starch pathway, energy related protein such as the ATP β Synthase subunit, some gliadins, and antioxidant enzymes (ascorbate, peroxidase).

Very recent investigations (Hurkman *et al*., 2009) on the albumins and globulins protein (KCl-soluble) compound have revealed that many other proteins are involved in the heat stress response in the hexaploid wheat (cv Butte) during different developmental phases. Under the regimen initiated to the early stage of the grain filling to the maturation seed, HSP16.9, HSP70, peroxidase, triosephosphate isomerase, and globulins (legumin-like protein, seed globulin), increased transiently. A striking feature

of the high temperature regimens is the increase in the levels of stress/defense and globulin storage proteins in the endosperm of the mature grain. The stress/defense proteins (i.e serpins) include those known to respond to desiccation (glyceraldehyde-3-Pdehydrogenase, LEA protein) and pathogen related protein, PR, (chitinase, xylanase inhibitor protein, α-amylase/subtilisin inhibitor, barwin/ PR-4 protein, LipidTransferProteins, polyubiquitin, 20S proteosome α-subunit, nucleoside diphosphate kinase, and globulin-2). Some of these proteins, notably α-amylase inhibitor, glyceraldehyde-3-P dehydrogenase (GADPH), triosephosphate dehydrogenase, and serpin are known as allergens (Tatham and Shewry, 2008). A recent investigation has established that the Glubulin-2, a member of the cupin superfamily of proteins with similarity to known food allergens, resulted up-regulated from high temperature, reinforcing the hypothesis that there is a connection between the heat stress and the accumulation of some classes of wheat allergens (Althenbach *et al*. 2009).

Moreover the results from heat stress-responsive transcriptome analysis in heat susceptible and tolerant wheats confirmed that also the heat responsive genes belong to a large number of important factors and biological pathways, including HSPs, transcription factors, phytohormone biosynthesis/signaling, calcium and sugar signal pathways, RNA metabolism, ribosomal proteins, primary and secondary metabolisms, and biotic and abiotic stresses (Qin *et al*. 2008).

1.4.2 Cold stress and acclimation response in wheat

Exposure of plants to cold stress, which includes chilling (<20°C) and freezing temperature (<0°C), adversely affects their growth and development and significantly constraints the spatial distribution of plants and agriculture productivity.

Cold stress prevents the expression of full genetic potential of plants owing to its direct inhibition of metabolic reactions and, through chilling induced inhibition of water uptake and cellular dehydration, oxidative and other stresses. Many plants increase freezing tolerance acting a process named cold acclimation, which is associated with biochemical and physiological changes, such the modification of the lipid composition, and the accumulation of some cryprotectants (proline, sucrose, and some simple sugars).(Steponkus *et al*., 1993, Carpenter and Crowe, 1988).

Winter habit plants (winter wheat, barley, oats, etc) have naturally a vernalization requirement, which prevents premature transition to the reproductive phase before the threat of freezing stress during the winter has passed, but on the other hand many important crops such as rice, maize, soybean cotton and tomato are chilling sensitive and they are not able to tolerate ice formation in their tissues.

The molecular basis of the acquired chilling and freezing tolerance are not understood in all the plants, but a large number of studies have substantially contributed towards the understanding of this process in *Arabidopsis* using transgenic and mutant individuals. The cold regulated genes (about 1000 genes) in this species have been estimated to constitute from 4 to 20% of the entire genome, some of which are transient or up-regulated and others are constitutively expressed or down-regulated. Among all those genes, CBF (C-repeat binding factor, also known as dehydration-responsive element binding protein) family plays a very import role in the cold acclimation, as well as transcription factor known as ICE1(Inducer of CBF Expression). They act coordinately in a very complex transcriptional cascade network (ICE1-CBF) with different transcriptional factors involved in the positive, negative and indirect regulation of the CBF regulon during the cold acclimation. In addition to their direct induction of CBF expression, ICE1 appears to regulate negatively a CBF negative upstream transcriptional factor (MYB15), reinforcing an indirect and positive role of ICE1 for the up-regulation of CBF.

Recently, post transcriptional regulatory mechanism, such as pre-mRNA splicing, mRNA export and mRNA degradation by small RNAs have been found implicated, especially in the gene down-regulation, in the cold response in *Arabidopsis*. Moreover, because the 86% of the cold-induced genes are not shared between roots and leaves, the cold-regulated transcriptional networks might also differ in different tissues.

Because wheat cultivars have a remarkable range of freezing tolerance, they are an excellent model to study this trait. One of the first studies to identify the durum wheat genes involved in the response to low temperature has been carried out from Gulick and coworkers (2005). By using microarray analysis, they compared two different bread wheat cultivars, and identified more than 300 genes differentially regulated by cold, 65 of them differentially expressed between the two cultivars at a specific time point. These genes encode potential regulatory proteins and proteins that act in plant metabolism,

including protein kinases, putative transcription factors, Ca^{2+} binding proteins, a Golgi localized protein, an inorganic pyrophosphatase, a cell wall associated hydrolase, and proteins involved in photosynthesis.

Compared with the transcriptome, analysis of the protein complement of plant tissues in response to cold stress were limited, but recently a relevant number of studies has been published. Indeed the analysis of the cellular proteome complement is required in order to understand the cellular processes operating in response to environmental and cold stresses. Different families of proteins are known to be associated with a plant's response to cold stress by being newly synthesized, accumulating or decreasing. These proteins are involved in signalling, translation, host-defense mechanisms, carbohydrate metabolism and amino acid metabolism. These proteins include antifreeze proteins (AFPs) (Griffith and Yaish 2004), heat shock proteins, (e.g. heat-shock protein 70 (HSP70); Sung *et al*. 2001, Wisniewski *et al*. 1996) dehydrins and other late-embryogenesis-abundant (LEA) proteins (Close 1997, NDong *et al*. 2002, Welling *et al*. 2004, Wisniewski *et al*. 1999), other cold-regulated (COR) proteins (Jaglo-Ottosen *et al*. 1998), PR proteins, H_2O_2 mediated proteins involved in the gene regulation in response to the low temperature and enzymes involved metabolic pathways.

AFPs have been shown to accumulate primarily in the apoplast under different stress such as cold, dehydration and short-daylength in both cereals and carrots. It has been demonstrated that the AFPs are involved in the lowering of the freezing temperature in cold-acclimated leaves and that after the leaves have frozen, they inhibit the recrystallization of intercellular ice (Griffith and Yaish 2004).

Another protein family involved in the cold response and acclimation are the LEA and dehydrins (a subclass of LEA proteins), that stabilize cell membranes and protect other proteins from denaturation when cellular water content is reduced during dehydration. They are reported to be involved in different stresses and in different plant species (Tunnaclife and Wise, 2007). Also involved in different stress responses are a group of evolutionarily conserved polypeptides known as HSPs. Their accumulation under several environmental stressor (heat, drought, salinity, low temperature) is not surprising because, integrated with the chaperonins, they are involved in several processes such as translation, translocation into organelles, refolding of stress-denatured proteins, prevention of aggregation of denatured proteins and membrane protection.

Although PR proteins are mainly expressed in plants in response to pathogen infection, environmental stress, chemical compounds and wounding, several of them such as β-1,3-glucanases, chitinases, taumatin-like proteins, Lipid Transfer Proteins (LTP) are responsive to low temperature in bittersweet nightshade, carrots and winter rye (Griffith and Yaish 2004).

Also the accumulation and the activity of some enzymes involved in several different metabolic pathways have been shown to occur in response to low temperature in wheat and other species (Guy 1990, Hurry *et al.* 1995). They are involved in the carbohydrate metabolism (2,3-bisphosphoglycerate, GADPH, sucrose synthase and enolase), in photosynthesis (e.g. RuBisCo subunits, RuBisCo activase and polypeptides of the photosystem II oxygen-evolving complex), in detoxifying enzymes (e.g. ascorbate peroxidase and superoxide dismutase), in proline metabolism (e.g. glutamine synthetase and proline dehydrogenase) and in lignin metabolism (e.g. caffeic acid 3-O-methyltransferase).

1.4.3 Biotic stress on wheat.

Another serious and affecting damage in term of yield and quality losses that occurs on the cereals is represented by the biotic stress (pathogens such as bacteria, fungi, virus, pests and weeds).

The worldwide crop losses to wheat productivity owing to pathogens were estimated to be 12.4%, but as high as 16.7% without chemical treatments or cultural practices (Oerke *et al*. 1994). A large number of fungal (such as rust caused by *Puccinia* spp., smut and bunt caused by *Tilletia* and *Ustilago* spp., blotch caused by *Septoria* spp., *Fusarium* blight/scab, *Helminthosporium* leaf blight, powdery mildew caused by *Blumeria graminis*, etc.), bacterial (such as leaf streak caused by *Xanthomonas translucens*) and more than 50 viral diseases are known to cause considerable worldwide damage to wheat production (Curtis *et al*. 2002).

Pests (aphids, Hessian fly, locusts, beetles, moths, etc.) cause additional losses during post-harvest storage and the worldwide crop losses to wheat productivity are estimated to be 9.3%. An increase rate of losses due to the weeds has been estimated in the wheat productivity (from12.3% to 23.9 without crop protection with herbicides) (Oerke *et al*. 1994).

1.4.3.1 Fusarium graminearum: life cycle and damages on crop.

The fungal pathogen *Fusarium graminearum* Schwabe [teleomorph *Gibberella zeae* (Schweinitz) Petch], is the most common causal agent of *Fusarium* head blight (FHB), mainly known as scab, in North America and many other parts of the world. The disease is caused by five major species, *Fusarium avenaceum* (teleomorph, *Gibberella avenacea*), *F. culmorum*, *F. graminearum* (teleomorph, *G. zeae*), *F. poae* and *Microdochium nivale* (teleomorph, *Monographella nivalis*) (Parry *et al*., 1995). FHB has been identified by CIMMYT as a major factor limiting wheat production in many parts of the world (Stack *et al*. 1999). The disease symptoms appear as slightly brown water-soaked spots present on the glumes and for wheat they evolve in brown, dark purple to black necrotic lesions form on the exterior surface of the florets and glume (Fig. 1.5). Although these lesion symptoms sometimes are referred to as scab, they are not formally related to the hyperplasia and hypertrophic epidermal growth associated with other scab diseases, such as apple scab. Peduncles immediately below the inflorescence may become discoloured brown/purple. With time, tissue of the inflorescence often becomes blighted, appearing bleached and tan, while the grain becomes atrophic. Awns often become deformed, twisted and curved downward.

The life cycle of the fungus starts as saprophytic micelya overwinter. In spring, warm moist weather conditions are favorable for the development and maturation of conidia and perithecia that produce ascospores concurrently with the flowering of cereal crops, and the deposition of the ascospores on or inside spike tissue initiates the infection process. The fungus initially does not penetrate directly through the epidermis. Rather, hyphae develop on the exterior surfaces of florets and glumes, allowing the fungus to grow toward stomata and other susceptible sites within the inflorescence (Bushnell *et al*., 2003). Hyphae may also form peculiar lobed structures between cuticle and epidermal cell wall on the surface of inoculated glumes (Pritsch *et al*., 2000). Such subcuticular growth on the glume, lemma and palea is thought to serve as a mechanism for fungal spread and, in the case of adaxial floret surfaces, probably leads to direct penetration of epidermal cells (Bushnell *et al*., 2003). Other avenues for direct entry include stomata and underlying parenchyma, partially or fully exposed anthers, openings between the lemma and palea of the spikelet /floret during dehiscence (Bushnell *et al*., 2003; Lewandowski and Bushnell, 2001) and through the base of the

wheat glumes where the epidermis and parenchyma are thinwalled. Once inside the floret, the anthers, stigmas and lodicules are most easily colonized.(Fig 1.4)

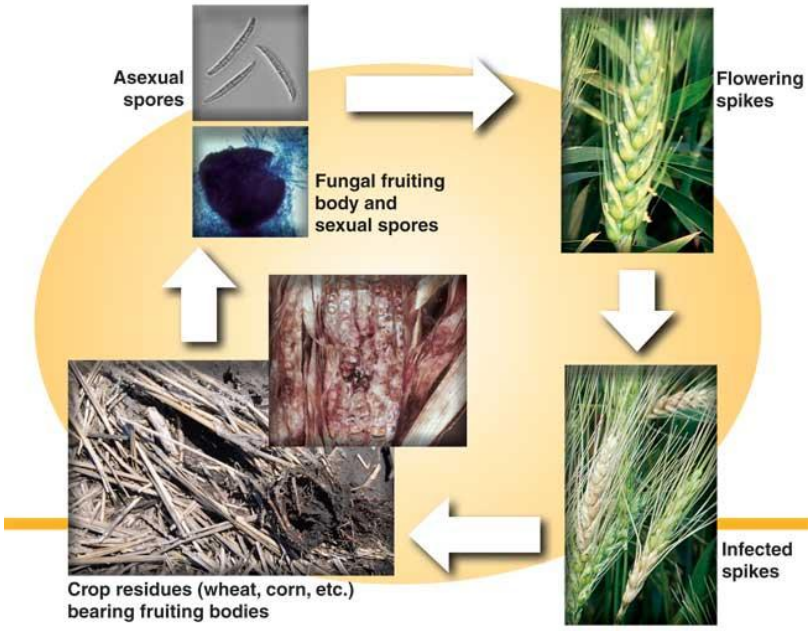


Fig. 1.4 Fusarium head blight disease cycle (photos by NDSU Department of Plant Pathology).

The principal mode of fungal spread in wheat from floret to floret inside a spikelet and from spikelet to spikelet is through the vascular bundles in the rachis and rachilla (Ribichich *et al*., 2000).

Several changes in the vascular bundles have been noted that may cause the xylem and phloem tissues in the infected rachis to become at least partially dysfunctional leading to premature death of the spikelet. Under wet conditions mycelia can spread over the exterior surfaces of the glume, lemma and palea in both wheat and barley (Bushnell *et al*., 2003). The fungus appears to have a brief biotrophic relationship with it host before switching to the necrotrophic phase. This necrotrophic stage is associated with an increase in vigor of colonization by the fungus and eventually plant death leads to thorough colonization of the host substrate. In some instances, *F. graminearum* also may colonize plant tissues asymptomatically, such as stalks of corn (Bushnell *et al.*, 2003), or can be isolated from non symptomatic grass hosts (Farr *et al.*, 1989; Inch and Gilbert, 2003).

The presence of *Fusarium* spp. in wheat can cause deleterious effects on grain processing qualities. Bechtel and coworkers, for example, found that *F. graminearum* was capable of destroying starch granules, storage proteins and cell walls during invasion of wheat grains (Betchel *et al*. 1985). Additionally, experimental results (Dexter *et al*. 1997) showed that Canadian hard red spring wheat grain samples that contained *Fusarium* damaged grains exhibited weak dough properties and unsatisfactory baking quality. Following a study of the effects of fungal proteases on wheat storage proteins, suggested that *F. graminearum* and *F. avenaceum* produced proteolytic enzymes (Nightingale *et al*. 1999). These enzymes hydrolyse endosperm proteins during dough mixing and fermentation and result in weaker dough and decreased loaf volume.

Focusing on the effect on the grain yield, FHB caused losses estimated in $2.6 billion in 9 US states in 5 years and a previous study reported that under experimental conditions, 1000-grain weight, the number of grains per head and the head weights in four triticale cultivars were reduced by 15%,18% and 22%, respectively (Arseniuk *et al*. 1993).

Apart from the effects on seed and grain processing qualities, *Fusarium* species produce a range of toxic metabolites. These include a number of mycotoxins belonging to the trichothecene group. The different trichothecenes produced by members of the *Fusarium* are classified as type A or type B according to the structural components (Krska *et al*., 2001). Type A trichothecenes includes T-2 and HT-2 toxins whilst type B trichothecenes are represented by deoxynivalenol (DON), nivalenol (NIV), 3-acetyldeoxynivalenol (3-AcDON), 15-acetyldeoxynivalenol (15-AcDON) and fusarenon-X (FUS-X). *F. graminearum*, *F. culmorum* and *F. crookwellense* also produce an oestrogenic mycotoxin zearalenone (ZEN), also known as F-2 toxin which does not belong to the trichothecene group of toxins (Hussein and Brasel, 2001).

If grain contaminated with *Fusarium* toxins is used as feed for animal or human consumption, a range of adverse toxicosis as well as other health disorders are observed, such as fever, necrotic angina, leukopenia, haemorrhaging and exhaustion of bone narrow.

On these bases, in order to reduce the risk of FHB epidemics, several cultural control techniques can be employed, including suitable crop rotation, appropriate use of fertilizers and weed control. Although cultural control strategies such as rotation, land

preparation and weed control can have an effect on inoculums load, the ubiquitous nature of the causal pathogens means that such control measures will always be limited. Also the use of fungicides is a strategy against FHB, but is significantly influenced by the dose rate used the time of application.

The most reliable and consistent strategy for controlling FHB and mycotoxins is the exploitation or creation of cultivars with good resistance.

Fig. 1.5 Field-grown wheat inflorescence showing symptoms of Fusarium head blight. The third spikelet from the bottom shows a darkened necrotic lesion ('scab') whereas the second and fifth spikelets demonstrate tissue bleaching ('blight') symptoms. From Gowsami and Kistler, 2004.

1.4.3.2 Transgenic wheat: a strategy to increase resistance to fungal pathogens

Resistance breeding for major wheat pathogens, such as rusts and smuts, has been the most important challenge for wheat breeders during the past six decades. These efforts have led to the identification of a number of resistance genes and their use to develop resistant cultivars. However, resistance breeding is a continuing and difficult process as resistance in most cases appears to be under polygenic control, and even when resistant cultivars are developed, they do not provide long term relief due to ever-evolving or mutating pathogens.

Development of the hugely successful biolistics technology by John Sanford and his colleagues (Sanford 2000; Sanford *et al*. 1987), that involved the high velocity bombardment of DNA-coated microprojectiles into regenerable cells or tissues (direct DNA delivery) rapidly became the method of choice for the transformation of cereals, particularly wheat.

In general, even though modest progress has been made in engineering resistance to major fungal and viral pathogens of wheat, the strategies that were used to confer resistance to dicot species, such as tobacco, have been applied successfully to wheat and other cereals. These include the use of viral gene sequences (pathogen-derived resistance) to disrupt viral life cycles, and pathogenesis-related (PR) proteins like chitinases, glucanases. For examples transgenic wheat plants stably expressing an antifungal barley seed class II chitinase gene (*pr3*) showed increased resistance to powdery mildew (Bliffeld *et al*. 1999), but even more significant protection was obtained with the introduction of the gene for an apoplastic ribosome-inactivation protein (RIP) from barley (Bieri *et al*. 2000, 2003, Yahiaoui *et al*. 2006). The expression of genes for an anti-fungal protein from *Aspergillus giganteum* and a barley class II chitinase were shown to significantly reduce the formation of powdery mildew and leaf rust (Oldach *et al*. 2001).

Symptoms of scab development were found to be slower in transgenic wheat plants expressing the rice thaumatin-like protein (TLP) gene (Chen *et al*. 1999), but no resistance was observed under field conditions (Anand *et al*. 2003).

To confer resistance to the wheat another possibility, recently developed, is the improvement of natural plant defense mechanisms against the tools that pathogens commonly use to penetrate and colonize the host tissue, exploiting the PolyGalacturonase-Inhibiting Proteins (PGIP). This subclass of protein, containing leucine-rich repeats, is able to inhibit the fungal Endo-PolyGalacturonase (PG), and this interaction leads to the accumulation of oligogalacturonides (OGs), which elicit a wide range of defense responses. Janni and coworkers produced transgenic wheat lines expressing a bean PGIP (PvPGIP2) having a wide spectrum of specificities against fungal PGs, in order to assess the effectiveness of these proteins in protecting wheat from fungal pathogens. Transgenic wheat leaves showed increased resistance to digestion by the PG of *Fusarium moniliforme* and significant reductions in symptom

progression. These new properties were also confirmed at the plant level during interactions with the fungal pathogen *Bipolaris sorokiniana* (Janni *et al*. 2008).

1.5 Comparative proteomics to study the plant stresses

Approximately 250 papers appeared in the Plant Proteomics field between 2007 and September 2008, dealing with the proteome of *Arabidopsis thaliana* and rice (*Oryza sativa*), and focusing on profiling organs, tissues, cells or subcellular proteomes, or studying developmental processes and responses to biotic and abiotic stresses using a differential expression strategy. Actually the platform based on 2-DE/MS is still the most commonly used, but the use of gel-free and second generation Quantitative Proteomic techniques, such as multidimensional protein identification technology (MudPIT), and those for quantitative proteomics including DIGE, isotope-coded affinity tags (ICAT), iTRAQ and stable isotope labeling by amino acids in cell culture (SILAC) have increased in according with the required Minimal Information about a Proteomic Experiment (MIAPE) standards (Jorrín Novo *et al*. 2009).

Comparative proteomics studies have been largely used to investigate the processes that take place in plant response to biotic and abiotic stresses. The proteins observed to respond to pathogen attack are those involved in detoxifying reactive oxygen species (ROS), along with the pathogenesis-related proteins (PRs). In general, the majority of the differential proteins present in resistant and susceptible genotypes to biotic stresses belongs to two major categories:

- defense- or stress-related
- enzymes associated with C- and N-metabolism and secondary metabolism.

Within the first group are the PRs (such as the glucanases, chitinases, proteases, protease inhibitors), antioxidants (catalases, SODs, peroxidases, enzymes of the ascorbate-glutathion cycle), late embryogenesis abundant (LEA) proteins, chaperones, and HSPs. The second group includes a number of enzymes associated either with carbohydrate assimilation and metabolism (photosynthesis, glycolysis, and Krebs cycle), nitrogen assimilation, or secondary (phenolics) metabolism.

The characterization of signaling events has been detected using proteomic or subproteomic comparisons in cell cultures treated with microbial elicitors, or exposed to key defense-related signaling molecules such as salicylic acid (SA), hydrogen peroxide

(H_2O_2), and nitric oxide (NO). It has also been suggested a hypothetical role of the glyceraldehyde-3-phosphate dehydrogenase in the mediation of reactive oxygen species (ROS) signaling in Arabidopsis as target for H_2O_2 (Hancock *et al.* 2005). The role of the glyceraldehyde-3-phosphate dehydrogenase has been recently reported in response to the oxidative stress in wheat and maize (Bustos *et al*. 2008).

In 2005 Wang *et al* carried out a differential proteomic analysis of proteins in wheat spikes induced by *Fusarium graminearum* The authors identified thirty protein spots classified in four groups on the basis of the time points accumulations after the inoculation, showing 3-fold change in abundance when compared with treatment without inoculation. Many of the proteins identified are related to carbon metabolism (fructose-bisphosphate aldolase, GBSS precursor), photosynthesis (protochlorophyllide reductase) and to stress defense (heat shock protein, hydrolase) of plants, indicating that proteins associated with the defense reactions were activated or translated shortly after inoculation (Wang *et al*. 2005).

As abiotic stresses are the most common environmental limitations to plant productivity, a number of important crop plants, in particular Arabidopsis as a model plant, rice, wheat, barley, maize, and sugar beet have been investigated using a proteomic approach. Especially in the last decade the attention of many research groups focused on the study of the comparative proteome profiling of the response to abiotic stress in plants by Difference In Gel Electrophoresis (DIGE). DIGE is a prelabelling technique using separate Cy dyes for different samples which then can be analyzed on one gel, avoiding shifts in gel patterns normally occurring when samples conventionally separated on two gels are compared. This technique permits to make quantitative analysis based on 2-DE much more reliable than the classic protein staining techniques.

DIGE has been used to study in detail the Arabidopsis leaf proteome changes in response to cold stress (Amme *et al*. 2006). These authors identified 18 spots with a different abundance (2-fold higher) in Arabidopsis after the treatment mainly belonging to the dehydrin. Bae *et al*. (2003) recognized 54 proteins which responded to the cold stress in the Arabidopsis nuclear proteome by using DIGE technology.

All those experimental evidences confirm the potentiality of this technique coupled with the advantages offered by mass spectrometry analysis in order to identify both the

cellular plant response to different external stimuli and the characterization of lower represented cellular subproteomes.

2. AIM OF THE PROJECT

The aim of the project is to study the proteomic response to abiotic and abiotic stresses in wheat.

The first target was the identification of the proteins of the durum wheat cv Svevo involved in the response to two different abiotic stresses: the heat stress and the cold stress. Using a classical proteomic approach based on 2DE and MS analyses, the effects of "heat shock", applied during the grain filling, on the metabolic proteins of the mature caryopsis has been evaluated. In Italy, the durum wheat is mostly grown in Central and Southern areas where it represents one of the most important crops. In this areas, grain filling occurs in April and May, a period in which sudden increases in temperature may occur, thus altering grain protein composition which interfers negatively with the semolina quality.

At the opposite the durum wheat could "suffer" the cold stress conditions during the seedling phase before flowering that, in the same area, may occur from December to March. The cold stress determines significant changes in plant metabolism and in gene regulation. The changes in the wheat leaf proteome, to integrate the information already available deriving from the transcriptome profiling in the cold stress response and acclimation, represented another interest of this project.

Moreover, we investigated the possible changes in the wheat seed proteome (bread wheat cv Bobwhite) due to the *Fusarium graminearum* infection. The proteome profiling in wheat transgenic plants (encoding a PolyGalacturonase Inhibitor Protein) more resistant to the fungal infection versus susceptible plants (relative null-segregant) was evaluated after infection.

The study of effects, due to abiotic and biotic stress, on protein regulation in the mature seed can be useful to determine the safety, nutritional and quality traits of wheat final products (fluor and semolina), that are widely used for human consumption

3.MATERIAL AND METHODS

3.1 Heat stress: Plant material and application of the heat treatment

Durum wheat (*cv* Svevo) was grown in a climate chamber in a medium composed of soil, sand, peat (6:3:1) at 10°C (9 h day)/ 7°C (15 h night) with 60% relative humidity and photon flux of 500 μmol m^{-2} s^{-1} until appearance of the third leaf. At this point, conditions were gradually (according to the development stage) switched to 20°C (13 h day)/ 17°C (11 h night) with 55% relative humidity and photon flux of 500 μmol m^{-2} s^{-1}. These conditions were maintained until five days after anthesis.

Then, while control plants were maintained in the same conditions, stressed plants were subject to heat-shock treatment, carried out at 37/17 °C (13 h day/11 h night) with 55% relative humidity for 5 days. Following the heat shock, the temperature was decreased to 28°C for 4 h, and then the growing cycle was set up at 20°C (13 h day)/ 17°C (11 h night) with 55% relative humidity and 500 μmol m^{-2} s^{-1} photon flux. Starting from the milk maturity stage, both control and stressed plants were brought to complete maturity at 25°C (16 h day)/ 20°C (8 h night) with 45% relative humidity. In both control and stress treatments, seeds were collected from four biological replicas.

3.2 Cold stress: Plant material and application of the cold treatment

Durum wheat (*cv* Svevo) was grown in a climate chamber in a medium composed of soil, sand, peat at 22°C (14 h day/10 h night) with approximately 50% relative humidity until appearance of the third leaf. These conditions were maintained constant for control plants, whereas treated plants were submitted to cold stress by bringing temperature at 4°C during day and night for 36 days. The temperature and the relative humidity were monitored using a LogTag Humidity and temperature Recorder (HAXO-8, LOGTAG, New Zeland). Leaves were collected from seedlings, by discarding those present on the border to avoid the boarder effect, frozen in liquid nitrogen and stored at –80°C. Harvest was performed at the following time-points: 1 Days After Start of Cold Treatment (DASCT), 6 DASCT, 36 DASCT (Fig. 3.2) The untreated plant material (control) were collected at the same time-points (Fig.3.1). The time-points were established according to Gulick et al (2005) in order to evaluate the early and medium cold stress response and the acclimation response. Based on seedling dry mass of 10

individuals, the comparison was at the same physiological developmental phase. In both control and stress treatments, leaves were collected from six biological replicas.

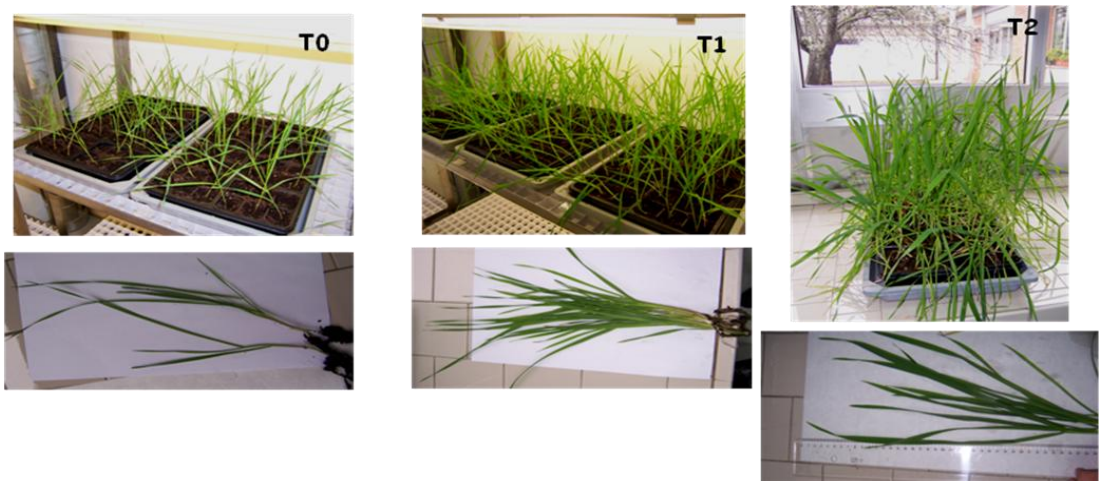


Fig. 3.1 Samples of durum wheat grown at T= 23°C (controls) collected at different time points: T0= 1 DAHSTC 1 day after the hypothetical of cold treatment start, t1= 6 DAHSTC, t2=36 DAHSTC.

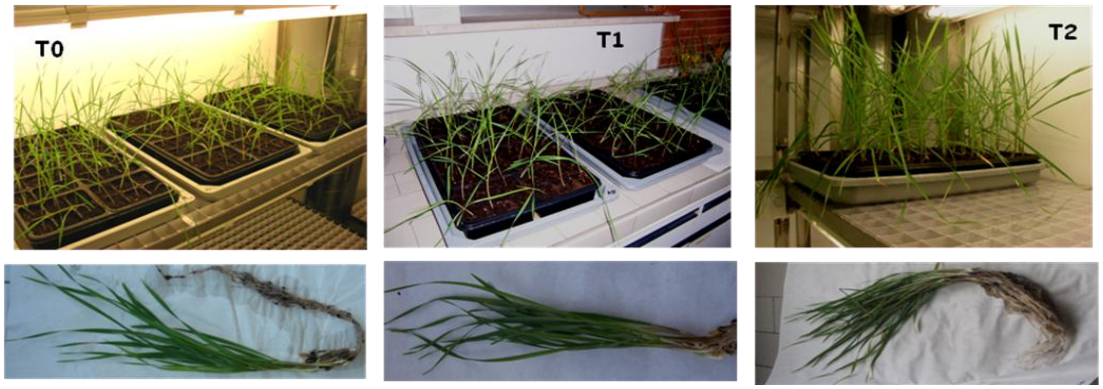


Fig. 3.2 Samples of durum wheat grown at T= 4°C (treated samples) collected at different time point: T0= 1 DASTC, t1= 6 DASTC, t2=36 DASTC.

3.3 Biotic stress: Plant material and *Fusarium graminearum* infection

For the transgenic (encoding the bean PGIP) and null-segregant (control materials that lacks for segregation the transgene) bread wheat individuals (*cv*. Bobwhite), the green house infections with *F. graminearum* and the fungal activity assay have been carried out as described in Janni et al (2008). Briefly the fungus was grown on synthetic nutrient agar and after 2-3 weeks, conidia were recovered by gently scraping plates with sterile water. Infection experiments of wheat plants were performed by single-spikelet

inoculation. T_5-T_9 generations of one transgenic line (J82-23a), homozygote for the transgene *Pvpgip2* , and their relative null-segregant lines, were used. Disease symptoms were previously assessed by counting the number of visually diseased spikelets 18 days after inoculation and by relating them to the total number of spikelets of the respective head, resulting in a percentage of symptomatic spikelets. The transgenic plants showed significant reductions in symptom progression and, for this reason, this plant material was chosen for performing the comparative proteomic analyses.

From each transgenic and null-segregant genotype, the seeds have been collected and pooled in four different groups (Fig.3.3) (control individuals, transgenic individuals, control infected and transgenic infected) and used for the further proteomic analyses. Five different infection experiments have been carried out and they represent the biological replicates for the DIGE experiment.

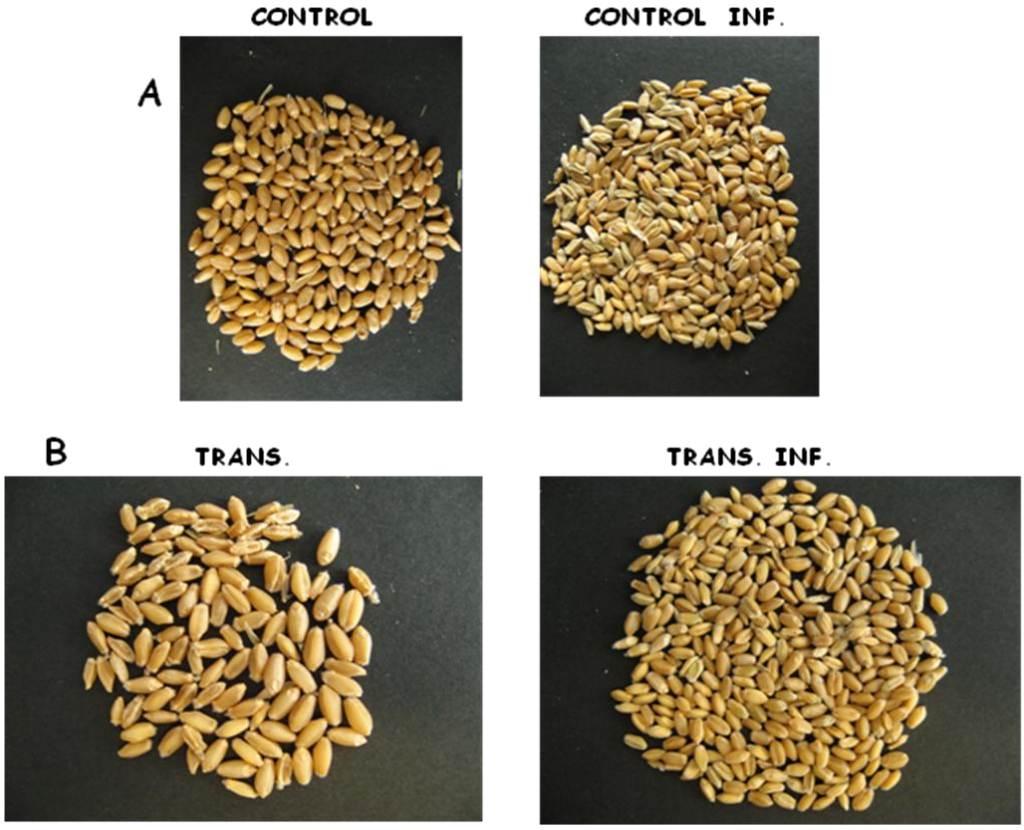


Fig.3.3 Mature wheat seeds (cv Bobwhite) collected and pooled after the a single experiment of infection with *F. graminearum*. **A** represents the pool of seeds from the null segregant individuals not inoculated with the pathogen (left) and infected (right). **B** represents the pool of the seeds deriving from the transgenic lines: left (not infected), right (infected).

3.4 Albumins and globulins extraction

In all the experiments, metabolic seed proteins were extracted according to Hurkman *et al* (2004), but amounts were scaled up in order to perform larger 2D gels. Briefly flour (150 mg) was suspended in 600 µl of cold KCl buffer (50 mM Tris–HCl, 100 mM KCl, 5 mM EDTA, pH 7.8). The suspension was incubated on ice for 5 min with intermittent mixing and centrifuged (14,500*g*, 15 min, 4 °C). The KCl-soluble fraction was collected and 5 volumes (v/v) of cold 0.1 M ammonium acetate in methanol were added at room temperature. Following incubation overnight at -20 °C, the methanol-insoluble fraction was pelleted by centrifugation as above. The pellet (containing metabolic proteins) was rinsed with cold acetone, dried down and stored at -20°C until further use.

3.5 Gliadin isolation

This analysis was performed only on samples submitted to the biotic stress. Pools of mature kernel collected from the four above mentioned groups (control individuals, transgenic individuals, control infected and transgenic infected) were separately crushed and gliadins were extracted for 2 hours at room temperature with a buffer containing 10% (v/v) dimethylformamide in a 10 mg : 100 ul ratio. After a centrifugation at 13.000 RPM of 10 minutes, aliquots of the supernatant were loaded on a acid PAGE gel, according to conditions reported in par. 3.10.2. Analysis was either performed immediately or the samples stored at -20 °C.

3.6 HMW-GS and LMW-GS isolation

Also this analysis was performed only on samples submitted to the biotic stress After the gliadin isolation, the remaining pellets were washed in a solution of 1-Propanol 50% (v/v) in a ratio (1:10 w/v) for 10 min and centrifuged at 13000 rpm for 10 min at 4°C. The HMW and LMW-GS were extracted in ratio 1:5 (w/v) in Tris-HCl 80mM pH 8.5, 1-Propanol 50%, DTT 1% for 30 min at 65°C. After a centrifuge step as above described the supernatant was alkylated with excess of 4-Vinilpirydin for 30 min at 65°C. The proteins were successively precipitated overnight in 1 mL of cold Acetone and centrifuged. The dried protein pellets were dissolved in a buffer containing Tris–HCl 70 mM, pH 6.8, SDS 2%, glycerol 10%, pyronine Y 0.02% and DTT 1%,

centrifuged as above and aliquots of the supernatant were loaded on an SDS-PAGE for the analysis (see par. 3.10.1).

3.7 Protein extraction from leaves

This analysis was carried out only on samples submitted to cold stress. Different procedures were applied in order to use the most efficient in separating the leaf proteome. Because ribulose bisphosphate carboxylase/oxygenase (RuBisCO) represents 30–60% of the total protein content in green leaf tissue, it masks the low abundant proteins (LAP), so different attempts were made to get rid of this protein in order to increase resolution of the other leaf proteins.

3.7.1 TCA-Acetone UREA

Wheat leaf tissue (5 g) was ground in liquid nitrogen with a ceramic mortar and pestle. The resulting powder was suspended (1 g/5 mL) in chilled 10% TCA in acetone containing 0.07% β-mercaptoethanol (ME) and 1X protease inhibitor cocktail (Complete EDTA-Free, Roche). The resulting mixture was then incubated 12 hours at -20°C and centrifuged at 13000 rpm for 1 h at 4°C. The pellet was washed three times in pure and chilled Acetone, centrifuging at 13000 rpm for 30 min between rinses. The fluid was removed and the pellet was freeze dried and stored at -20°C.

The freeze dried pellet was then solubilized in 8 M urea, 2% Triton X-100, and 60 mM DTT (1:30 w/v) *via* incubation at room temperature for 1 h, vortexing every 15 min, and sonicated with 6 boasts of 30 s on ice. After an incubation of 30 min at room temperature the solution was centrifuged for 1 h at 13000 rpm and the supernatant containing the leaf proteins was used for the 2DE analysis (Donelly et al 2005).

3.7.2 Salt buffer extraction (TBS)

In order to use a specific Kit (par. 3.7.3) for the depletion of the RuBisCO, an extraction in a compatible saline buffer was first carried out. The wheat leaves (5 gr) were ground in liquid nitrogen with a ceramic mortar and pestle. The obtained powder was then added of different dilutions of a solution containing 10 mM TRIS-HCl, 150 mM NaCl, pH 7.4 (TBS) and 1X protease inhibitor cocktail (Complete EDTA-Free, Roche) in two

different ratios (1:20 and 1:50 w/v). The mixture was then incubated on ice for 30 min vortexing every 10 min, sonicated as described in the par. 3.7.1 and centrifuged at 13000 rpm at 4°C. The supernatant has been collected and stored at 4°C (no more than 2 days) for the further RuBisCO purification step. An aliquot (5 µl) of the proteins extraction has been checked on a SDS-PAGE for each dilutions.

3.7.3 RuBisCO depletion: Seppro IgY Spin Column Kit

A Seppro™ RuBisCO IgY Spin Column Kit from GenWay Biotech (San Diego, CA) was used to remove the RuBisCO from the total protein extract, following the customer's instructions. Briefly the column (that contains beads coupled with Anti-RuBisCO IgY) was centrifuged at 2000 rpm for 30 s to remove suspension buffer, and then a cap for the tip of the column was attached to seal it. The beads were resuspended in 500 µLof sample (TBS extraction about 0.8 mg of proteins) and incubated at room temperature for 15 min with agitation. After 15 min, the cap on the column's tip was removed, and the unbound (Flow-throught, FT 1) fraction was collected by centrifugation at 2000 rpm for 30 s. An additional step with 500 µL of dilution buffer (TBS) has been performed in order to collect as much of unbounded fraction as possible (FT 2). Four wash steps using 500 µL of dilution buffer each were performed to elute remaining unbound proteins. The column was agitated for 1 min with dilution buffer and then centrifuged for each wash step. The wash and unbound fractions were pooled. Bound RuBisCO was then eluted off the column with five, 500 µL volumes of stripping buffer (250mM glycine, pH 2.5, supplied by GenWay as a 1M glycine concentrate with the spin column kit). The column was agitated for 3 min and then centrifuged for each strip. The stripped fractions were pooled prior to concentration and neutralized with 500 µL of neutralization buffer (250mM Tris–HCl, pH 8.0). The column was then immediately neutralized with 600 µL neutralization buffer under agitation for 5 min. Neutralization buffer was removed by centrifugation, and the beads were resuspended in TBS and sodium azide 0.02%. An aliquot of every fractions have been checked on SDS-PAGE.

3.7.4 PolyEthylene Glycol (PEG) fractionation

The wheat leaves (5 gr) were ground in liquid nitrogen with a ceramic mortar and pestle. The obtained powder was homogenized in 10 mL of ice-cold Mg/NP-40 extraction buffer containing 0.5 M Tris-HCl, pH 8.3, 2% v/v NP-40, 20 mM MgCl2, 2% v/v β-mercaptoethanol, and 1% w/v polyvinylpolypyrrolidone (PVPP). After centrifugation at 13000 rpm for 15 min at 4°C, proteins in the supernatant were subjected to PEG fractionation by adding a 50% w/v PEG stock solution. The 4% PEG sample was incubated on the ice for 30 min and then centrifuged at 1500g for 10 min. The precipitant was saved. The supernatant was adjusted with the 50% w/v PEG stock solution to make up 8%, 16%, 24% w/v PEG. Each mixed samples were incubated on ice for 30 min and then centrifuged at 12 000 g for 15 min. The final pellet was used for the 24% w/v PEG fraction precipitant and the final supernatant was recovered and precipitated with acetone (Kim et al 2001). All the fractions were analyzed on SDS-PAGE and in 2DE after the solubilization in an appropriate 2DE lysis buffer.

3.8 Protein quantification

The seed protein content was determined by Kjeldahl nitrogen analysis (Nx5.7).

3.8.1 Bradford assay

The protein extracts were quantified using the Biorad Protein Assay Kit following the customer's instructions. Five dilutions (0-1.0 mg/mL) of Bovine Serum Albumin (BSA) provided in the kit (2mg/mL) were used as standards. In order to avoid the interference effect of the Urea and detergents (CHAPS, Triton X-100) different dilution of the samples dissolved in the rehydration buffer (RB) for IEF (7M UREA, 2M Thiourea, CHAPS 2-4%, Triton X-100 2%) in the dye solution and ultrapure water (1:10, 1:20) were performed. The extracted metabolic proteins and the gluten proteins were quantified and the final proteins concentrations of the heat stressed and control samples were compared.

3.8.2 2-D quant Kit Assay

2-D quant Kit Assay (GeHealthcare) 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 (2mg/mL) were used as standards. Five and ten µL of samples (containing the protein extractions dissolved in RB) have been assayed in each test.

3.8.3 DC protein assay

The Bio-Rad *DC* Protein Assay is a colorimetric assay for protein concentration following detergent solubilization, based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. This Kit has been employed prior the DIGE labeling, because it permits to avoid the interference effect of the substance in the lysis buffer (par3.12.1) and it is more handling than the 2-D quant kit.

For this quantification, nine dilutions (0-1.6 mg/mL) of BSA provided in the kit (5mg/mL) were used as standards and two different sample dilutions in the lysis buffer (1:5, 1:10) were tested.

3.9 Total proteins and starch estimation from caryopses

This analysis was performed only on samples submitted to the biotic stress. Starch content was determined using a Total Starch Amyloglucosidase/α-Amylase Assay Kit (Megazyme International Ireland Ltd, Bray, Ireland). One hundred mg of wheat flour sample was weighed into a glass tube (16X120 mm). The samples were wetted with 0.2 ml of 80% (v/v) aqueous ethanol to aid dispersion, and stirred on a vortex mixer. Three ml of thermostable α-amylase in sodium buffer (100mM sodium acetate, pH 5.0) was added to the samples and vigorously stirred on a vortex mixer. The samples were incubated in a boiling water bath for 6 min with stirring after 2 min and 4 min. The samples were placed in a water bath at 50 °C; sodium acetate buffer (4 ml, 200 mM, pH 5.0) was added followed by amyloglucosidase (0.1 ml). The samples were stirred on a vortex mixer and incubated at 50 °C for 30 min. The entire contents of the test tubes were transferred to glass tubes and the volume was adjusted with 10 ml distilled water. The sample was mixed thoroughly and centrifuged at 3000 rpm for 10 min. Aliquots (1 ml) of the diluted solution were transferred in duplicate to the bottom of glass test tubes

(16X100 mm) and 3.0 ml of glucose oxidase peroxidase 4-aminoantipyrine (GOPOD) reagent was added to each tube (including the glucose controls and reagent blanks), and incubated at 50 °C for 20 min. Glucose controls consisted of 0.1 ml of glucose standard solution and 3.0 ml of GOPOD Reagent. Reagent Blank Solutions consisted of 0.1 ml of distilled water and 3.0 ml of GOPOD reagent. The absorbance was read against the reagent blank at 510 nm for each sample including the glucose control.

3.10 Electrophoresis

3.10.1 SDS-PAGE of HMW-GS and LMW-GS

Five µL aliquots of the proteins extracts obtained in the par. 3.6 were loaded under reducing conditions. The main gel was T 12, C 1.28 and the stacking gel was T 3.67 C2.68. The SE 600 apparatus (Hoefer) was used. Run was performed at 30 mA per gel at 10 °C. The electrophoresis was stopped 45 min after the tracking dye (bromophenol blue) reached the bottom of the gel. All the gel were stained with Coomassie BBR 250 (Sigma). All the proteins extracts were run in triplicate. Sixty glutenin subunits extracts were analyzed (5 biological replicated X 3 technical replicates X 4 different groups) by densitometry analysis with the image software 1D (Kodak).

3.10.2 Acid polyacrilamide gel electrophoresis (Acid-PAGE) of gliadins.

The gliadin extracts (5.5 µL) from mature seeds were analysed by polyacrylamide gel electrophoresis in aluminium lactate buffer, pH 3.2. In this system, migration is mainly determined by the net positive charge on the protein molecules. The procedure was described by Khan *et al*. (1985), with minor modifications and all the samples were run in triplicate. Sixty gliadins extracts were analyzed (5 biological replicated X 3 technical replicates X 4 different groups) by densitometry analysis with the image software 1D (Kodak).

3.10.3 2DE of metabolic seed proteins

Metabolic proteins (albumins and globulins) were dissolved in 800 µl of strip rehydration buffer containing 7 M Urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (v/v) Triton X100, 1.2% (v/v) Destreak reagent (GE Healthcare), and 0.5% (v/v) IPG buffer pH 3–10. IEF linear IPG strips (18 cm, GE Healthcare) pH 3–10 were used as first

dimension. Strips were rehydrated overnight at 20°C with 200 µL of dissolved proteins (about 300 µg protein) and 150 µl of rehydration buffer was added. The same extract volume (200 µL) was loaded. Focusing was performed at 20°C for 80 kVh (200 V 4 h, 500 V 2.5 h, 1000 V 3 h, 5000 V 2 h, 8000 V 9 h) using IPGphor™ Isoelectric Focusing System (GeHealthcare). The gel strips were subsequently equilibrated for 25 min in 0.1 M Tris-HCl pH 8.8 containing 6 M Urea, 30% (w/v) glycerol, 2% (w/v) SDS, 1% (w/v) DTT and bromophenol blue as tracking dye. For the second dimension, the strips were transferred onto 18 × 20 cm polyacrylamide gels (15%T, 1.28%C) (Protean II X-Cell, BioRad) and run at 40 mA *per* gel for 3–4 h at 11°C until the dye front left the gel. Three technical replicas were performed for each of the four biological replicas from each treatment, giving a total of 24 gels.

3.10.4 2DE of total leaf proteins

The total leaf protein extracts (100 µg, 300 µg and 400µg) were loaded on IEF linear IPG strips (GeHealthcare) pH 3-10 18 cm long in a rehydration buffer containing 8 M urea, 2% Triton X-100, 1,2% DeStreak e 0,5% IPG buffer 3-10 L and bromophenol blue. Strips were rehydrated overnight at 20°C in a final volume of 340 µL. Focusing was performed at 20°C for 81 kVh (100 V 3 h, 500 V 2.5 h, 1000 V 3.5 h, 8000 V 30 min gradient, 8000 V 9 h) using IPGphor™ Isoelectric Focusing System (GeHealthcare). The gel strips were subsequently equilibrated for 25 min in 0.1 M Tris-HCl pH 8.8 containing 6 M Urea, 30% (w/v) glycerol, 2% (w/v) SDS, 1% (w/v) DTT and bromophenol blue as tracking dye. For the second dimension, the strips were transferred onto 18 × 20 cm polyacrylamide gels (15%T, 1.28%C) (Protean II, BioRad) and run at 40 mA *per* gel for 3–4 h at 11°C until the dye front left the gel.

Alternatively IEF linear IPG strips pH 3-10 (GeHealthcare) 7 cm long were used. In this case 60 µg of proteins samples were loaded at the same conditions as above reported. Focusing was performed at 20°C for 38 kVh (100 V 1.5 h, 500 V 1 h, 1000 V 3.5 h, 8000 V 15 min gradient, 8000 V 4.5 h) using IPGphor™ Isoelectric Focusing System (GeHealthcare). The strips were transferred, after the equilibration as above described, onto polyacrylamide minigels (15%T, 1.28%C) (Mini-Protean, Biorad) and run at 200V until the dye front left the gel.

3.11 Protein staining and Image acquisition

Both Coomassie R-G 250 and silver staining were performed as follows. All the albumins and globulins 2DE gels were stained in a solution containing 100 gr/L of ammonium solphate, 1 gr/L of Commassie G250, 11% of phosphoric acid and 20% methanol for 16 h and destained for 1 h with distilled water before image acquisition.

The 2DE gels in which leaf proteins were separated were visualized using Coomassie G250 and silver staining. The gels were fixed in a solution containing 30% methanol and 10% Acetic acid for 30 min and dept in a solution containing 30% methanol for 10 min. After two washes in ddH_2O the gels were stained in a solution of $AgNO_3$ (1gr/L) and formaldehyde 0.15% (v/v) for 30 min in the dark. The gels were developed in a solution containing (25gr/L) and formaldehyde 0.05% (v/v) until the spots develop. The staining was blocked in the same solution used for the fixing (30% methanol, 10% acetic acid).

Except for DIGE acquisition, all the gels were scanned with Image Master Labscan v 3.00. (Amersham Pharmacia Biotech) at 16 Bit and 300dpi resolution in grayscale.

3.12 DIGE experimental design

To evaluate the metabolic protein profiles of the mature seeds in the experiments relative to the biotic stress, a two Dye scheme (DIGE) was applied. As reported in Karp and Lilley (2005), a two-dye system based on the use of Cy3 and Cy5 only, was found to be more reproducible than the three-dye system, because Cy3 *versus* Cy5 “was marginally less variable and more reliable than other dye combination”. Additionally the Cy2 dye is a weaker fluorophore and hence the signal is closer to the background noise. On those bases the experiments were designed to perform an appropriate Biological Variation Analysis (B.V.A) with a total of 20 maps in which both the samples (labeled with Cy3) were represented and the pool or internal standard (a mixture of equal amounts of metabolic protein extracts from each sample) labeled with Cy5 (Tab. 3.1). The inclusion of an internal standard allows experimental errors to be corrected and therefore improves the quantitative comparison of protein expression. This experimental design allows to compare quantitatively (trough the B.V.A) the differences in the protein abundances between the four groups of interest (Fig.3.4)

GEL	Cy3	Cy5
1	CONTROL1	POOL
2	CONTROL INF 1	POOL
3	TRANS 1	POOL
4	TRANS INF 1	POOL
5	CONTROL 2	POOL
6	CONTROL INF 2	POOL
7	TRANS 2	POOL
8	TRANS INF 2	POOL
9	CONTROL 3	POOL
10	CONTROL INF 3	POOL
11	TRANS 3	POOL
12	TRANS INF 3	POOL
13	CONTROL 4	POOL
14	CONTROL INF 4	POOL
15	TRANS 4	POOL
16	TRANS INF 4	POOL
17	CONTROL 5	POOL
18	CONTROL INF 5	POOL
19	TRANS 5	POOL
20	TRANS INF 5	POOL

Tab. 3.1 Dye experimental scheme in which the number of gels and the dyes used for each samples are reported, along with the internal Standard or pool (the mixture of all the samples analyzed in the experiments).

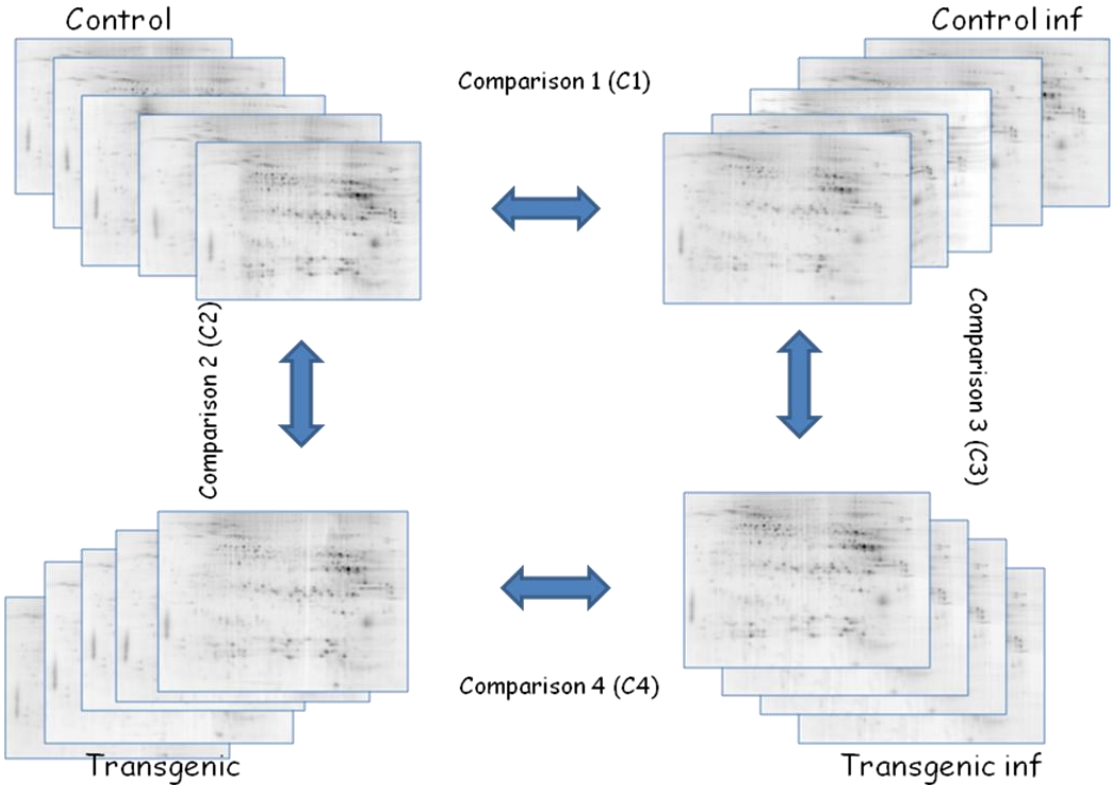


Fig.3.4 Experimental scheme of the DIGE study where all the comparisons of interest in the proteomic analysis are reported.

3.12.1 Metabolic protein labeling

The albumins and globulins pellets obtained as described in the par (3.4) were dissolved in 500 µL of lysis buffer (8M Urea, 10mM Tris HCl, 5mM Magnesum acetate, pH 8.8) and the impurity were removed using 2-D Clean-Up kit (GE Healthcare) following the manufacturer' s procedures. All the sample were solubilised again in Lysis buffer to a final protein concentration comprised between 5.5 and 7.0 mg/mL. Samples were labelled using the fluorescent Cyanine dyes developed for DIGE (GE Healthcare) following the manufacturer's recommended protocols. Fifty micrograms of protein were labeled with 400 pmol of amine reactive Cyanine dyes, freshly dissolved in anhydrous dimethyl formamide. The labelling reaction was incubated on ice in the dark for 30 min and the reaction was terminated by addition of 10 nmol lysine. Equal volumes of 2X sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mg/mL DTT and 2% Pharmalytes IPG 3–10N.L.) were added to each of the labeled protein samples and the two samples were mixed. Rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2

mg/mL DTT and 1% Pharmalytes IPG 3–10 N.L.) was added to make up the volume to 450 mL prior to IEF.

3.12.2 2-DE and image acquisition

Nonlinear IPG strips (24 cm long), pH 3–10 (GE Healthcare) were rehydrated with CyDye-labelled samples for 12 h at 20°C at room temperature. IEF was performed in a IPGphor II apparatus (GE Healthcare) for a total of 92 KVh (200 V for 8 h, 300 V for 3 h, 3500 V 30 min in gradient, 3500 V 3.5 h in gradient, 8000 V 2.30 h in gradient, 8000 V 8 h) at 20°C. Prior to SDS-PAGE, the strips were equilibrated first for 15 min in 100 mM Tris pH 6.8, 30% glycerol, 8 M urea, 1% SDS, 1% DTT, 0.2 mg/mL bromophenol blue and second for other 10 min in 100 mM Tris pH 6.8, 30% glycerol, 8 M urea, 1% SDS, 2.5% IAA, 0.2 mg/mL bromophenol blue on a rocking table. The strips were loaded onto a 15%, pH 8.8, 24 cm polyacrylamide gel with about 1 cm 5%, pH 6.8, stacker gel. The strips were overlaid with 1% agarose in SDS running buffer containing 5 mg of bromophenol blue. The gels were run in an Ettan Twelve apparatus (GE Healthcare) at 5Watt/gel for 15 min and then at 20 Watt/gel at 15°C until the bromophenol blue dye front had run off the bottom of the gels (6-7 hours). A running buffer of 25 mM Tris pH 8.3, 192 mM glycine, and 0.1% SDS was used. As previously described a total of 20 gels (5 biological replicates x 4 groups) were run.

Labelled proteins were visualized using a Typhoon™ 9410 imager (GE Healthcare). The Cy3 images were scanned using a 532 nm laser and a 580 nm band pass (BP) 30 emission filter. Cy5 images were scanned using a 633 nm laser and a 670 nm BP30 emission filter. All gels were scanned at 100 µm resolution. The PMT was set to ensure a maximum pixel intensity between 50000 and 80000 pixels.

3.13 Gel Analysis

The gel analyses were performed using two different software: SameSpots Progenesis (vers. 1.0.2602.33289, Nonlinear Dynamics, UK) for the Coomassie or Silver stained gels, and DeCyder BVA V5.0 (GEHealthcare) for DIGE samples.

3.13.1 Gel analysis of the albumins and globulins involved in the heat stress response

The 24 gels (12 control samples, and 12 heat stressed samples) were analyzed using the software SameSpots Progenesis (vers. 1.0.2602.33289, 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). Only the spots with *p* value lower than 0.05, *q* value lower than 0.05 and a power of test higher than 80% were considered as significant differentially regulated.

3.13.2 DIGE multigel analysis of the albumins and globulins involved in *Fusarium graminearum* response.

Gel analysis was performed using DeCyder^{TM} Biological Variation Analysis Version 5.0 (GE Healthcare), a software package designed specifically to be used for DIGE, following the manufacturer's recommendations. Data were normalized within the software using a ratiometric approach, and a Log_{10} transformation was used on the standardized abundance to stabilize variance. The estimated number of spots for each co-detection was set to 2500. The software automatically calculates the Student's t-test (*p*) and the spots were selected on a *p* values lower than 0.01. Additionally the *q* value was calculated, using the *p* values calculated in DeCyder, via a point-and-click tool provided by Storey and Tibshirani (2003). The Principle Component Analysis of the Log_{10} standard abundance of the spots matched on the 80% of the maps was performed using the SIMCA-P (Soft Independent Modeling of Class Analogy, Umetrics).

3.14 Protein identification by mass spectrometry

The metabolic proteins involved in heat response were identified using MALDI-TOF-TOF (Bruker Daltonics) in collaboration with the Department of Systems Biology, Enzyme and Protein Chemistry Technical University of Denmark.

The identifications of the proteins involved in the *Fusarium graminearum* response were obtained using LC-ESI-Orbitrap (Thermo Fisher) in collaboration with Cambridge Centre for Proteomics.

3.14.1 MALDI-TOF-TOF

Protein identification was done by picking gel spots from two independent gels to minimize technical variability. CBB-stained spots were excised from the preparative gels (400 µg), cut to pieces and washed twice with 40% ethanol until colorless. The destained gel pieces were dehydrated with acetonitrile (ACN), treated with 10 mM DTT in 50 mM NH_4HCO_3 for 1 h at 56°C and finally alkylated with 55 mM iodoacetamide in 50 mM NH_4HCO_3 for 30 min in the dark. Gel pieces were dehydrated with ACN, rehydrated with 1 pmol trypsin (Sigma) solution in 50 mM NH_4HCO_3 and incubated at 37°C overnight for digestion. The peptides were extracted twice from gel slices with 5% formic acid in 50% ACN. The peptide solution was then desalted, concentrated and applied to an Anchorchip target™ (Bruker Daltonic) using α-cyano-4-hydroxycinnamic acid as matrix, according to the manufacturer's instructions and Zhang *et al.* (2007). Mass spectrometric analysis was performed on a MALDI TOF-TOF Ultraflex II in positive ion reflector mode and spectra were processed and analysed using the software FlexAnalysis and BioTools (Bruker Daltonics). Database searching was carried out using an in-house MASCOT server (Matrix Science, London, UK) to search NCBInr (ftp://ftp.ncbi.nih.gov/blast/db/) and the Wheat Gene Index (http://compbio.dfci.harvard.edu/tgi/tgipage.html) release 10. Proteins were identified by PMF. Matrix contaminants and predominant keratin peaks were removed from peak lists using PeakErazor (http://www.protein.sdu.dk/gpmaw/Help/PeakErazor/peakerazor.html). In order to identify a protein unambiguously the following criteria were used: MASCOT score with $p \leq 0.05$; protein sequence coverage > 15%; at least five independent peptides matching with a mass tolerance of 50 ppm and maximum one trypsin miscleavage site. The Oxidation (M) and the Gln->pyro-Glu were selected as variable fragment modifications. Where the PMF-based identification was uncertain, fragment ion spectra were obtained for at least three peaks with a signal:noise ratio > 5 and m/z > 1400. Each fragment ion

spectrum was checked against the same database as used for PMF and the identification was confirmed if correspondence was found.

3.14.2 LC-ESI-ORBITRAP

The gel spots were excided manually from two independent silver stained gels (150-200 µg) and identified (as a service) in collaboration with Cambridge Centre for Proteomics.

4.RESULTS AND DISCUSSION

4.1 Abiotic stress: heat stress and comparative proteomic analysis

As described above, heat stress can be an important factor affecting yield and quality of durum wheat. Although quality is mostly determined by gluten proteins, that are the major protein components of wheat seeds, also the soluble metabolic protein fraction (albumins and globulins) plays a role, especially in terms of nutritional/anti-nutritional properties. Most of these polypeptides, in fact, show allergenic properties in sensitive individuals (Tatham and Shewry, 2008). Albumins and globulins are distributed mainly in the outer layers of wheat kernels, and thus are important components in whole-wheat flours and semolina, towards which there is an increasing consumer interest, because they have a higher amount of fibers, proteins and functional components (Dewettinck *et al*, 2008).

In the present section of the project, the durum wheat cultivar Svevo, widely grown in Italy and moderately resistant to cold stress, was subjected to two thermal regimes, *i.e.* heat stress and control conditions during grain filling (as described in the Materials and methods section).

Firstly, protein contents in the seeds were compared between stressed and control samples (Tab. 4.1), which revealed that heat stressed samples accumulated significantly higher amounts of protein, the protein content being 19.30% (±1.43) in stressed and 13.85% (±1.87) in control samples ($p=0.004$). A correlation between heat stress and increase in protein content was previously reported in bread wheat (Shewry *et al*, 1995). In our case, the increase in protein content was due mostly to gluten proteins, that were about 50% more abundant in heat stressed samples with respect to control samples, whereas the metabolic fraction increased by about 20% (Tab. 4.2).

The 2-D gels (24 gels) were highly reproducible and showed about 1000 spots in the pI range 3–10 both in the control and heat treated maps.

The comparison revealed differential expression of proteins ($1.2<\text{fold change}<2.5$) in 132 spots (Fig. 4.1), 65% were up-regulated in heat-treated samples (Fig. 4.2). Only those spots satisfying ANOVA ($p<0.05$) and FDR ($q<0.05$) values were chosen, and the Principal Component Analysis (PCA) performed on the differentially expressed spots

(O'Gorman *et al,* 2007) indicated two separate groups corresponding to the two thermal regimes (Fig. 4.3). In addition, all the spots selected satisfied the threshold of the test power (higher than 80% of confidence). Those data deriving from univariate and multivariate statistic tests gave reason that the observed differences between the control and treated groups were significant.

Kjekdahl			
SAMPLE	**protein content (%)**	**Average Protein Cont.**	**Standard deviation**
Control 1	18.84		
Control 2	17.51	19.30	1.43
Control 3	20.73		
Control 4	20.10		
Heat treated 1	13.42		
Heat treated 2	15.62	13.85	1.87
Heat treated 3	11.41		
Heat treated 4	14.93		
T-test		0.004	

Tab. 4.1. Determination of the protein content of the control seeds and heat treated seeds (4 biological replicates) by Kjekdahl. The average of the protein content from the different biolocical replicates, the standard deviation and the t-test value (Control *vs* Heat treated samples) are reported.

	GLUTEN PROTEINS		**METABOLIC PROTEINS**	
	CONTROL	**HEAT**	**CONTROL**	**HEAT**
Protein conc. (mg/mL)	1.39	2.05	3.3	4.05
Standard Dev.	0.62	0.71	0.05	0.02
Fold change	+ 1.5		+1.2	
T-test	0.0046		0.0015	

Tab. 4.2. Estimation of the protein concentrations (par. 3.8.1) of the gluten and metabolic proteins from the control and heat treated seeds. The protein concentrations are reported as mean of the biological replicates. In the table the standard deviations, the fold change between "heat seeds" and control seeds and the t-test values are also reported

Fig. 4.1. 2-D PAGE map of metabolic proteins in the 3−10 pH range: circles indicate the differential spots.

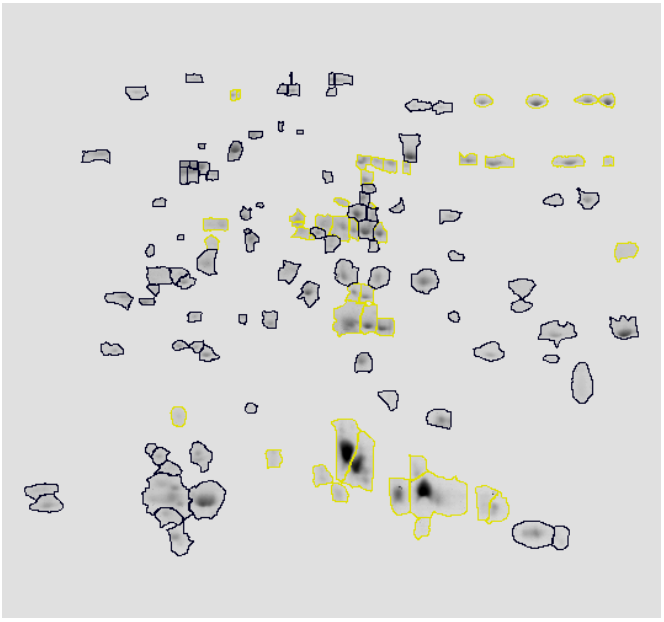


Fig. 4.2 2D-PAGE of the metabolic proteins that are differentially regulated after the heat treatment: the yellow selection is referred to spots down-regulated, whereas the black one to up-regulated (fold change between 1.2 and 2.5). In the map all other proteins not involved in the heat response have been removed.

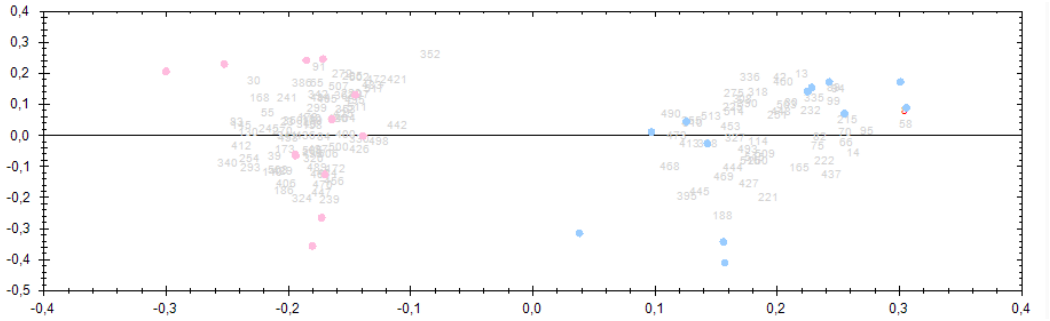


Fig.4.3 PCA representation in which differential spots relative to each gel analysed are reported. Red spots: heat stressed samples; blue spots: control samples. Numbers represent the differentially expressed spots.

4.1.1 Protein identification in the metabolic seed proteome

MALDI TOF analysis identified 47 proteins from the picked varying spots (Tab. 4.3; Fig. 4.4). Among the identified proteins, 85% were up-regulated and 15% down-regulated. Proteins identified by mass spectrometry were classified based on to their main activity, although most are involved in different pathways or signaling. As expected, heat stress increased expression of many proteins related to desiccation and oxidation stress, e.g. Late Embryogenesis Abundant (LEA)-proteins, the heat shock proteins HSP70 and HSP26, (Treglia *et al*, 1999; Renault *et al*, 2006. Queresci *et al*,2007; Timperio *et al*, 2008). These proteins, through binding or interaction with other proteins, prevent damage of proteins and cell membranes (reviewed in Buitink *et al*, 2000). Also glyoxalase I, one of the enzymes of the glyoxalase pathway related to the detoxification pathway of methylglyoxal in plants (Hossain *et al*, 2009) was up-regulated.

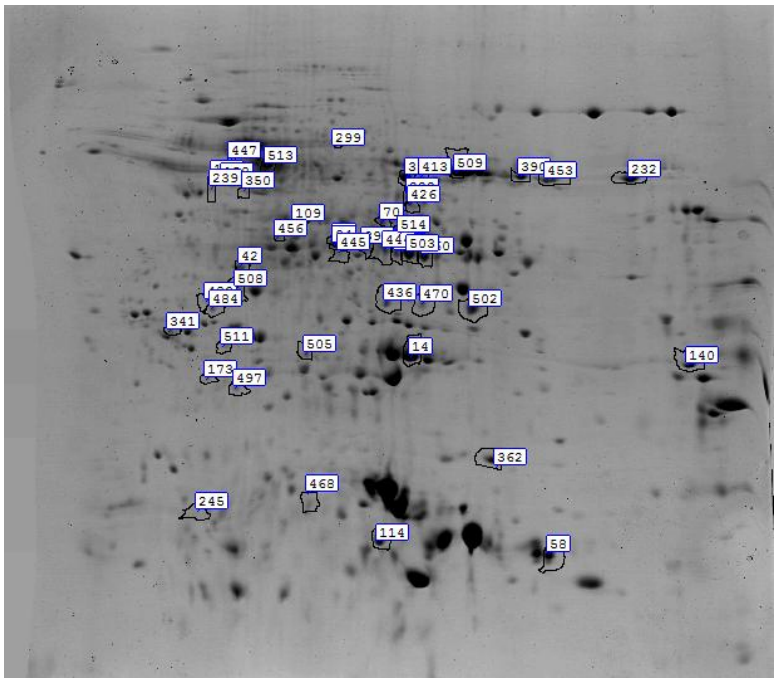


Fig. 4.4. 2-D PAGE map of metabolic proteins in the 3-10 pH range: circles indicate the identified proteins by MALDI-TOF and MALDI-TOF-TOF.

Spot	Anova	Fold Change	Protein	Accession	Specie	Score/coverage%	Theor Mw (kDa)
Up-regulated proteins							
14	1.58E-05	1.7	1-Cys peroxiredoxin PER 1	gi/1710077	*T. durum*	87/35	24.1
42	0.004	1.2	Isoflavone reductase homolog	TC235506		148/30	42.8
58	1.21E-08	2.5	Homologue to Embryo globulin	TC234172		135/27	71.3
70	3.58E-05	1.6	HSP70	gi/2827002	*T. aestivum*	83/21	71.4
89	0.001	2.2	Serpin	gi/5734506	*T. aestivum*	96/21	43.3
94	4.61E-04	2	Serpin	gi/5734506	*T. aestivum*	109/33	43.3
114	0.035	1.3	Nucleoside diphosphate Kinase	TC262718		96/26	19
222	2.45E-04	1.4	Tritin	gi/147744620	*T. aestivum*	111/38	29.5
232	0.002	1.9	Globulin-like protein	TC246874		356/45	71.1
336	0.015	1.3	Globulin-like protein 57%	TC246703		133/22	68.2
350	0.002	1.4	Globulin-like protein	TC246874		76/18	71.1
362	0.008	1.5	Endogenous α amylase inhibitor (WASI)	gi/123975	*H. vulgare*	232/85	14.7
390	0.033	1.2	Globulin-like protein	TC246703		257/27	68.2

395	0.033	1.3	Serpin	gi/5734506	*T. aestivum*	74/29	43.3
413	0.008	1.4	Globulin-like protein	TC246703		133/19	68.2
426	0.014	1.2	HSP70	gi/476003	*H. vulgare*	87/30	71.4
427	0.002	1.2	GAPDH cytosolic	gi/32478662		103/53	18.2
436	0.023	1.5	Glucose and ribitol dehydrogenase	TC233140		113/17	31.6
444	0.007	1.3	GAPDH	gi/148508784	*T. aestivum*	125/42	36.6
445	0.027	1.5	Flavonoid 7-O-Methyltransferase-like (52%)	TC252404		141/26	49.7
447	0.026	1.3	Rubisco large subunit binding protein	gi/2493650	*Secale*	141/38	53.7
453	0.014	1.2	Globulin-like protein	TC246874	*T. aestivum*	383/47	71.1
456	0.049	1.2	Serpin	TC236181		87/34	34.8
460	0.01	1.2	GAPDH cytosolic	gi/32478662	*T. aestivum*	103/53	18.2
468	0.047	1.4	Embryo globulin	TC234134		94/16	76.5
470	0.022	1.3	Glucose and ribitol dehydrogenase	gi/7431022	*H. vulgare*	89/28	31.6
483	0.032	1.3	Late embryogenesis abundant (LEA)	TC268629		97/32	30.5
484	0.003	1.3	Late embryogenesis abundant (LEA)	TC268629		126/34	30.5

497	0.004	1.6	HSP26	gi/147225072	*T. aestivum*	110/45	26.6
502	0.006	1.2	Glucose and ribitol dehydrogenase homologue	gi/7431022	*H. vulgare*	95/31	31.6
503	0.005	1.2	GAPDH	gi/148508784	*T. aestivum*	123/61	36.6
505	0.01	1.4	Embryo-specific protein	TC235043		98/14	37.6
508	0.012	1.4	Glyoxalase I	TC264636		91/36	44
509	0.006	1.7	Globulin-like protein	TC246874		290/50	71.1
511	0.009	1.3	Globulin-like protein	TC246703		112/26	68.2
513	0.033	1.3	Hypothetical protein Oryza with Enolase Domain	gi/115451911	*O .sativa*	69/22	51.1
514	0.046	1.2	GAPDH cytosolic	gi/120680 TC264316		119/28	36.6

Down regulated proteins							
109	0.018	-1.3	Phosphoglycerate Kinase	gi/129916	*T. aestivum*	176/43	42.1
110	0.016	-1.6	ATP Synthase β subunit	gi/525291 and TC264886	*T. aestivum*	123/21	77.5
110	0.016	-1.6	Globulin-like protein	TC246874		104/23	71.1
140	8.82E-04	-1.6	Tritin	gi/391929	*T. aestivum*	112/24	29.5
158	0.002	-1.4	ATP Syntase β subunit	gi/525291	*T. aestivum*	81/13	59.3
173	0.007	-1.3	Glycosyl hydrolase 85	gi/30695320	*A. thailiana*	85/29	40.7
239	0.022	-1.2	Homologue to Embryo globulin	TC234045		163/41	77.3
245	0.008	-1.3	Single stranded nucleic acid binding protein	gi/974605, TC249148		100/19	19.3
299	0.003	-1.2	Fructose-6-P 1 phosphotransferase	TC248170		71/13	82.3
341	0.007	-1.4	14-3-3 homologue	gi/22607 and TC233195	*H. vulgare*	172/47	29.4

Tab.4.3 Proteins responsive to heat shock identified by PMF and MS-MS analysis. The following criteria were used: MASCOT score ($p<0.05$),minimal coverage 13%, at least 6 independent peptides should match with a mass tolerance of 50 ppm and 1 miss cleavage site. The database search was applied in NCBInr and TIGR wheat. For all the identifications three peptide fragmentations were done (MS-MS) and used in combination in the database search. The ANOVA value for each spot, the fold change of the normalized volumes between the spots in the control and heat stressed maps, the proteins names, accession numbers, the MASCOT scores, the protein coverage and the theoretical molecular weight of the proteins identified are reported.

4.1.1.1 Carbohydrate metabolism or energy related proteins

Nucleoside diphosphate kinase (NDPK), which is required for the synthesis of nucleotide triphosphate precursors of DNA and RNA, was up-regulated along with some housekeeping enzymes involved in glycolysis and the pentose phosphate pathway (glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, and glucose and ribitol dehydrogenase), in agreement also with Hurkman *et al* (2009) that have studied the effect of high temperature on soluble proteins of developing bread wheat grains. NDPKs are housekeeping enzymes that catalyze the transfer of γ-phosphates mainly from ATP to cognate nucleoside diphosphates (NDPs). This is done to maintain the balance between cellular ATP and other nucleoside triphosphates (NTPs). Altered expression pattern of NDPK was previously found in response to abiotic (including heat stress) and biotic stresses in rice and other plant species (Salekdeh *et al* 2002, Hajheidari *et al*, 2005), suggesting that NDPK plays a regulatory role in addition to its primary metabolic functions.

Five spots (427, 444, 460, 503, 514) which were up-regulated by heat stress contained glyceraldeyde 3-phosphate dehydrogenase (GADPH), which is involved in glycolysis and has been identified as one of the allergens implicated in bakers' asthma (Tatham and Shewry, 2008). This enzyme is mainly involved in the glycolysis and is responsible for catalyzing the reversible conversion of glyceraldehyde 3-phosphate (GAP) and inorganic phosphate into 1,3-bisphosphoglycerate. However also secondary functions has been reported. Indeed Bustos and Iglesias (2003) reported that wheat endosperm and shoot GAPDH undergoes posttranslational phosphorylation enabling interaction with 14-3-3 family proteins, thus exerting a regulation aimed at maintaining the levels of energy and reductants in the cytoplasm. Recently, using a proteomics approach it was established that GAPDH activity in *Arabidopsis* was inhibited by H_2O_2, suggesting that GAPDH is a direct target of H_2O_2 and might have a role in mediating ROS signaling in plants (Hancock *et al*, 2005).

Three up-regulated spots (436, 470, 502) belonged to the glucose and ribitol dehydrogenase protein family.

Among the polypeptides down-regulated after heat shock, ATP synthase β subunit (spot 110) was identified, in agreement with observations in bread wheat (Majoul *et al*,

2004). The decrease in amount of ATP synthase may affect energy-dependent processes involved in heat stress resistance. It has been reported that energy consuming processes are the primary cellular targets for a decreasing of ATP demand in response to stresses, such as anoxia (Boutilier and St-Pierre, 2000), and it is known that heat shock and anoxia are abiotic stresses eliciting similar cellular responses (Wu *et al*, 2002).

Also phosphoglycerate kinase (spot 109), glycoside hydrolase family 85 (spot 173), and fructose 6 phosphate-phosphotransferase (spot 299), all involved in glycolysis, were down-regulated. The glycoside hydrolase family is present in essentially all living organisms and has been implicated in a diversity of roles, such as biomass conversion in microorganisms and activation of defense compounds, phytohormones, lignin precursors, aromatic volatiles, and metabolic intermediates (Opassiri *et al*, 2006). Phoshoglycerate kinase was previously found to be over-expressed in the nuclear proteome of Arabidopsis in the cold stress response (Bae *et al*, 2003).

4.1.1.2 Stress related proteins

Two members of the heat shock protein family, HSP70 (spots 70, 426) and HSP26 (spot 497) were up-regulated after the heat stress. HSP70s functions as molecular chaperones. Under normal physiological conditions, they participate in protein biogenesis and turnover. HSP70s bind to nascent peptides emerging from ribosomes, preventing irreversible aggregation prior to folding, stabilize precursors in unstructured forms that are competent for membrane transport, assist in the assembly and disassembly of oligomeric complexes, and play also a role in protein degradation. HSP70s have been linked to the development of acquired thermotolerance in heat stress, although they seem also to correlate with tolerance to low temperature stress (Guy *et al*, 2006). HSP26 belongs to the family of small heat shock proteins (sHSP) exhibiting chaperone activity and thought to protect proteins from irreversible aggregation (Nguyen *et al*, 1993).

A polypeptide of the 14-3-3 protein family was slightly down-regulated by heat stress (spot 341). Also Hurkman *et al*, (2009) observed down-regulation of members of this protein family after heat stress in bread wheat. Transcripts encoding proteins belonging to the 14-3-3 family accumulate in barley after biotic stresses (Brandt *et al*, 1992; Gregersen *et al*, 1997) and in wheat a region of chromosome 4AL containing genes coding for 14-3-3 is associated with a resistance QTL against specific fungal diseases

(Faris *et al*, 1999). The up-regulated polypeptides after heat treatment moreover included flavonoid *O*-methyltransferase (spot 445), a 14-3-3-binding protein involved in production of antimicrobial secondary metabolites, thus showing a potential role in response to a pathogen attack in barley (Christensen *et al*, 1998). Furthermore these compounds have antioxidants properties and could thus be involved in oxidative stress.

Serpins (*ser*ine *pr*otease *in*hibitors) were identified in multiple spots (89, 94, 395, 456) as up-regulated in response to high temperature. The same observation was made by Hurkman *et al*, (2009). Serpins, found in the soluble fraction of wheat seeds, undergo differential regulation in response to environmental stress (Sancho *et al*, 2008). The serpins are widespread in the plant kingdom and represent up to 4% of the total protein in the mature endosperm of cereal grains (Rasmussen *et al.*, 1996; Østergaard *et al*, 2000). While the precise physiological role of serpins remains unclear, their activity suggests that they are involved in inhibition of endogenous proteases, or proteases from grain pests. Because of their high concentration in the endosperm, serpins have potential to influence grain quality traits (Roberts *et al*, 2003; Salt *et al*, 2005, Roberts and Hejgaard 2008).

Other proteins with a defense role have a modified expression profile in response to heat shock. Such proteins are typically identified in multiple forms on 2-D gels (Østergaard *et al.*, 2004) and include: tritins (spots 140, 222), α-amylase inhibitors (spot 362), and some 14-3-3 related or binding proteins. Most of these proteins besides having a metabolic role, are also considered as storage proteins. Furthermore, some are also considered wheat grain allergens along with the serpins (Sotkovsky *et al*, 2008).

Another enzyme found in response to the heat treatment is glyoxalase I (spot 508). The glyoxalase system is a set of enzymes that carry out detoxification of methylglyoxal and other reactive aldehydes that are produced during normal metabolism. This system has been studies in both bacteria and eukaryotes. This detoxification is accomplished by the sequential action of two thiol-dependent enzymes; firstly glyoxalase I, which catalyses the isomerisation of the spontaneously formed hemithioacetal adduct between GSH and 2-oxoaldehydes (such as methylglyoxal) into S-2-hydroxyacylglutathione. Secondly, glyoxalase II hydrolyses these thiolesters and in the case of methylglyoxal catabolism, produces D-lactate and GSH from S-D-lactoyl-glutathione. In plants it has been demonstrated that different kinds of stress, such as salt and metal stress, elicits enhance

the expression of glyoxalase I (Veena *et al*, 1999; Singla-Pareek *et al*, 2003; Yadav *et al*, 2005).

Late Embryogenesis Abundant (LEA) proteins (spots 483 and 484) were up-regulated and are involved in stress responses. They are typically correlated with cellular dehydration in response to cold stress. Other LEA functions include roles as antioxidants and membrane and protein stabilisers during water stress (Tunnacliffe and Wise, 2007).

Finally, 1-Cys peroxiredoxin (spot 14), also observed to be up-regulated, has antioxidant and chaperone activity (Bystrova *et al*, 2007).

4.1.1.3 Storage proteins

Up-regulated proteins with a storage role here identified in the heat response mainly belong to the globulin-like protein family (spots 110, 232 350, 509, 511) or protein homologous to embryo globulin (spots 58, 239, 468, 505). Spot 239 (Homologue to Embryo Globulin) was found to be down-regulated by heat stress. A role for these proteins in thermotolerance is not known. They may be directly related to heat stress response by an unknown mechanism or, indirectly, be a target for other proteins involved in the heat shock response. This is in agreement with Hurkman *et al* (2009).

4.2 Abiotic stress: cold stress application and thermal regime

In the present section of the project, the durum wheat cv Svevo was submitted to two thermal regimes (see par. 3.1) to evaluate the effects of the short (1 day of cold stress), medium (6 days) and long response or acclimation (36 days) to the temperature of 4°C in the wheat leaf proteome. The comparison had to be made between the treated and the control samples, namely the same genotype grown at 23°C.

Unfortunately the temperature conditions of the chamber were stable for the temperature set as control, but they were unstable for the cold stress application. The temperature indicated by LogTag measurements was 19°C (± 0.8°C) for the control individuals, and 7.2°C (±1.8°C) for the stressed samples. The relative humidity (RH%) was 86.0% (±12.03) in the control conditions and 80.3% (±21.00) during the cold stress application (Fig. 4.5). Because during the application of the cold stress there were great temperature ranges (between 5°C and 10°C, see Fig 4.5B), we have deemed that the

experimental conditions were not appropriate, although we observed that wheat plants showed a reduced growth at 6 and 36 days after application of the presumed cold stress conditions. These latter were in fact approximately half-size than their relative control (Fig.4.6). On those bases we did not consider the plant material suitable for the comparative proteomic analysis to study the effect of the cold stress response in the wheat leaf proteome.

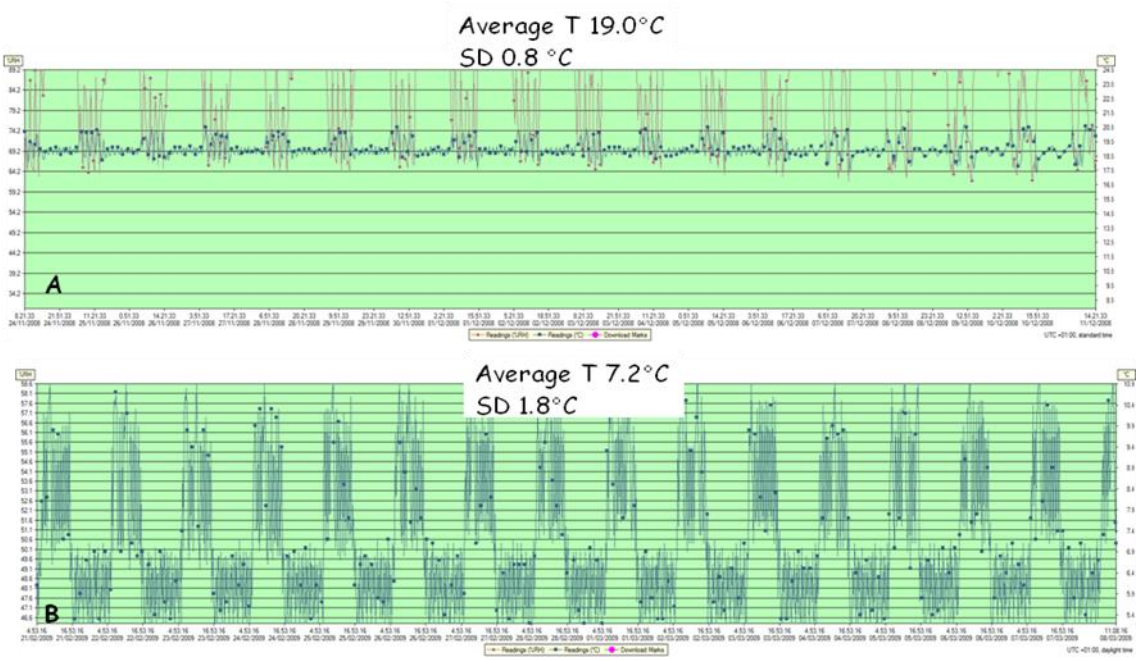


Fig. 4.5 Temperature applied to the Svevo control plants (A) and to the cold stressed individuals (B). The average temperature and the standard deviation are reported in both the cases.

Fig.4.6 Wheat plants growth at control temperature (A) collected at T2 (36 DAHST) and stressed plants collected at the same time point after the cold stress application (B).

4.2.1 Set up of the procedure for the efficient separation of the durum wheat leaf proteome

In order to have a suitable procedure to analyse the leaf proteome of durum wheat, once the sample for the cold stress study were ready, we previously tried different extraction procedures.

4.2.1.1 RuBisCO depletion

Both the Seppro IgY-RuBisCO Spin Column Kit and the PEG fractionation were evaluated to deplete the RuBisCO from the wheat leaf proteome. The presence of the RuBisCO Large and Small subunits were confirmed on 1D-PAGE in both TCA-Acetone UREA and in TBS (1:10 w/v) extractions methods (Fig.4.7).

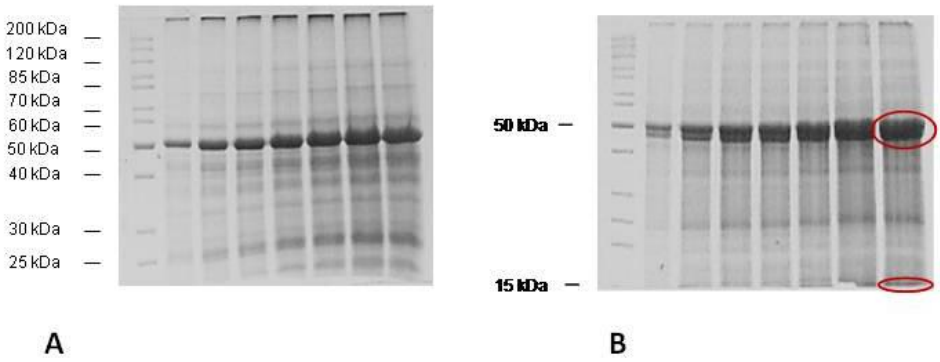


Fig. 4.7 SDS-PAGE of the wheat total leaf proteins extractions in TCA-Acetone UREA (A) and in TBS (B). Increasing amount of proteins (from 4 µg to 30 µg) were loaded in the lanes and the protein were visualized with Coomassie staining. The red circles indicate the RuBisCO LSU and SSU

This first step has allowed to evaluate, using the leaf proteins extraction in TBS, the effectiveness of the spin column that does not support caotropic agents, detergent and reducing substances, such as UREA, SDS, and DTT or β-Mercaptoethanol. The immunopurification of the RuBisCO, starting from 1.0 mg/mL of total protein extraction, has been validated on 1D-SDS-PAGE. (Fig.4.8). In the flow through the presence of the RuBisCO Large Subunit (LSU), approximately 51 KDa, was not detectable, but it could be visualized in the stripped fraction. These results are in according with the previous results published from Cellar et al. (2008), where the authors demonstrated the effectiveness of this method to deplete about 90% of the RuBisCO from the leaf proteome of Spinach, Arabidopsis and Canola.

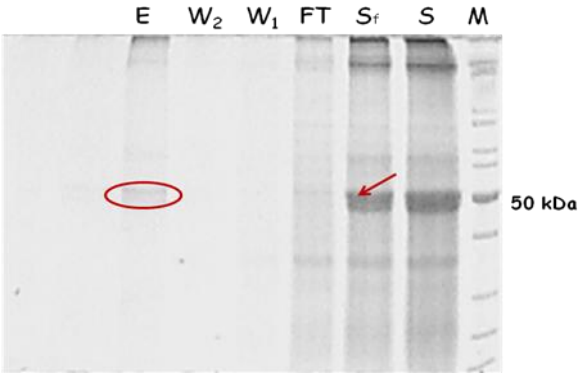


Fig. 4.8 1D SDS-PAGE of the wheat leaf protein extraction in salt buffer after the RuBisCO immunoaffinity purification. M: molecular marker S: salt extraction, Sf: filtered extraction, Ft: flow through, W1 and W2: washes, E: Elution. The arrow and the circle represent the Large Subunit RuBisCO (LSU). The proteins were visualized with Coomassie staining.

This method was not evaluated and reported for the 2DE applications, that need additional steps, especially a desalting step to remove the presence of the salt that interferes during the IEF. To evaluate the efficacy of the spin column for a 2DE comparative proteomic study, the TCA-Acetone-UREA extraction method and a method suitable for the spin column purification combined with IEF/SDS-PAGE were compared (Fig.4.9).

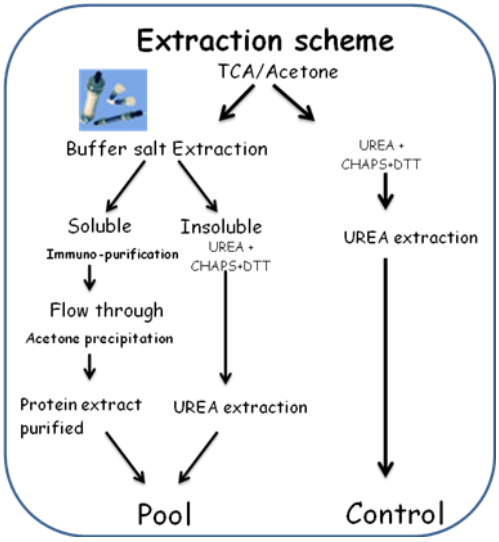


Fig. 4.9 Schematic representation of the extractions method comparison used for the 2DE maps

The purification protocol was efficient for RuBisCo Large subunit depletion in 2DE analysis (Fig. 4.10) but, compared with the TCA-UREA control protocol, it showed limitations due to technical differences in the solubilization of a group of proteins.

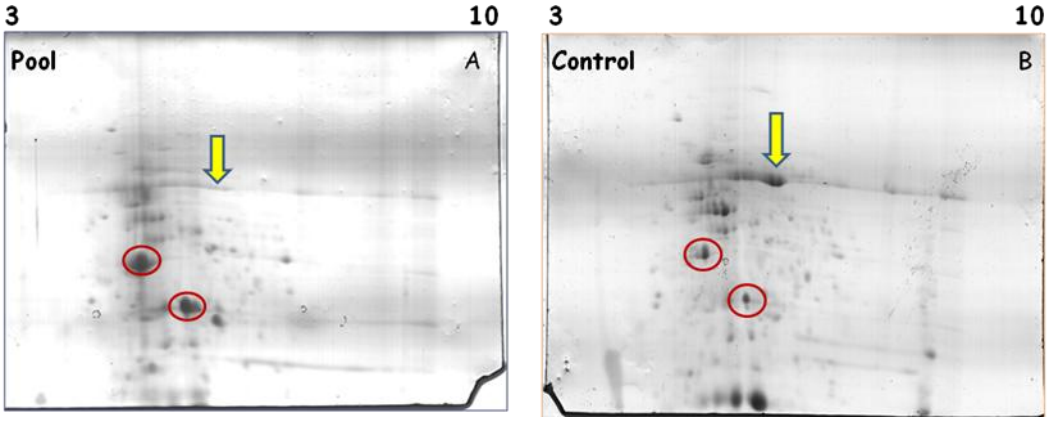


Fig4.10 2 DE maps in the pH range 3-10 (7 cm strips) of the total leaf proteins extraction after the RuBisCO purification (A) in comparison with the TCA-UREA protocol as control (B). The red circles indicate the difference in the spots visualization and the yellow arrows the LSU map position. The proteins were visualized with Coomassie staining.

Also the PEG fractionation technique applied on the total leaf protein extracts was validated by SDS-PAGE and the RuBisCO LSU and SSU resulted mainly compartmentalized in the fraction corresponding to PEG 16% (Fig. 4.11).

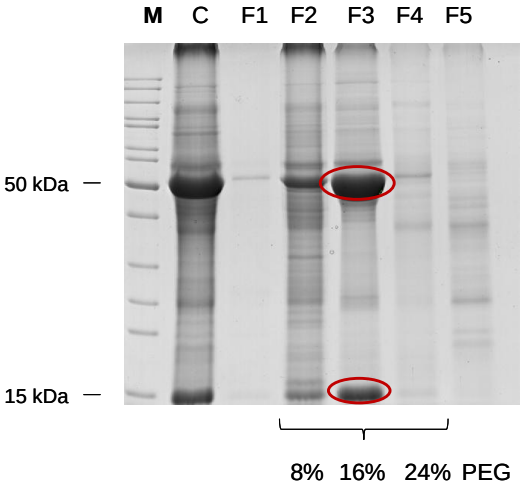


Fig. 4.11. SDS-PAGE of the wheat leaf proteins in a gradient PEG concentration (F1 4%, F2 8%, F3 16%, F4 24%, F5 24% supernatant). C: TCA-UREA protocol; M: molecular marker. The selected bands correspond to LSU and SSU of RuBisCO. The proteins were visualized with Coomassie staining

This confirms the potentiality of this method to solubilised both the RuBisCO subunits, but the presence, in the 16% PEG fraction, of a considerable number of polypeptides, besides RuBisCo, did not make this the method of choice for a comparative proteomic analysis. Moreover, a more detailed 2DE protein separations of the PEG fractions in comparison with the control conditions of the reference gel (Fig. 4.12) (TCA-Acetone-UREA method), showed that the 24% PEG fraction (Fig. 4.13) contained a group of low abundant proteins, masked by RuBisCO in the reference gel, but this fraction was not suitable for a proteomic comparison because of the large number of spots selectively lost in the 16% PEG fraction (Fig. 4.14).

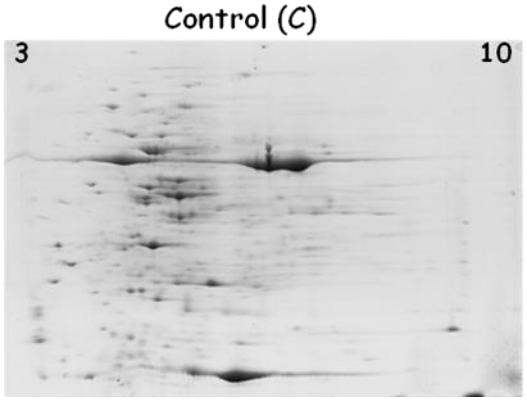


Fig. 4.12. 2D-PAGE in the 3-10 pH range of the total leaf protein extraction based on TCA-UREA used as control reference gel. The proteins were visualized with Coomassie staining

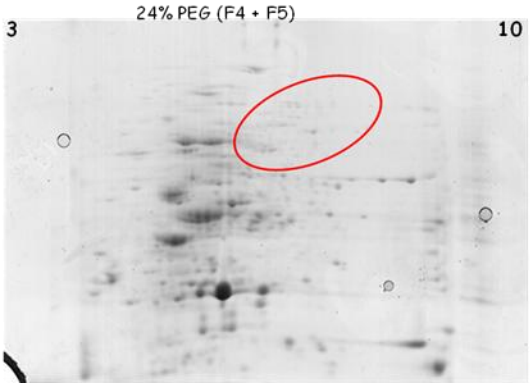


Fig. 4.13. 2D-PAGE in the 3-10 pH range of the leaf proteins solubilized in 24% PEG. The RuBisCO Large Subunit is not present and some low abundant proteins are visible (red selection).

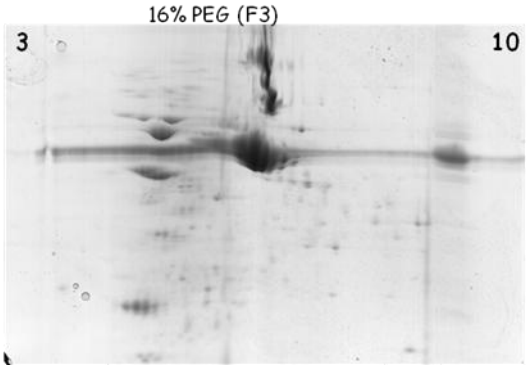


Fig. 4.14. 2D-PAGE in the 3-10 pH range of the leaf proteins extracted in 16% PEG. The RuBisCO Large Subunit is predominant in this fraction. The proteins were visualized with Coomassie staining.

All these evidences gave reason that this method is not optimal for a whole comparative proteomic approach, because is not highly reproducible.

4.2.3 Optimization of the total wheat leaf protein extraction for comparative proteomic study

Based on the lack of reproducibility of the protocols allowing depletion of RuBisCO, the TCA-Acetone-UREA extraction previously described, (Donelly et al 2005), was used for the analysis of the wheat leaf proteome. The leaf proteins (400 µg and 500 µg) were extracted using TCA-Acetone-UREA and visualized on 2DE gels in the broadest range of pH (3-10), showing very well resolved spots patterns and quantity linearity. The presence of RuBisCO slightly interferes on the gel quality especially when the amount of proteins loaded on the IPG strip is higher (Fig 4.15.). This effect could be due to the binding capacity of the RuBisCO with some contaminants such as phenolic compounds (Redone et al 1995).

Three independent gels were aligned using Samespots Progenesis and 580 spots were visualized on the resulting 2DE map (Fig. 4.16). All the spots were manually checked and validated. This result confirms the optimization of the extraction and protein separation for the durum wheat leaf proteome increasing the number of spots detectable on 2DE map in relation to these published (Donelly et al 2005) in bread wheat (404 spots).

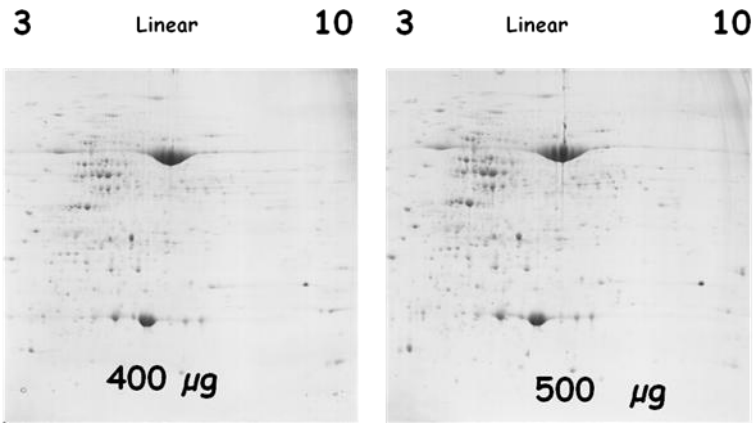


Fig.4.15 2 D-PAGE in the 3-10 pH of 400 µg (left) and 500 µg (right) of the total durum wheat leaf proteins with the TCA-Acetone-UREA based protocol.

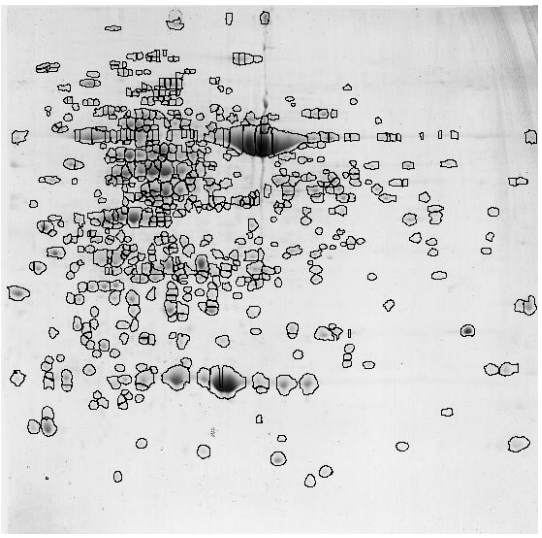


Fig.4.16 2 D-PAGE in the 3-10 pH of the total durum wheat leaf proteins with the TCA-UREA based protocol after the gels alignment with the software Samespots Progenesis (vers. 1.0.2602.33289, Nonlinear Dynamics, UK). Every spots selected has been checked manually and validated. The proteins were visualized with Coomassie staining.

4.3 Biotic stress: proteomic response to *Fusarium graminearum* infection in tolerant and susceptible transgenic bread wheat lines

In this section of the project I will present data about the proteomic comparison of the metabolic fraction extracted from transgenic lines of bread wheat kernels (cultivar Bobwhite) transformed with a bean PGIP gene with the corresponding null-segregant line, after and without infection with *F. gramineaurum*.

This bean PGIP gene have a wide spectrum of specificities against fungal PGs and it was previously demonstrated that the expression of this transgene conferred a significant reductions in symptom progression in the transgenic line, whereas the null-segregant line, genotypically identical to the transformed lines for the exception of the lack of the transgene, did not differ significantly (Janni et al, in preparation).

Because wheat based foods are prepared from wheat flour, we were interested in studying the effect on the accumulation of the main components of wheat kernel, in order to understand if the tolerance-susceptibility shown by these transgenic lines was correlated to specific changes in protein (metabolic and gluten proteins) and starch composition, that affect both nutritional and technological quality.

4.3.1 DIGE on metabolic proteins.

Regarding the metabolic protein fraction, DIGE was performed by comparing five biological replicas of each group considered (Null-segregant, Null-segregant infected, Transgenic, and Transgenic infected), following the scheme reported in Fig. 3.4.

To reduce the presence of contaminant, to reach a good representation of the wheat metabolic seed proteome and to confirm the effectiveness of the protein quantification method, the metabolic protein extraction protocol was validated by both 1D SDS-PAGE and 2-DIGE (Fig. 4.17).

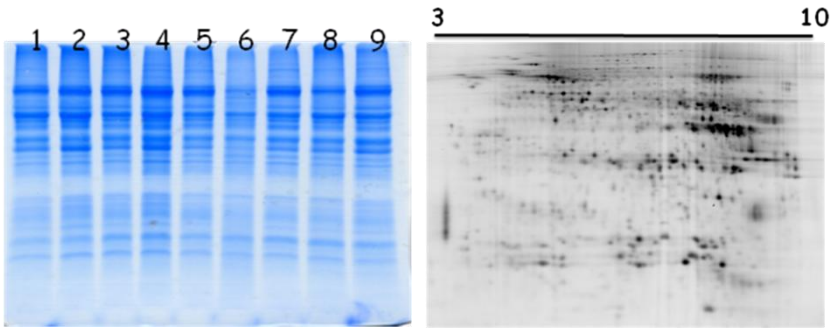


Fig. 4.17, Left: SDS-PAGE of the metabolic protein extractions. Lane **1-2** Transgenic and control samples from Replica 1, lane **3-4** Transgenic and Control infected from Replica 2, lane **5-6** Control e Transgenic infected from Replica 3, lane **7-8** Control e Control infected from Replica 4, lane **9** Transgenic infected from Replica 5. Right: 2DIGE of the control infected sample labeled with Cy5 in the pH 3-10 (strip 24 cm).

The protein extractions resulted optimal for the labelling; indeed they did not show presence of any contaminants reaching a protein concentration ranging between 5.15

mg/mL to 7 mg/mL. Additionally, all the 2-DIGE maps proved a well resolved spot pattern in the 3-10 pH range for both the infected and the control samples. In total about 2000 spots were detected and the Differences In gel Analysis (D.I.A) of the matched maps (more than 2 fold of change) were estimated between 5 and 10% in all the gels. An example of the D.I.A on the gel corresponding to an infected sample and another example of the gels quality are reported in Fig. 4.18.

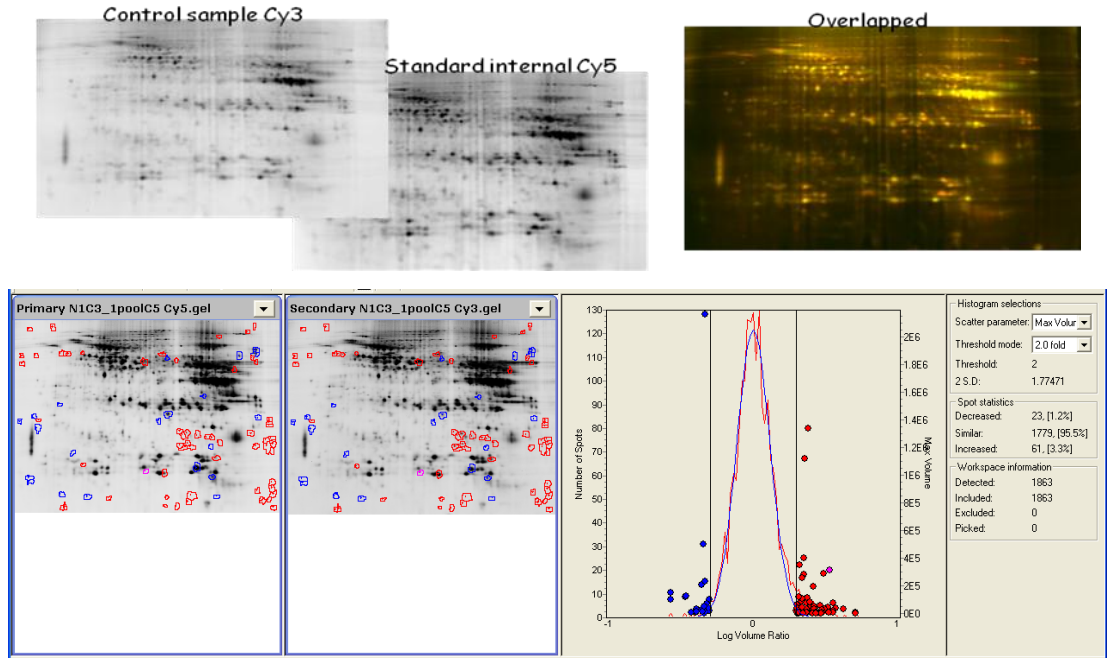


Fig. 4.18 Example of a DIGE map with one control sample labeled with Cy3 and the Standard Internal (the mixture of all the samples) with Cy5 and the two images overlapped . On the bottom the D.I.A is report. The red dots are the spots increased in the same map of 2fold-change and the blue dots represent the spots decreased. The 95.5% of the spots are comprised between 1 and 2 fold-change.

Master No.	Appearance	T-test	Av. Ratio	1-ANOVA
1059	36 (40) A, M	0,0049	-2,47	0,0047
1240	36 (40) A, M	0,0051	1,84	0,062
1120	40 (40) A, M	0,0053	-1,35	0,084
158	40 (40) A, M	0,0058	1,66	0,035
1417	40 (40) A, M	0,0061	-1,53	0,045
168	38 (40) A, M	0,007	1,91	0,024
684	40 (40) A, M	0,0074	-1,38	0,011
1015	38 (40) A, M	0,0076	-1,44	0,032
891	40 (40) A, M	0,0094	-2,09	0,0033
1543	38(40)A	0.02	-4,63	0,048

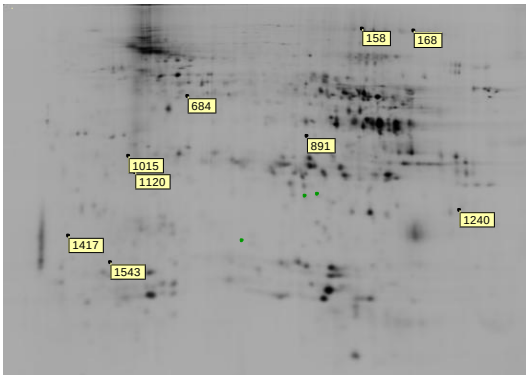


Fig. 4.19. 2DIGE map of the spots differentially regulated in the comparison between the control and control infected samples. In the tab the spots numbers with their relative t-test values are reported, along with the appearance of the spots on the total number of gel in the study, the ANOVA value and the average ratio.

The Biological Variation Analysis revealed 10 spots differentially regulated (Fig. 4.19) in the comparison between the control and control infected groups. The spots of interest were selected according to the following criteria: t-test (p) lower than 0.01, fold change higher than 1.2, and spot appearance at least on the 80% of the total maps. Respectively 1 spot, 2 spots and 1 spot, in the comparison between transgenic and control, transgenic infected and control infected, and the transgenic and transgenic infected groups were detected as differentially regulated (Fig. 4.20).

In general these spots were of low intensity and were very difficult to visualize on the gels. Because only a few spots were shown to have changed in abundance in a statistically significant manner, PCA was performed on the gel groups to determine if there was any clustering in the data derived from the same sample groupings. The Log of the standard abundances of all the spots plotted in P.C.A graph did not discriminate any clusters (Fig.4.21).

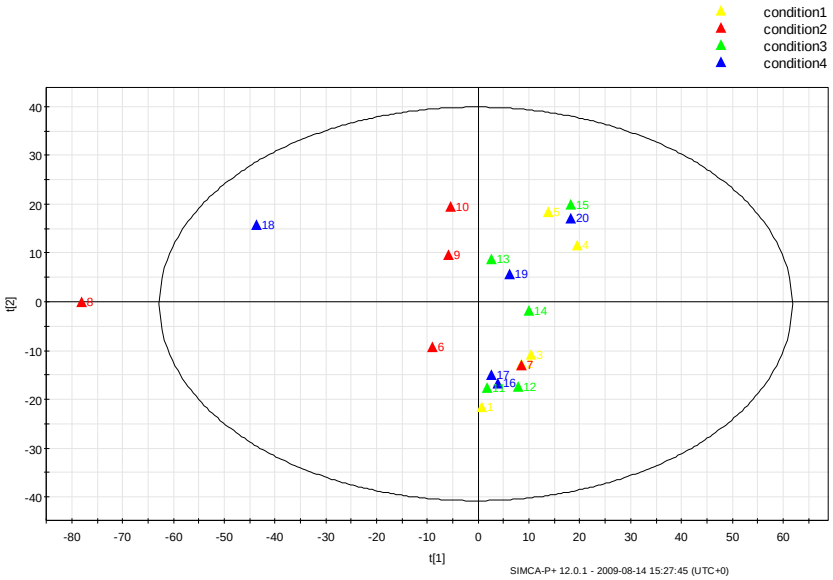
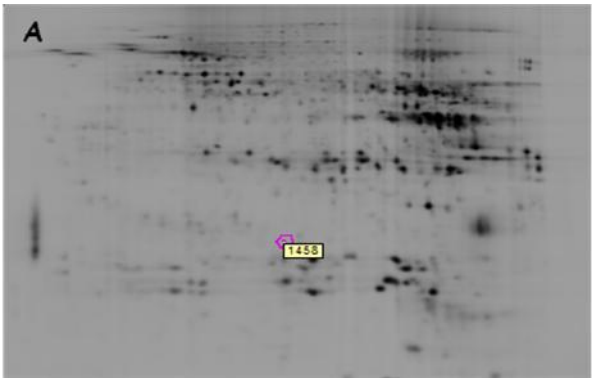
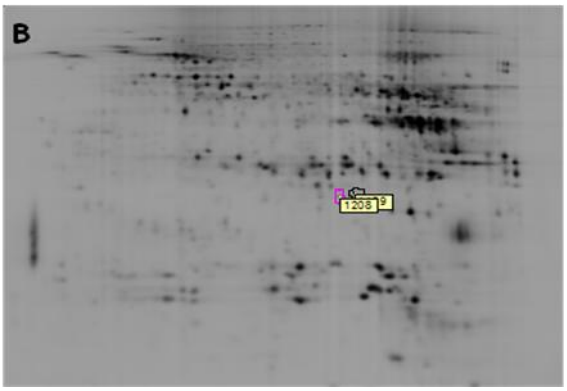


Fig. 4.21. Principal Component Analysis plot of the Log standard abundance of the spots present on the 80% of the maps performed using the SIMCA-P (Soft Independent Modeling of Class Analogy, Umetrics). The yellow triangles represent the control gels, the control infected gels are reported in red, the transgenic in green and the transgenic infected in blue.

Master No.	Appearance	T-test	Av. Ratio	1-ANOVA
1458	40 (40) A, M	0,0074	-1,21	0,12



Master No.	Appearance	T-test	Av. Ratio	1-ANOVA
1208	40 (40) A, M	0,0083	1,38	0,015
1199	40 (40) A, M	0,014	1,5	0,056



Master No.	Appearance	T-test	Av. Ratio	1-ANOVA
1827	40 (40) A, M	0,0041	1.98	0,011

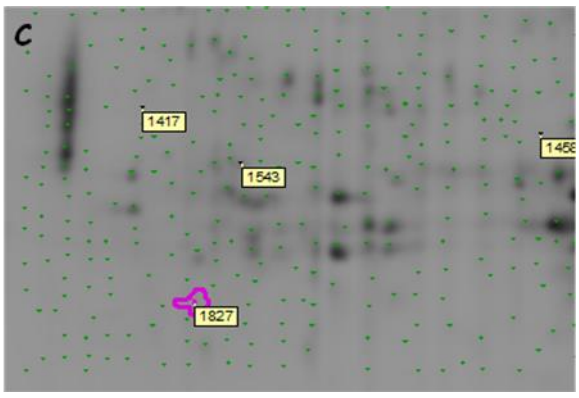


Fig. 4.20. 2DIGE maps of the spots differentially regulated in the comparison between the control and transgenic samples (A), control infected and transgenic infected (B) and transgenic and transgenic infected (C). In the tabs the spots number with their relative t-test value, the appearance of the spots on the total number of gel in the experimental design, the ANOVA value and the average ratio are reported.

Furthermore, the q-value estimation, in order to establish the percentage of calls of change of expression which were false positives in the analysis, was applied. The q-value calculated for all the spots resulted higher than the threshold of confidence (0.05), thus indicating that the spots considered significant from the t-test were false positive identifications.

All the pictured spots from two preparative silver stained gels (150, 200 µg) were excised from the gels and identified by Thermo Fisher Orbitrap MS. Analysis of uninterpreted tandem mass spectra using the MASCOT search engine did not show hits versus the green plant database, but underlined the presence of keratins contaminants. This is probably the consequence of the low abundance of the protein of interested on the gels in relation to the presence of keratins.

This analysis showed that the mature seeds metabolic proteome was not affected by the *Fusarium* infection. This is not surprising because the effects of the *F. graminearum* infection are more significant during wheat seed development, and less visible at maturity. Additionally, the effect of the fungal infection is probably masked by the asymptomatic seeds present in the infected spikelet (the proteome of the pathogen has not been found) to be detected by DIGE, and the effect of the *Pgip* transgene in the resistant response is not recognizable in the metabolic fraction of the mature seed.

4.3.2 Acid-PAGE of gliadins

In order to see if *F. graminearum* infection affected gluten protein accumulation, we analysed both gliadins and glutenins amount in the same groups above mentioned. Gliadins and glutenins are mostly responsible for the quality and technological traits of the dough.

Twelve gliadins extractions (4 groups x 3 technical replicates) were run on A-PAGE gels for all the biological replicates.. One dimensional patterns of gliadin separations from the control, control infected, transgenic and transgenic infected groups is illustrated in Fig. 4.22 .

No significant differences in the total (α/β, ω, γ) gliadins pattern were detectable comparing the mean value of each group by densitometry analysis of the lanes. This demonstrates that there are not effects due to the *F. graminearum* infection or to the transgene in the gliadins synthesis.

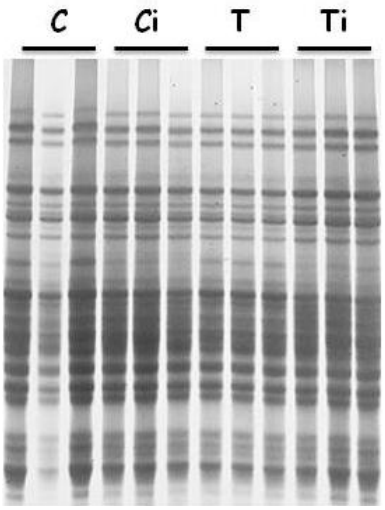


Fig.4.22 A-PAGE of the gliadin extracts from the control (C), control infected (Ci), transgenic (T) and transgenic infected (Ti) pool of seeds. The gliadins were visualized with Coomassie and every sample was run in triplicate.

Also the graph (Fig. 4.23), in which the absolute values from the densitometry analysis of the different groups were plotted, clearly showed that inter group variance is higher than the variance observed intra-group. Those data were confirmed from the direct comparison of each couple of group using the t-Student test. The observed changes in the gliadin synthesis are wider in intra-group comparison than among the groups. Moreover, there is not a significant trend of differences in gliadin accumulation, by comparing the different groups and considering all the biological replicates of the same sample group. In the mature seed, where the pathogen influence is less evident than during the seed development, the gliadin composition is not significantly affected from the infection with *F. graminearum* in both transgenic (more tolerant) and null-segregant (more susceptible) plants.

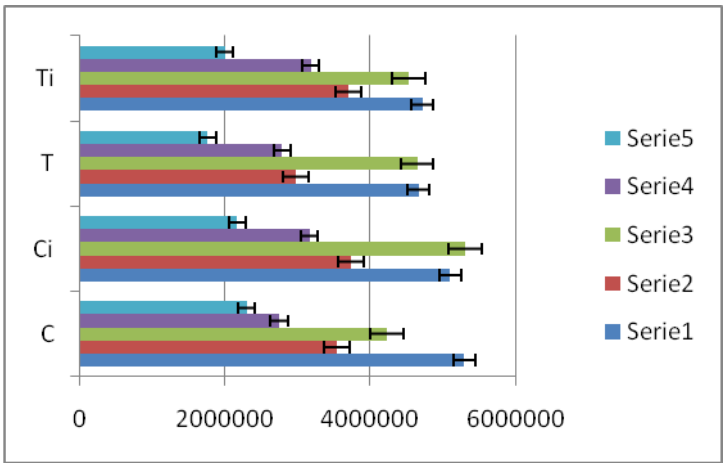


Fig.4.23 Graph bar of the absolute values from densytometry analysis of the gliadins in the control (C), control infected (Ci), transgenic (T) and transgenic infected (Ti) pool of seeds. The biological replicates are reported as series from 1 to 5 and the the black bars represent the standard errors

4.3.3 SDS-PAGE of HMW-GS and LMW-GS

The same samples used in the gliadins analysis (illustrated in the previous paragraph) were investigated to find out the possible differences in the protein profiling of the glutenin subunits (GS). (Fig. 4.24).

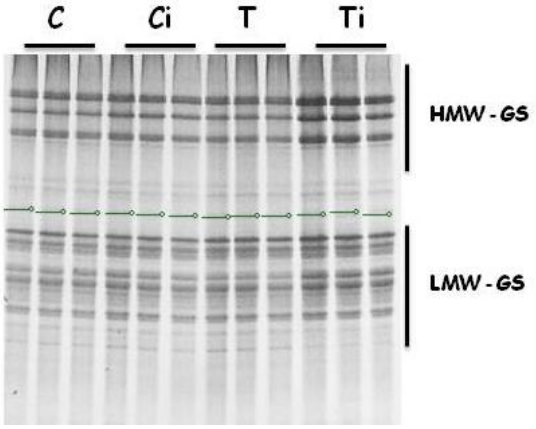


Fig.4.24 SDS-PAGE of the HMW-GS and LMW-GS extracts from the control (C), control infected (Ci), transgenic (T) and transgenic infected (Ti) pool of seeds. The GS were visualized with Coomassie and every sample was run in triplicate.

Only the comparison between the transgenic infected samples and the control infected samples showed a slightly decrease (1.09) in the accumulation of the total glutenin subunits and HMW-GS (1.12) with a significant value of the t-student test (p=0.05). On

the other hand this trend was not confirmed for the LMW-GS that did not result differentially regulated in any of the comparison performed. Additionally the ratio between the HMW-GS and LMW-GS, that is closely associated with quality parameters, did not show significant changes, in all the groups in comparison to the control plants, thus confirming that the quality traits of the flours are not affected significantly by the infection in the transgenic plants.

Also the graphs relative to the accumulation of the total glutenin subunits (both HMW-GS and LMW-GS) (Fig.4.25, 4.26 and 4.27) showed that, except for the above mentioned comparison between the control infected and the transgenic infected genotypes, the variance observed into every single infection experiment was higher than the variance due to the infection with *F. graminearum* in susceptible genotype.

We do not have any reliable explanation about the observed slight decrease in HMW-GS (and consequently in total glutenin subunits) in the infected transgenic genotype and we think that it might not be a consistent result, since all the other gluten protein classes were not affected.

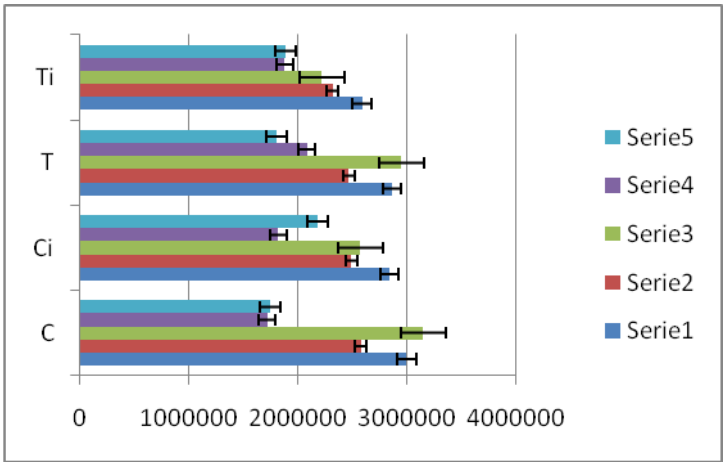


Fig.4.25 Graph bar of the absolute values from densytometry analysis of the total Glutenin subunits (GS) in the control (C), control infected (Ci), transgenic (T) and transgenic infected (Ti) pool of seeds. The biological replicates are reported as series from 1 to 5 and the the black bars represent the standard errors.

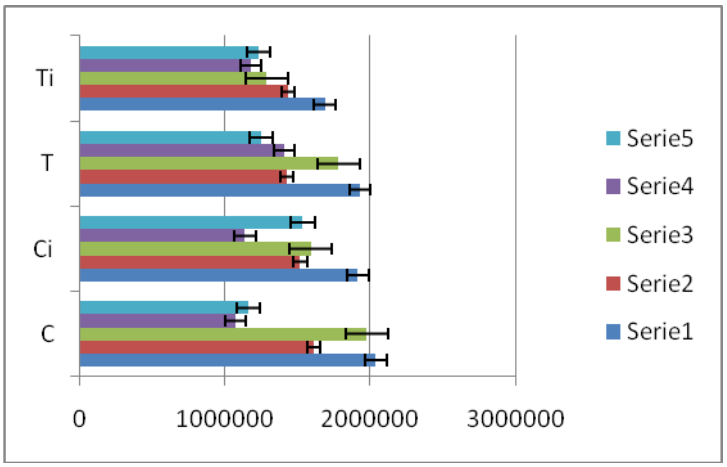


Fig.4.26 Graph bar of the absolute values from densytometry analysis of the HMW-GS in the control (C), control infected (Ci), transgenic (T) and transgenic infected (Ti) pool of seeds. The biological replicates are reported as series from 1 to 5 and the the black bars represent the standard errors.

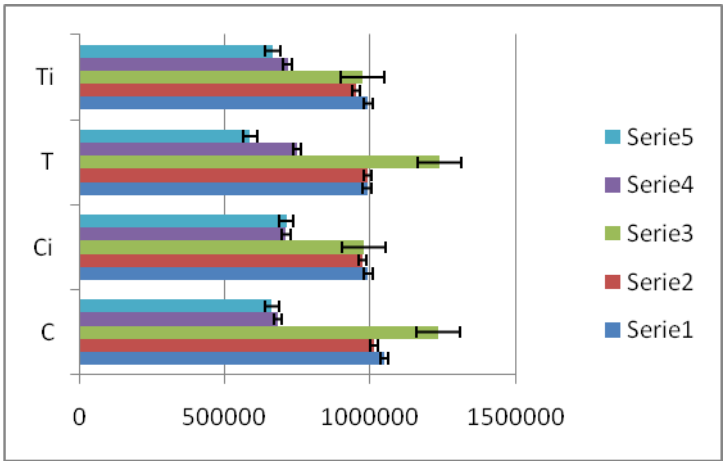


Fig.4.27 Graph bar of the absolute values from densytometry analysis of the LMW-GS in the control (C), control infected (Ci), transgenic (T) and transgenic infected (Ti) pool of seeds. The biological replicates are reported as series from 1 to 5 and the the black bars represent the standard errors.

4.3.4 Starch accumulation

To provide a total picture of the biotic effect of *F. graminearum* infection, the total starch in the mature seeds was analyzed in the different wheat genotypes (transgenic and control), investigating if the pathogen alters the starch accumulation and if the transgenic plants maintain the same nutritional characteristics after the fungal infection.

The results showed that the starch content varied between 37% to 58 % and was not significantly altered, considering the biological replicates, both after the infection with the pathogens and in the transgenic individuals. The graph illustrates (Fig. 4.28) the

starch accumulation in all the different biological replicas of each groups compared. Moreover, comparing the average value (the mean of the biological replicates) of the starch accumulation, the graph (Fig. 4.29) showed, although not significantly, the reduction trend in the starch content in the control plants in relation to the control infected, transgenic and transgenic infected individuals.

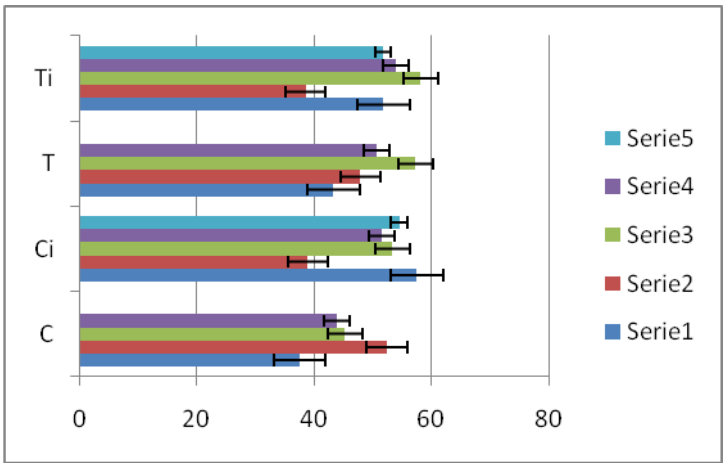


Fig.4.28 Graph bar of the percentage of the total starch presents in the control (C), control infected (Ci), transgenic (T) and transgenic infected (Ti) pool of seeds. The biological replicates are reported as series from 1 to 5 and the the black bars represent the standard errors.

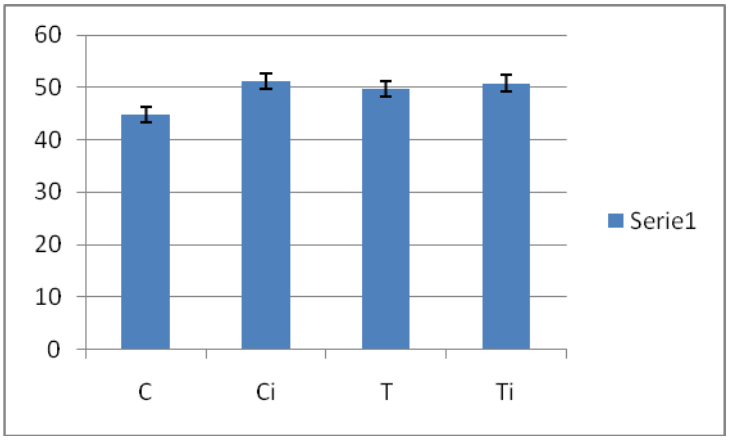


Fig. 4.29 Hystogram of the total starch mean values (%) of the biological replicates in the control (C), control infected (Ci), transgenic (T) and transgenic infected (Ti) pool of seeds.

These data show that there is no significative difference between the four groups here analyzed, thus confirming the same safety, quality and nutritionals traits of the transgenic materials in comparison with the wild type ones, at least at the mature stage.

5.CONCLUSIONS

The results reported offer a picture of the consequences of heat stress occurring during grain filling on the accumulation of the soluble proteins in the mature seeds of the widely grown Italian durum wheat *cv* Svevo. The results provide a basis for understanding how this environmental change influences protein synthesis and consequently the metabolic and quality traits of the durum wheat kernel. Moreover, since the durum wheat kernel is of primary interest because the semolina obtained by crushing mature seeds is the basis of many products of common use, among which pasta is the most important, it is critical to understand if the different types of stresses that wheat plants can potentially undergo, may alter protein composition, and, consequently, qualitative and nutritional properties of the derived products.

In the present study 47 metabolic proteins were identified from the soluble seed fraction of durum wheat which were induced or repressed by heat shock. The fold changes observed were between 1.2 and 2.5. In agreement with previous studies performed in bread wheat cultivars (Majoul *et al* 2003, Hurkman *et al* 2009) both HSP70 and LEA proteins were up-regulated in response to heat stress. Other proteins, such as ATP synthase β subunit and nucleoside diphosphate kinase (NDPK) related to the energy metabolism also responded to heat treatment. This agrees also with the findings of Hurkman (Hurkman *et al* 2009). Interestingly, we did not find any difference in the level of expression of starch related enzymes, although this was a finding of Majoul (Majoul *et al* 2004) in bread wheat. This might be a result either of the different protein extraction procedures, or of a different protein turnover in the plant material, or the enzyme forms that are regulated in bread wheat may be coded by genes present on the D genome, that is absent in durum wheat.

GAPDH, a housekeeping enzymes involved in glycolysis, was found to be up-regulated. Others recently suggested a relationship between this enzyme and certain abiotic stresses (Bustos *et a*l 2008). Proteins reported to be influenced by abiotic stresses, e.g. oxidative and drought stresses, and also found as differentially regulated in the present work include 14-3-3 proteins, serpins, LEA proteins, 1-Cys peroxiredoxin, glyoxalase I, and proteins with a storage function (e.g. globulin-like proteins). It is noteworthy that some of the differentially regulated proteins are considered as allergens (α-amylase

inhibitors, serpins, tritins, GAPDH) and were found to be up-regulated after heat stress, which obviously represents a disadvantage for sensitive individuals.
These results illustrate that there is a common network "response" to different types of abiotic stress, such as drought, oxidative, cold and heat stress.

In addition the results obtained in the study of the durum wheat leaf proteome (cv Svevo) for the cold stress investigation provided an optimized method to discriminate on 2DE the total leaf proteins. We compared the 2DE protein pattern obtained using two different methods to deplete the RuBisCO, based on the immunopurification by affinity column (Cellar et al 2008) and the PEG fractionation (Kim *et al* 2001), with a classical protein extraction method based on TCA and UREA (Donnelly *et al* 2005). Both the RuBisCO depletion strategies showed limitations due the technical variability in the solubilization or precipitation of some groups of spots. Those methods resulted not enough reproducible for comparative proteomic analyses. In contrast using TCA and UREA extraction protocols, that does not get rid of the RuBisCO, we visualized, on 2DE maps, 580 spots well resolved in the pH 3-10 narrow. This result improved (about 20% more) the number of spots resolved on 2DE gels in relation to the results published in the study of the bread wheat leaf proteome (Donelly *et al* 2005).
Unfortunately, it was not possible to perform the analysis of the proteomic response to cold stress, because it was impossible to reach the requested optimal thermal regimes, due to technical problems..

Finally, the results regarding the study of the *Fusarium graminearum* infection performed on bread wheat transgenic plants, encoding a bean PoliGalacturonase Inhibitor protein (Janni *et al* 2008), suggested that there were not significant alterations in the metabolic proteins profile in the mature seed, both between infected and not infected plants, and between transgenic and null segregant genotypes. The DIGE investigation did not discriminate any proteins differentially regulated in the transgenic individuals in relation to the their relative control (null-segregant); moreover, the infection with *Fusarium graminearum* did not influence the protein expression in the metabolic protein compound of the seed collected at the maturation phase.

In accord with the situation observed in the metabolic protein fraction of the grain, also the gliadins synthesis was not modified both by the fungal infection and by the genetic transformation at seed maturation. Only the high molecular weight glutenin subunits (HMW-GS) resulted slightly down regulated (≈1.10 fold change) comparing the control individuals infected with the transgenic plants infected, although we do not have any reliable explanation to this observation.
Also the total starch accumulation in the infected caryopses did not differ comparing the seeds deriving from the transgenic plants and their relative control individuals.
All these experimental evidences confirmed that, even if the quantity of the micotoxins (such as DON) were not taken in exam for the high dose of material requested, the major parameters responsible for the flour quality were not altered in the transgenic grains, after the *F graminearum* infection, in relation to the mature grains collected in "unstressed" conditions.

In conclusion this study provides a global picture of the effects of both the heat stress and the *Fusarium graminearum* infection (on susceptible and resistant genotyopes) on the wheat seed proteome, underlying the consequences of those "stressors" on the quality traits of the derived flour and semolina that are the principal compounds of the human diet.

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