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Coordinatore: Prof.ssa Annunziata Giangaspero

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STUDY OF RESPONSES TO HEAT STRESS IN DURUM WHEAT, AND THEIR RELATIONSHIP WITH NUTRACEUTICAL QUALITY

Dottorando:
De Leonardis Anna Maria

Relatore:
Dr. Mastrangelo Anna Maria

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ABSTRACT

Durum wheat [*Triticum turgidum* (L.) subsp. *turgidum* (L.) convar. *durum* (Desf.)] is an important crop for human nutrition, especially in the Mediterranean area, where it is the main source of semolina for the production of pasta, traditional/typical bread, couscous, and burghul. Environmental stress can strongly limit the yield potential and affect qualitative characteristics of grain. The stress conditions that are commonly experienced by crops are extreme lack or excess of water (i.e., drought, flooding), presence of salt or contaminants (e.g., heavy metals), and temperature (i.e., cold, heat). In particular, environmental temperatures have increased since the beginning of the last century and they are predicted to further increase under the present conditions of climate change.

Wheat is very sensitive to high temperatures during the reproductive phase, due to direct effects of temperature on grain numbers and dry weight. These phenotypic effects are due to molecular modifications at the different levels of gene expression, and to changes in metabolite accumulation levels.

The aim of this study was to investigate the transcriptomic and metabolomic variations in response to heat stress in grains of two durum wheat genotypes, and their relationship with variation in grain composition that can affect the nutraceutical quality of the final product. The study was carried out on the durum wheat cv Primadur and T1303 (PI 352395 USDA code); the first is an elite cultivar with high grain yield and yellow index and the second one an anthocyanin-rich purple cultivar. Heat shock was imposed at 37°C from 5 up to 10 days after flowering (DAF). Immature seeds (14 DAF) and mature seeds (seeds at physiological maturity stage), collected from heat shocked and control plants (20°C), were analyzed. The response to stress was investigated with different approaches. Genome–wide gene expression analysis, carried out using Illumina HiSeq2000, showed that 1202 genes were differentially expressed in response to stress in the two genotypes. The number of up-regulated genes belonging to stress-related categories was higher in Primadur with respect to T1303, whereas the number of genes with nutrient reservoir activity was larger in T1303 compared to Primadur. These results were according to the higher sensitivity to stress of Primadur with respect to T1303, measured as grain weight loss.

The profile of polar metabolites was investigated by gas chromatography-mass spectrometry (GC-MS). The effects of heat stress were genotype dependent. Although some metabolites (e.g., sucrose, glycerol) increased in response to heat stress in both genotypes, clear differences were observed. Following the heat stress, there was a general increase in most of the

analyzed metabolites in Primadur, with a general decrease in T1303. Heat shock applied early during seed development produced changes that were observed in immature seeds, and also long-term effects that changed the qualitative and quantitative parameters of the mature grain. The level of anthocyanins increased significantly in response to the heat stress whereas the levels of carotenoids were not affected. Only for T1303, the Trolox equivalent antioxidant capacity (TEAC) method assessed an increased antioxidant capacity of ground grains, in response to heat stress. Protein content increased only in T1303 that showed lower grain weight loss with respect to Primadur, in response to stress. Therefore, short heat-stress treatments can affect the nutritional and nutraceutical value of grain of different genotypes of durum-wheat in different ways.

The present study increased the knowledge about the effect of mechanisms of adaptation to stress of wheat plants on nutritional and quality traits of grain and provides a global picture of the effects of the heat stress on metabolies and transcriptome of two different genotypes of durum wheat.

Moreover, although the genetic backgrounds of two durum-wheat genotypes were different, it cannot be excluded that some of the differences observed in the responses to heat stress can be due to anthocyanin accumulation, which can be considered a trait of interest for future breeding activities for durum wheat.

Keywords

durum wheat, heat stress, metabolic profiling, transcriptomic analysis, antioxidant activity, anthocyanins

1. INTRODUCTION

1.1 Wheat

Cereals are the base of world agriculture and therefore they are a key element for food nutrition. Currently, the global area planted with cereals amounts to nearly 700 million hectares for a total production of 2.2 billion tonnes (FAO, 2009). Rice, wheat and maize are the main cereal cultivated in the world, with respectively 745, 713 and 1,016 million tonnes produced in 2013 (http://faostat3.fao.org/).

Wheat is grown over a large area and under a wide range of conditions; it is easily transported and safely stored over long periods of time and can be consumed by humans with minimal processing.

Durum wheat [*Triticum turgidum* (L.) subsp. *turgidum* (L.) convar. *durum* (Desf.)] is an important crop for human nutrition, especially in the Mediterranean area, where it is the main source of semolina for the production of pasta, traditional/typical bread, couscous, and burghul. Wheat contributes carbohydrates, essential minerals, vitamins, beneficial phytochemicals, dietary fibres and proteins to the human diet. Recently, the focus has been on the possibility of increasing the content of bioactive compounds to improve healt and prevent desease (Gani et al., 2012). Some bioactive compounds can be specific to certain cereals such as γ-oryzanol in rice and β-glucans in oat and barley; whereas anthocyanins and carotenoids are present in pigmented cereal grains of rice, corn, bread (*Triticum aestivum* L.) and durum wheat (Finocchiaro et al., 2010; Escribano-Bailon et al., 2004; Ficco et al., 2014).

The main components of the grain are the starch (65-79% of whole grain) and the proteins (7-22%), that are mostly responsible for the derived products quality. Due to the high content of starch wheat grain is a good source of calories. However, despite its relatively low protein content, wheat still provides protein for human and livestock nutrition, estimated at about 60 m tonnes per annum (calculated by Shewry, 2000).

The grain proteins, in particular the storage proteins that form a network in the dough called gluten, determine the visco-elastic properties of dough (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. Moreover, 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.

1.1.1 The origin of the wheat genomes

The first cultivation of wheat occurred 10,000 years ago, the 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 belong to the genus *Triticum* of the family *Graminaceae* and has a basic chromosome number (x) equal to 7; it includes diploid (2n=2x=14), tetraploid (2n=4x=28) and hexaploid (2n=6x=42) wheat species. 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 from 1 to 7. Wheat genomes 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 and B genomes of tetraploid and hexaploid wheats are clearly related to the genomes of *Triticum urartu* and *Aegilops Speltoides*, respectively. The D genome of hexaploid wheat is derived from that of *T. tauschii.* The S genome of *Aegilops Speltoides* is also close to the G genome of *T. timopheevi*, a tetraploid species with the A and G genomes (Feldman, 2001); whereas, the formation of hexaploid wheat occurred so recently that little divergence has occurred between the D genomes present in the hexaploid and diploid species.

A schematic representation of the origins of the different types of wheat is reported in Fig. 1.1. The large part of cultivated wheat have evolved from the wild type group *T. dicoccoides* (AABB), whereas *T. timopheevii* is a second tetraploid species cultivated only in Armenia and Transcaucasia. The wild progenitor of *T. timopheevii* is *T. araraticum* (G genome), which is closely related to the genome of *Ae. speltoides* (S genome). 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. Durum wheat is particularly suited to the production of pasta products, but also of some types of bread and couscous in the Mediterranean Region; it is getting more and more importance with the increase of pasta products demand in these last years. The hexaploid wheats *T. aestivum* (the common "bread wheat") originated 6,000-7,000 years ago by natural hybridisation of tetraploid wheat, most likely *T. dicoccum* (AABB) with the diploid wild grass

Ae. squarrosa (DD), also known as *T. tauschii* (Miller, 1987). 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.

Few other domesticated 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", and spelt continues to be grown in Europe, particularly in Alpine areas. Several tetraploid wheats with purple grain have been identified, including *Triticum dicoccum* accessions, which originate from Ethiopia. The trait was then introgressed into hexaploid wheats, where this trait has been widely investigated (Zeven 1991; Eticha *et al*. 2011).

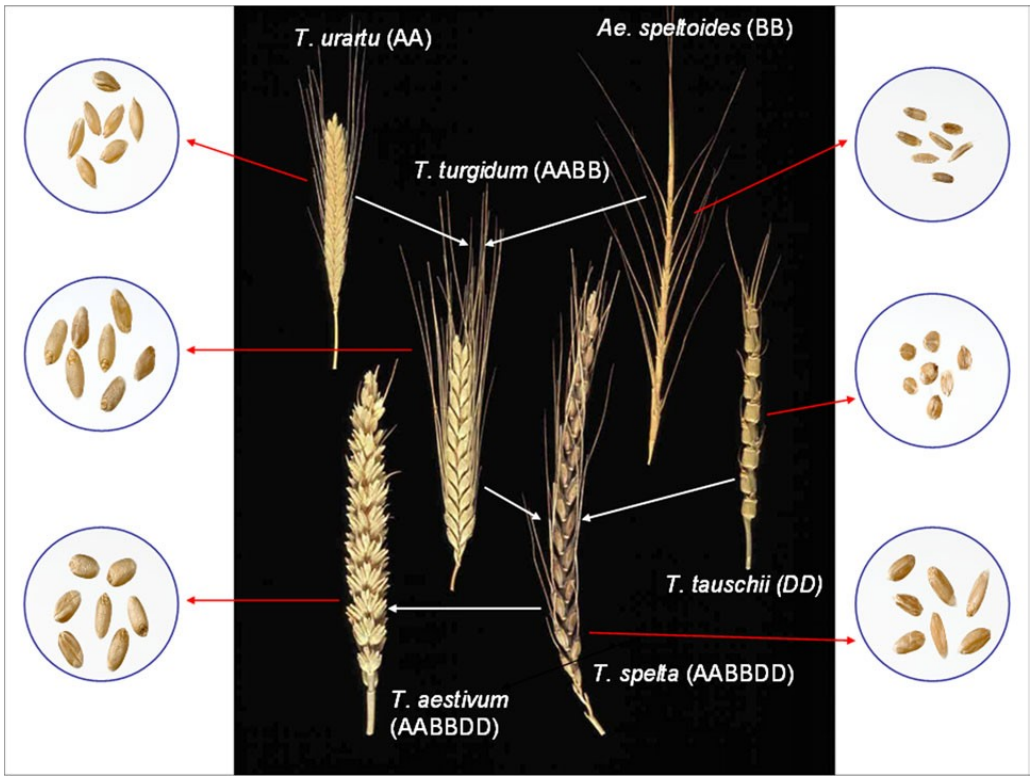


Fig. 1.1. The evolutionary and genome relationships between cultivated bread and durum wheats and related wild diploid grasses, showing examples of spikes and grain (from Shewry, 2009).

1.1.2 The grain structure and composition

Wheat is widely consumed by humans. Due to the high content of starch, the wheat is considered a good source of calories; nevertheless, it contributes essential amino acids, minerals, and vitamins, and beneficial phytochemicals and dietary fiber components to the human diet, and these are particularly enriched in whole-grain products (Shewry et al., 2009). Wheat and other cereals are significant sources of iron and zinc minerals.

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. 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.

The bran of the wheat grain is composed by a series of different cell layers. The "pericarp" (fruit coat) 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 contains significant quantities of fiber, phenolic compounds, pigments (carotenoids and anthocyanins) and dietary minerals.

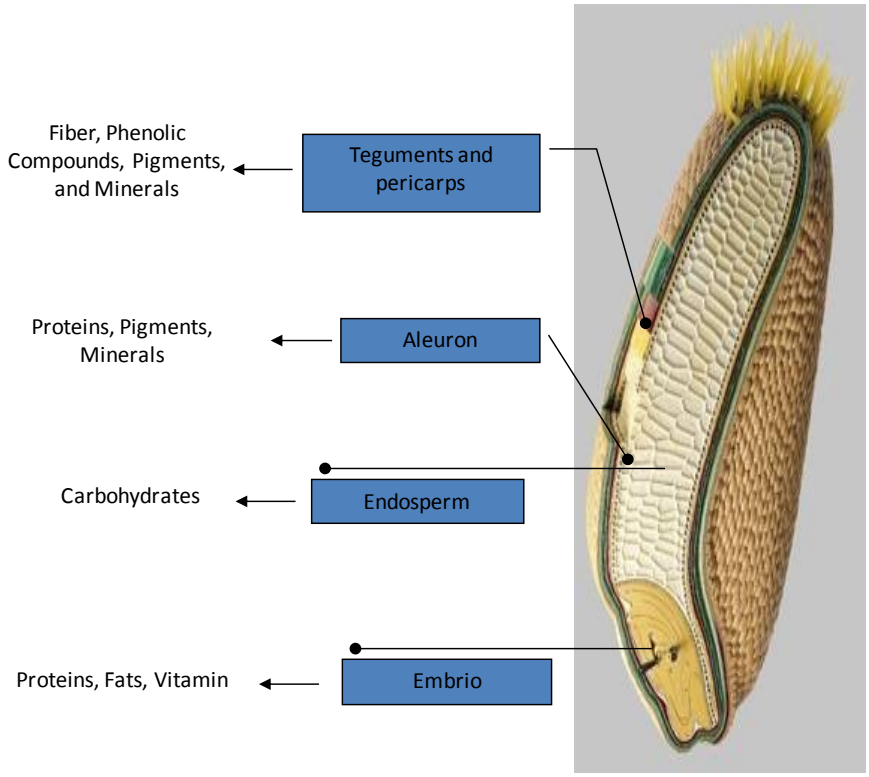


Fig 1.2. Structure of the wheat grain and chemical composition

The major compounds of the wheat mature grain are the endosperm and the embryo or germ. They derive from two different fecundation processes that generate the diploid zygote (embryo or germ) and the triploid endosperm; the first generates the scutellum and the embryonic axis, the second develops and differentiates in the starchy endosperm and in the aleurone. The starchy endosperm is about 80% of the grain and is the first source of carbohydrate. The aleurone tissue has functions of storage; it secretes the amylase enzyme during germination and is source of proteins, pigments and dietary mineral. The wheat embryo is rich in proteins, fats and vitamins. Cereals are significant sources of tocols which include vitamin E (27.6–79.7 μg/g) (Lampi et al., 2008) and sterols (670–959 μg/g) (Nurmi et al., 2008) and are considered to account for about 22% of the daily intake of folate (vitamin B12) in the UK (Goldberg, 2003).

The seed protein content is the most important factor determining wheat quality and are a good source of essential amino acids. Wheat kernel proteins are divided according to their solubility properties into prolamins (gliadins and glutenins, collectively known as gluten proteins), soluble in dilute acid or alkali or alcohol–water mixtures, and albumins and globulins, which are water and salt soluble, respectively (Osborn et al., 1924). Gluten proteins represent about 80% of wheat seed proteins, and are the most important determinant of the dough properties. Out of the 20 amino acids commonly present in proteins, 10 can be considered to be essential in that they cannot be synthesized by animals and must be provided in the diet. Recommend levels of essential amino acids for adult human compared with those present in wheat grain and flour are reported in tab.1.1(Shewry, 2009).

Table 1.1 Recommended levels of essential amino acids for adult humans compared with those present in wheat grain and flour (expressed as mg/g protein)

Amino acid protein	FAO/WHO/UNU[a]	Wheat	
		Grain[b]	Flour[b]
Histidine	15	23	22
Isoleucine	30	37	36
Leucine	59	68	67
Lysine	45	28	22
Methionine + Cysteine	22	35	38
Methionine	16	12	13
Cysteine	6	23	25
Phenylalanine + tyrosine	38	64	63
Threonine	23	29	26
Tryptophane	6	11	11
Valine	39	44	41
Total indispensable amino acids	277	339	326

[a]FAO/WHO/UNU (2007).
[b] Calculated from literature values as described in Shewry (2007).

Wheats are also a good source of minerals such as zinc, iron and selenium (He and Ning, 2003). Plant selenium content is largely determined by the amount of Se and sulphur availability in the soil which competes to prevent selenium uptake (Gissel-Nielsen et al., 1984; Fan et al., 2008) and some wheat genotypes with high levels of anthocyanins in grains have also been found to have higher contents of amino acids and minerals including selenium (He and Ning, 2003). Selenium has been identified as an essential animal nutrient (Schwarz and Foltz, 1957) and many human health conditions appear to be affected by selenium status; these conditions include heart, immune, and endocrine functions (Rayman, 2008). Selenium in wheat grain has high bioavailability (83%) (Thomson et al., 2004) and the role of the selenium content of wheat in human nutrition has been discussed in a literature review (Tamás et al., 2010).

Iron is predominantly located in the aleurone and its bioavailability is restricted because it complexes with phytate (myo-inositolphosphate 1,2,3,4,5,6-hexa-kisphosphate). Some studies have been carried out to increase mineral bioavailability, in particular an heat-stable form of the enzyme phytase was expressed in the developing grain to allow hydrolysis of complexes to occur during food processing (reviewed by Brinch- Pedersen et al., 2007).

Lignans, a group of polyphenols with phytoestrogen activity, are present at levels up to about 20 μg/g in bran (Nagy-Scholz and Ercsey, 2009), while total phenolic acids in wholemeal range up to almost 1200 μg/g (Li et al., 2008). Ferulic acid is abundant among phenolic compounds and possesses antioxidant property (Sobrattee et al., 2005).

Carotenoid pigments are differently distributed in the wheat kernel: α- and β-carotene are mainly located in the germ while lutein, the most abundant pigment, is equally distributed across the kernel (Borrelli et al. 2008, Abdel-Aal et al. 2007; Digesù et al. 2009).

Anthocyanins are pigments for which a wide range of variability has been documented in wheat grains (Li and Beta, 2011; Varga et al. 2013); they are predominant in the external layers of grain (Adom et al. 2005) and in blue wheat pigments are prevalently located in the aleurone, whereas in purple wheat they are distributed in the pericarp (Zeven 1991; Abdel-Aal et al., 1999).

1.2 The responses to abiotic stress in plants

Stress is defined as any soil and climatic conditions or combination of both that can affect the full realization of genetic potential of plants, limiting their growth, development and reproduction. Plants use various mechanisms to cope with stress conditions including their morphology, physiology and metabolism at organ and cellular levels (Levitt, 1972).

Plants posses a number of escape, avoidance and tolerance strategies. Length of life cycle or developmental plasticity (Araus et al., 2002) are examples of escaping strategies that allow the plant to complete its life cycle before adverse conditions occurs. Avoidance strategies limit the exposition of tissues and cells to stress; as an example, in case of drought stress avoidance strategy helps the plant to maintain high water status either by a more efficient water absorption from roots or by reducing evapotranspiration from aerial parts (Chaves et al., 2002). Tolerance strategies adopted from plants include osmotic adjustment mechanisms, enhanced antioxidant capacity and physical desiccation tolerance of the organs that allow to withstand water deficit with low tissue water potential (Ingram and Bartels, 1996). The osmotic compounds, synthesized in response to stress, include proteins and aminoacids (like proline, aspartic acid and glutamic acid), methylated quaternary ammonium compounds (e.g. glycine betaine, alanine betaine), hydrophilic proteins (e.g. late embryogenesis abundant (LEA), carbohydrates (like fructan and sucrose) and cyclitols (e.g. D-pinitol, mannitol) (De Leonardis et al., 2012). Some of these mechanisms and the production of some osmotic compounds is common to different stress conditions.

Abiotic stress conditions which are commonly experienced by crops refer to extreme water amount (drought or flooding), presence of salt or contaminants like heavy metals, and temperature (cold and heat stress). Environmental temperatures in particular have increased since the beginning of the last century and are predicted to further increase under climate change (IPCC, 2007), also in the major wheat-producing regions (Alexander et al., 2006; Hennessy et al., 2008). Climate models predict an increase in global air temperature between 1.8 and 4.0°C by the end of this century (IPCC, 2007).

Plants detect changes in ambient temperature through perturbations in metabolism, membrane fluidity, protein conformation and assembly of the cytoskeleton (Ruelland and Zachowski, 2010).

Extreme temperatures also cause several metabolic changes associated to impairment in electron transport chains and production of ROS, such as at level of the membrane bound NADPH oxidase (McClung and Davis, 2010; Theocharis et al, 2012). In addition, another primary target of this stress is the photosynthetic system, which is sensitive to temperature changes for the high content of unsaturated fatty acids in the thylakoid membrane (Allakhverdiev et al., 2008).

The heat temperature stimulus generates a signal which is further transmitted to initiate appropriate physiological and biochemical changes for the plant survival under heat stress (Saidi

et al., 2009). Initial effects of heat stress are on plasmalemma which shows more fluidity of lipid bilayer under stress. This leads to the induction of Ca^{2+} influx and cytoskeletal reorganization, resulting in the upregulation of mitogen activated protein kinases (MAPK) and calcium dependent protein kinase (CDPK). Schematic representation of the temperature sensing machinery in plants is presented in Fig.1.3.

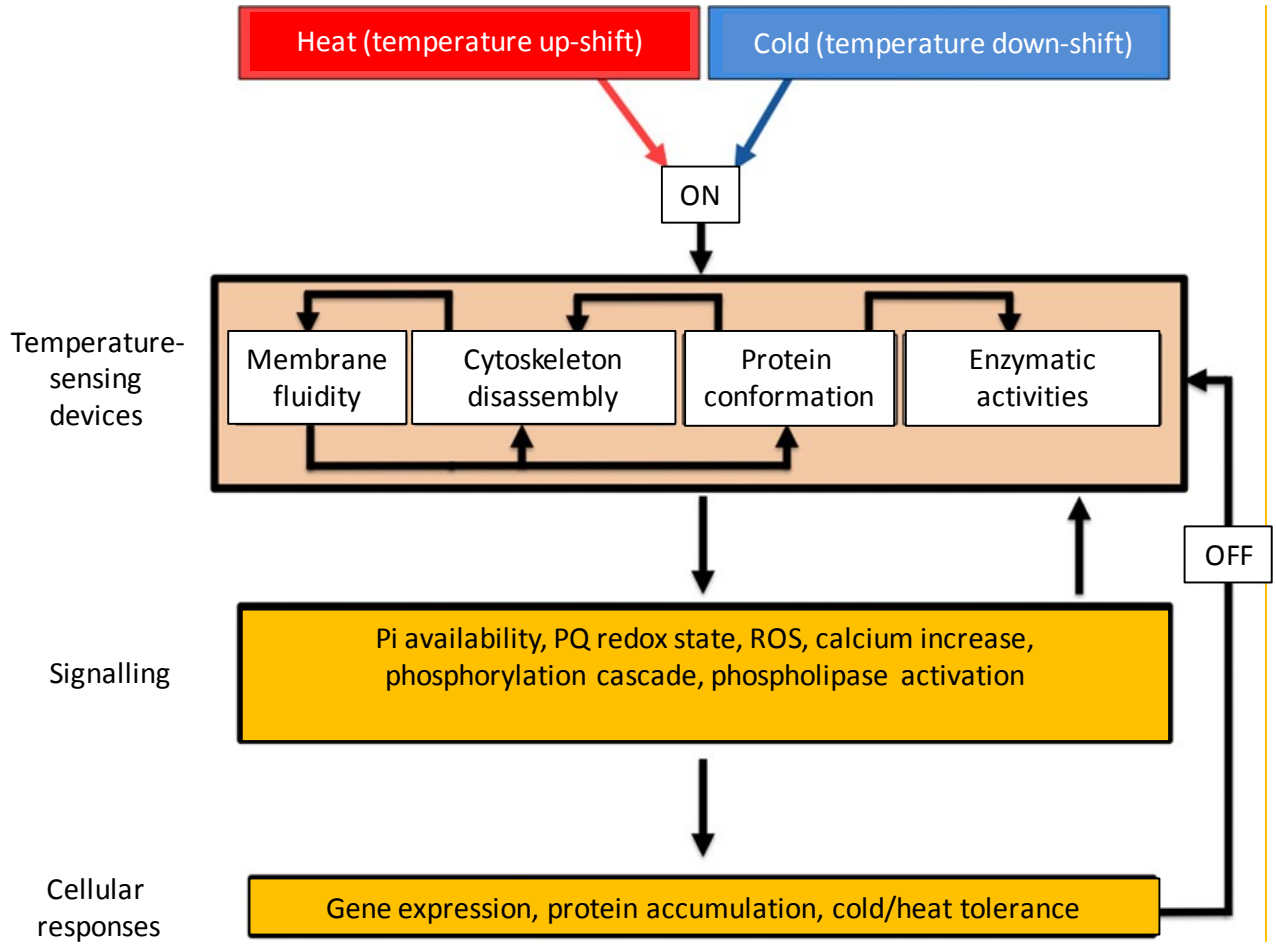


Fig.1.3 Schematic representation of the temperature sensing machinery in plants (Ruelland and Zachowski, 2010).
Cold and heat sensing mechanisms in plants are the very same cellular processes disturbed by a temperature change. Membrane fluidity, protein conformation, cytoskeleton assembly status and enzymatic activities can be interlinked: cytoskeleton depolymerization may be a consequence of monomer conformation changes and/or of the impact of a change in membrane fluidity; because of the link between membranes and cytoskeleton, cytoskeleton disassembly may also influence membrane fluidity; enzymatic activities are dependent on their kcat but conformational change of the enzymes can also impact on their activities. The signalling pathways downstream of the sensing steps can also influence these sensing steps. Ultimately, the cellular responses activated in response to heat or cold will participate in switching off the temperature sensing devices.

Ion transporters, proteins, osmoprotectants, antioxidants, and other factors are involved in signaling cascades and transcriptional control for the induction of gene expression and for metabolite synthesis to improve tolerance to heat stress (Mittler et al., 2012). For gene expression, heat shock proteins are up-regulated in plants, and tobacco plants overexpressing

heat shock proteins (HSPs) are better able to withstand heat stress as they protect proteins from heat-induced damage (Wang et al., 2015). At physiological level, plants manifest different mechanisms for surviving under elevated temperatures, including long-term evolutionary phenological and morphological adaptations and short-term avoidance or acclimation mechanisms such as changing leaf orientation, transpirational cooling, or alteration of membrane lipid compositions (Cossani et al., 2012). The severity of heat stress is a function of the magnitude and rate of temperature increase, as well as the duration of exposure to the raised temperature and photosynthesis is the most sensitive physiological process to elevated temperature (Wahid et al*.,* 2007).

Heat stress reduces plant photosynthetic capacity through metabolic limitations and oxidative damage to chloroplasts, with concomitant reductions in dry matter accumulation and grain yield (Farroq et al., 2011).

In many crop plants, early maturation is closely correlated with smaller yield losses under high temperatures, which may be attributed to the engagement of an escape mechanism (Adams et al., 2001). Environmental stresses can strongly limit yield potential and affect grain qualitative characters (Cattivelli et al., 2008; Balla et al., 2011) and wheat is very sensitive to high temperature during the reproductive phase due to the direct effect of temperature on grain number and dry weight (Wollenweber et al., 2003; Dias et al., 2011). The optimum temperature during grain filling is around 21°C (Porter and Gawith, 1999; Farooq et al., 2011). Maintaining grain weight under heat stress during grain filling is a measure of heat tolerance (Singha et al*.,* 2006). High temperature accelerates the rate of grain filling and Dias and Lidon (2009) proposed that high grain-filling rate and high potential grain weight can be useful selection criteria for improving grain yield under heat stress.

To adapt new crop varieties to the future climate, we need to understand how crops respond to elevated temperatures and how tolerance to heat can be improved (Halford, 2009; Mittler et al., 2012). The interactions among different elements (DNA, RNA, proteins and metabolites) with the environment (favorable or adverse conditions) and the developmental stage result in the phenotype of an organism. Therefore the plant responses to heat stress can be explained if the different levels of gene expression are considered in a multidisciplinary approach.

1.2.1 The effect of heat stress on transcriptome changes in plants

Different components of tolerance, controlled by different sets of genes, are critical for heat tolerance at different stages of plant development or in different tissues (Howarth, 2005; Bohnert et al., 2006). Despite advances in our understanding of genes of major effect conferring disease resistance in wheat (Krattinger et al., 2009), the genetic basis of heat adaptation is poorly understood.

The quantitative trait locus (QTL) approach has been used to study heat stress alleles because they show interaction with both environment and genetic background (Pinto et al., 2010); it identifies genetic markers that are associated with genes affecting whole plant stress tolerance or individual components contributing to it.

Another approach that can be used is the analysis of transcriptome in response to heat stress, despite different results can be obtained in relation to the modality of stress imposition, the stage of plant development and the tissue analysed (De Leonardis et al., 2007).

Heat stress is known to swiftly alter patterns of gene expression (Yang et al., 2006), regulating the expression of the heat shock proteins (HSPs). There are evidences of increased thermotolerance of transgenic lines expressing higher levels of HSP chaperones with respect to wild type (Sanmiya et al., 2004). Other genes with an important role in heat stress tolerance are chloroplast proteins, as shown for wheat (Fu et al., 2008). Heat stress may also affect splicing of some mRNAs that can led to the production of new proteins with a role in heat stress defense mechanisms (Mastrangelo et al., 2012).

There are several platforms available to perform the analysis of gene transcripts: the extremely accurate qRT-PCR that allows only a limited number of genes to be analyzed (Steibel et al., 2009); the gene microarray technology allowing the analysis of thousands of genes at a time (Brady et al., 2007; Aprile et al., 2009, 2013) and RNA-seq oriented to the performance of unbiased analysis of RNA transcripts (Filichkin et al., 2010) which generates gigabyte size readouts with virtually all of the RNA transcripts of a given cell, organism or tissue. The increasing amount of data generated pushed researchers to create databases of experiments covering different organs (Birnbaum et al., 2003), tissues and cell types (Iyer-Pascuzzi et al., 2011), developmental events (Schmid et al., 2005) and environmental cues (Dinneny et al., 2008), allowing to take a sneak peek into the expression of gene candidates in advance.

As an example, Genevestigator (https://www.genevestigator.com/gv/index.jsp) database allows a preliminary survey of the gene candidates before programming any experiment, and

Arabidopsis eFP Browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) shows gene expression at the organ level. Moreover, with RNA-seq is also possible to detect diverse variants of mRNA and other RNA molecules such as noncoding RNA or small RNAs in different organisms (Breakfield et al., 2012).

Molecular response to heat stress can be different if it occurrs in presence or absence of other environmental stresses such as water deficit. A number of studies described the transcriptional changes induced in response to drought (Aprile et al., 2009; Ergen et al., 2009) and heat (Qin et al., 2008) stresses. The results from heat stress-responsive transcriptome analysis in heat susceptible and tolerant wheat genotypes confirmed that 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).

Several studies indicate that the molecular response to the combination of heat and drought activate networks that are different from those activated by heat or drought stress taken singularly (Atienza et al., 2004; Mittler et al., 2006). Recently Aprile et al. (2013) reported a microarray-based transcriptomic analysis carried out on two durum wheat cultivars (Ofanto and Cappelli) subjected to heat, drought and to a combination of drought and heat stresses, conditions similar to the experience of a crop grown in Mediterranean environments and exposed to a terminal heat/drought stress. This study shows that the two genotypes showed a different expression for many agronomic and physiological traits including an early activation of the senescence in Cappelli with respect to the Ofanto (De Vita et al., 2007; Rizza et al., 2012). Moreover they were characterized by two opposite stress-responsive strategies. A large set of well-known stress-related genes were activated in Ofanto, while Cappelli showed the constitutive expression of several stress-induced genes and a modulation of a limited number of genes in response to stress. In Ofanto (lower water use efficiency) the combination of drought and heat stress led to an increased number of modulated genes, exceeding the simple cumulative effects of the two single stresses, whereas in Cappelli (higher water use efficiency) the same treatment triggered a number of differentially expressed genes lower than those altered in response to heat stress alone. This work provides clear evidences that the genetic system based on wheat genotypes with contrasting phenotypes represents an ideal tool for the genetic dissection of the molecular response to abiotic stresses. These genotypes were used to develop a segregating population that was used to construct a linkage map (Marone et al., 2012) on which QTL for leaf porosity and chlorophyll content have been localized (Panio et al., 2013).

1.2.2 The effect of heat stress on proteome changes in plants

Expression of stress proteins is an important adaptation to cope with environmental stresses. Most of the stress proteins are soluble in water and therefore contribute to stress tolerance presumably via hydration of cellular structures (Wahid and Close, 2007) but there is considerable evidence that acquisition of thermotolerance is directly related to the synthesis and accumulation of HSPs (Bowen et al., 2002). Heat stress affects synthesis and functionality of HSPs (Timperio et al., 2008) but other classes of proteins are also involved (Fu et al., 2008) and proteome analysis is an effective tool for investigation of changes in protein accumulation in response to heat stress, as shown for wheat kernels (Wahid et al., 2007; Laino et al., 2010). Increased production of HSPs occurs when plants experience an increase in temperature (Nakamoto and Hiyama, 1999), and prevent denaturation of other proteins. Induction of HSPs seems to be a universal response to temperature stress, being observed in all organisms ranging from bacteria to human (Vierling, 1991).

In higher plants, HSPs are usually induced under heat shock at any stage of development. HSP90, HSP70 and low molecular weight proteins of 15–30 kDa are the three principal classes of proteins, as distinguished by molecular weight. The special importance of small HSPs in plants is suggested by their unusual abundance and diversity. HSP70 has been extensively studied and is proposed to have a variety of functions such as protein translation and translocation, proteolysis, protein folding or chaperoning, suppressing aggregation, and reactivating denatured proteins (Zhang et al., 2005). Small HSPs are able to assemble into heat shock granules (HSGs) and their disintegration is a prerequisite for survival of plant cells under continuous stress conditions at sub lethal temperatures (Miroshnichenko et al., 2005). All small-HSPs in plants are encoded by six nuclear gene families, each gene family corresponding to proteins found in distinct cellular compartments like cytosol, chloroplast, endoplasmic reticulum (ER), mitochondria and membranes.

Five mitochondrial LMW-HSPs (28, 23, 22, 20 and 19 kDa) were expressed in maize subjected to heat shocks (42 °C), whereas only one (20 kDa) was expressed in heat shocked wheat and rye; the difference in the number of HSPs suggested the reason for higher heat tolerance in maize than in wheat and rye (Korotaeva et al., 2001). Two members of the HSP family, HSP70 and HSP26 showed increased protein abundance after heat stress in durum wheat grains (Laino et al., 2010).

Among other stress proteins, expression of ubiquitin (Mazzucotelli et al., 2006), Mn-peroxidase (Iba, 2002), late embryogenesis abundant (LEA) (Goyal et al., 2005; Laino et al.,

2010) and dehydrins has also been established under heat stress. These proteins act protecting cellular and sub-cellular structures against oxidative damage and dehydration. Protein synthesis elongation factor in chloroplast (EF-Tu) has also been related to heat tolerance in several crops. In wheat, transgenic plants over-expressing EF-Tu protein showed less aggregation of leaf proteins and damage to thylakoids, and higher rate of CO_2 fixation compared with non-transgenic plants under heat stress. During wheat-grain filling, high temperatures affect starch biosynthesis, while nitrogen metabolism is favored, thus leading to greater amino acid and protein accumulation in seeds (Asthir et al., 2013). The seed protein content is the most important factor determining wheat quality. Heat stress modifies the ratio between the different gluten proteins in the seed favoring the synthesis of gliadins, whereas glutenin synthesis decreases (Blumenthal et al., 1991). The gliadins increase after heat stress might be related to the presence of heat stress elements in the upstream regions of some gliadin genes (Blumenthal et al., 1990). Storage proteins of the 7S globulin (vicilin-like) and R-globulin families are also affected by environmental changes (Sancho et al., 2008).

In durum wheat grain, serpins (serine protease inhibitors) and tritins increased in amount in response to high temperature (Laino et al., 2010). 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); they are considered a wheat grain allergene (Šotkovský et al., 2008) and have the potential to influence grain quality traits (Salt et al., 2005). Their activity suggests that they are involved in inhibition of endogenous proteases, or proteases from grain pests. The amount of an enzyme closely related to the enzyme dihydroflavonol reductase (DFR) was also shown to increase in response to heat stress, in durum wheat grain (Laino et al., 2010). The dihydroflavonol reductase (DFR) is an important enzyme in phenylpropanoid metabolism, a branch of plant metabolism which has been previously shown to be stimulated in plants in response to thermal stress (Rivero et al., 2001) and is directly involved in the biosynthesis of flavonoids, potent antioxidants compounds.

1.2.3 The effect of heat stress on metabolite accumulation in plants

Metabolites reflect the integration of gene expression, protein interaction and other different regulatory processes and are therefore closer to the phenotype than mRNA transcripts or proteins alone. Metabolites are highly dynamic in time and space and represent end products of cellular regulatory processes, their levels can be regarded as the ultimate response of biological systems to genetic or environmental changes. Metabolomics can provide valuable

tools in a wide range of applications, including microbial biotechnology, food technology, pharmacology, toxicology, enzyme discovery, systems biology, and plant biotechnology.

Plants produce more than 200,000 metabolites (Mittler et al., 2012) and gas chromatography equipped with mass spectrometry (GC-MS) is one of the most widely used analytical techniques in plant metabolomics. It is utilized to analyze a qualitatively and quantitatively wide range of volatile and/or derivatized nonvolatile metabolites with high thermal stability (Fiehn et al., 2000). After separation, the eluted metabolites are identified by mass spectrophotometers. This technique has high analytical reproducibility and lower costs compared to liquid chromatography equipped with mass spectrometry (LC-MS) or liquid chromatography equipped with nuclear magnetic resonance (LC-NMR) (Bedair and Sumner et al., 2008). Metabolomics is being increasingly used for evaluating impact of stress/treatment on plant metabolism (Sanchez et al., 2008; Arbona et al., 2013) and for discovering bioprotective foods (Goodacre et al., 2007) in studies of nutrigenomics, which investigate the changes in gene expression that take place after the exposure to different nutrients (Riscuta et al., 2012).

Metabolomics of temperature stress has been reviewed from Guy et al. (2008). When plants are subjected to heat or cold, carbon assimilation and the primary metabolism are largely affected (Arbona et al., 2013). Among all primary metabolites: sugars, sugar alcohols and amino acids are the most important metabolites which concentration in plant tissues is affected by stress, usually as a downstream result of an impairment in the CO_2 assimilation process, but also as a result of a complex regulatory network (Krasensky et al., 2012). Changes in secondary metabolites have also been described; nevertheless, due to the great differences in concentration (usually several orders of magnitude) changes in secondary metabolites levels cannot be simply inferred from variations in their primary metabolite precursors.

1.2.3.1. Primary Metabolism and Osmoprotectants

Several aspects of carbohydrate and amino acid metabolisms were found to be affected by heat shock, in plants (Guy et al., 2008). During the stress period there is an increase of β-amylase activity and plants use starch and fructans as a source of energy instead of glucose (Kaplan et al., 2004). Carbohydrate metabolism plays an important role in the stress tolerance conditions as it is directly linked to photosynthetic performance. Sucrose, in particular, is a metabolite of primary metabolism that can act as signal molecule during response to stress. It increases very rapidly in response to temperature shock in Arabidopsis and its content is maintained high for the duration of the stress treatment in leaves (Kaplan et al, 2004).

Several studies described the effect of heat stress on vegetative tissues of plants (Hasanuzzaman et al., 2013). In particular, plants subjected to a combination of drought and heat stress accumulated several soluble sugars including sucrose, maltose, trehalose, fructose and glucose (Kaplan et al. 2004, Rizhsky et al. 2004). Furthermore, some studies performed on rice showed that heat stress induced the accumulation of sucrose also in developing caryopsis (Yamakawa and Hakata 2010). Many of the accumulated soluble sugars can act as osmolytes maintaining cell turgor, stabilizing cell membranes and preventing protein degradation and can be beneficial during heat stress conditions (Diamant et al., 2001; Rizhsky et al. 2004). Under water deficit, the concentration of soluble carbohydrates such as glucose and fructose increased in roots of stressed plants whereas sucrose, transported to the root tips, promoted growth and contributed to the increase in root-to-shoot ratio (Sicher et al., 2012). Moreover, high amounts of non-reducing disaccharides such as trehalose can accumulate in tolerant plants subjected to desiccation and a direct role of this metabolite in stress protection has been found (Iordachescu et al., 2008).

Other sugars with no energetic role, such as the oligosaccharides raffinose and stachyose accumulate in different plant species in response to a broad range of abiotic stress conditions such as drought, salinity or extreme temperatures (Krasensky et al., 2012). These compounds have been associated to a reduction in oxidative membrane damage and ROS scavenging (Nishizawa et al., 2008). Polyols are also implicated in stress tolerance due to its action as scavengers of hydroxyl radicals. In addition, accumulation of sugar alcohols like mannitol or sorbitol has been linked to stress tolerance (Conde et al., 2015).

Under stress also amino acids, tertiary and quaternary ammonium compounds, and tertiary sulphonium compounds contribute to turgor maintainance by osmotic adjustment (Szabados et al., 2010). Acclimation of plants to heat stress involves the accumulation of aminoacids (asparagine, leucine, isoleucine, threonine, alanine and valine) derived from oxaloacetate and pyruvate (Kaplan et al., 2004). Fumarate and malate (oxaloacetate precursors) were also increased. During the first days of osmotic stress, concentration of amino acids was greater in the tolerant wheat genotype than in the sensitive one, while the opposite relationship was observed at the end of the osmotic stress treatment (Kovàcs et al., 2012).

Glycinebetaine (GB), an amphoteric quaternary amine, plays an important role as a compatible solute in plants under various stresses, such as desiccating conditions of water deficit or high temperature (Quan et al., 2004; Whaid and Close, 2007). Like GB, proline is also known to occur widely in higher plants and normally accumulates in large quantities in response to

environmental stresses (Kavi Kishore et al., 2005). In assessing the functional significance of accumulation of compatible solutes, it is suggested that proline or GB synthesis may have cellular redox potential under heat and other environmental stresses (Wahid and Close, 2007; De Campos et al., 2011).

Molecules strictly related to the amino acid metabolism are the polyamines (PA) (Groppa et al., 2008), nitrogenous aliphatic molecules of low molecular weight which are present in most living organisms. Several abiotic stress conditions induce PA accumulation which has been positively correlated with stress tolerance (Bitrián et al., 2012). The most common PAs, found in higher plants, are putrescine (Put), spermidine (Spd) and spermine (Spm) and can be present as free and conjugated forms. Indeed, PA conjugation as hydroxycinnamic acid amides, such as coumaroylputrescine, feruloylputrescine, dicoumaroylspermidine, diferuloylspermidine or diferuloylspermine, contributes to regulate free PAs levels in plants (Hussain et al., 2011).

As a whole, concentration of free PAs is tightly controlled by balancing biosynthesis, catabolism and conjugation, which is especially relevant during adverse environmental conditions (Nayyar et al., 2004). There are evidences that putrescine and ABA are integrated in a positive feedback loop, in which they reciprocally induce each other's biosynthesis in response to abiotic stress (Alcázar et al. 2010 a, 2010 b). ABA enhances also polyamine accumulation and induces the polyamine oxidation pathway in grapevine in response to drought stress, resulting in secondary protective effects such as stomata closure (Toumi et al., 2010).

The specific way by which these compounds increase stress tolerance in plants still remains unknown. Exogenous application of PA to plants subjected to drought alleviated stress pressure by reducing H_2O_2 levels through the increase in peroxidase and catalase enzyme activity and Proline levels (Alcázar et al., 2010 a). Moreover, Put levels during stress conditions were positively correlated with reduced levels of H_2O_2 and lipid peroxidation and increased antioxidant enzyme activity and carotenoid concentration (Gill et al., 2010).

There is no clear agreement about the specific role and implication of each PAs in stress tolerance. This could be due to a different contribution of PAs in each species and stress condition (Hussain et al., 2011; Gill et al., 2010) or to the induction of a different set of genes involved in responses to abiotic stress by exogenous application of every PA.

Other metabolites with a role in stress response are γ-4-aminobutyric acid (GABA) and myo-inositol, whose levels after heat stress increased also in developing caryopsis in studies carried out in rice (Yamakawa and Hakata 2010). The enzyme that catalyzes the synthesis of GABA by glutamate is the glutamate decarboxylase (GAD) and, following high temperatures,

the increase of the citosolic level of Ca^{2+} can lead to the calmodulin-mediated activation of GAD (Taiz and Zeiger, 2006). It is also hypothesized that the degradation of GABA could limit the accumulation of reactive oxygen intermediates under oxidative stress conditions that inhibit certain enzymes of the TCA cycle. In fact the GABA shunt is also a way to assimilate carbons from glutamate and to generate C:N fluxes that enter the tricarboxylic (TCA) cycle (Breitkreuz et al., 1999), the GABA shunt can provide both succinate and NADH to the respiratory chain.

Myo-inositol and associated raffinose-family oligosaccarides are emerging as putative signalling compounds (Valluru and Van den Ende, 2011) and it is known that availability of myo-inositol regulates the production of galactinol (Smith et al., 1991; Hitz et al., 2002), which is a precursor of raffinose. This explain because myo-inositol and raffinose levels change in the same manner in response to variations of environmental conditions.

1.2.3.2 Secondary Metabolites: Antioxidants, *Defense Compounds and Regulatory Metabolites*

Most of the secondary metabolites are synthesized from the intermediates of primary carbon metabolism via phenylpropanoid, shikimate, mevalonate or methyl erythritol phosphate (MEP) pathways (Wahid and Ghazanfar, 2006).

Phenolics, including flavonoids, anthocyanins, lignins, etc., are the most important class of secondary metabolites in plants and play a variety of roles including tolerance to abiotic stresses (Wahid, 2007). Moreover high-temperature induces the production of phenolic compounds such as flavonoids and phenylpropanoids.

Accumulation of soluble phenolics under heat stress is accompanied with increased phenyl ammonia lyase (PAL), considered to be the principal enzyme of the phenylpropanoid pathway, and decreased peroxidase and polyphenol lyase activities (Rivero et al., 2001). Anthocyanins, a subclass of flavonoid compounds, are greatly modulated in plant tissues; on one hand, in some studies their levels have been shown to increase and decrease, in buds and fruits, in response to low and high temperature, respectively (Sachray et al., 2002). One of the causes of low anthocyanin concentration in plants at high temperatures is a decreased rate of its synthesis and stability (Sachray et al., 2002). On the other hand, vegetative tissues under high temperature stress showed an accumulation of anthocyanins in sugarcane leaves (Wahid and Ghazanfar, 2006). Anthocyanin pigments have been investigated in many species, like rice (Abdel-Aal et al. 2006; Sompong et al. 2011), maize (Moreno et al. 2005; Del Pozo-Insfran *et al*. 2006), colored grapes (He et al., 2010), potato (Payyavula et al., 2013) and wheat (Žofajovà et al., 2012; Ficco et al., 2014). In seeds they are accumulated in a variety-specific manner in cereal crops such as

maize, rice, wheat and sorghum, and also in cultivars of legumes, such as bean and soybean (Del Pozo-Insfran et al. 2006; Ranilla et al., 2007; Escribano-Bailon et al., 2004; Xu and Chang, 2008).

In wheat kernel, a greater effect of environmental conditions on anthocyanin accumulation was observed in a blue aleurone spring wheat line than in red and purple wheat cultivars (Abdel-Aal and Hucl, 2003) and in response to heat stress an increase of anthocyanin content was observed in purple wheat (Hosseinian et al., 2008). Anthocyanins contribute to the non-specific disease resistance in plants (Treutter 2006) and plant protection against biotic and abiotic factors (Lorenc-Kukula et al., 2005, Castellarin et al., 2007). They have antioxidant properties (Abdel-Aal et al., 2008) and partecipate to plant mechanisms of photoprotection (Azuma et al., 2012; Chen et al., 2013; Costa et al., 2015).

Carotenoids are widely known to protect cellular structures in various plant species (Wahid and Ghazanfar, 2006; Wahid, 2007). Carotenoids and xanthophylls are lipophilic compounds synthesized in plants from isopentenyl pyrophosphate (IPP) via the plastidial methyl erythritol phosphate (MEP) pathway. The carotenoid pathway is very well established and produces lycopene which is the precursor of β-carotene. In a two-step hydroxylation, β-carotene is converted to zeaxanthin and sequentially to violaxanthin by epoxidation. Finally, an arrangement in one epoxy ring of violaxanthin to form an allenic bond forms neoxanthin (Tanaka et al., 2008), the precursor of ABA in plants. These metabolites and others like α-tocopherol exert a positive effect against heat stress through the stabilization of the lipid phase of the thylakoid membranes (Demkura et al., 2012). Carotenoids have a protective role under UV radiation. But the overexpression of the gene coding for phytoene synthase, a key enzyme involved in the biosynthesis of these pigments, improved osmotic and salt stress tolerance, in transgenic tobacco plants, by channeling carotenoid flux to ABA biosynthesis which led to increased levels of this phytohormone (Cidade et al., 2012). Carotenoids are essential components of the human diet because mammals are incapable of *de novo* synthesis of vitamin A which precursor is β-carotene (Yeum and Russell 2002). β-carotene compounds are responsible for antioxidant activity (Mueller and Boehm, 2011) and this property has beneficial effects on human health, for this reason cereals like rice (Ye et al., 2000), potato (Römer et al., 2002) and tomato (Fraser et al., 2009) have been improved genetically for an increased accumulation of carotenoids.

Accumulation of pigments as carotenoids and anthocyanins in plants is affected by environmental conditions (Borrelli et al., 2011; Jing et al, 2007; Mpofu et al., 2006; Mori et al.,

2007) despite the strong genotypic component and low genotype×environment interaction, observed for carotenoid accumulation (Van Hung and Hatcher, 2011).

1.3 The effect of heat stress on accumulation of compounds with a beneficial or detrimental effect on human health in wheat grain.

Wheat is the dominant crop in temperate countries being used for human food. Its success depends partly on its adaptability and high yield potential but also on the gluten protein fraction which confers the viscoelastic properties that allow dough to be processed into bread, pasta, noodles, and other food products. However, wheat products are also known to be responsible for a number of adverse reactions in humans, including intolerances (notably coeliac disease) and allergies (respiratory and food). Coeliac disease (CD) is a chronic inflammation of the bowel which leads to malabsorption of nutrients. It results from an autoimmune response which is triggered by the binding of gluten peptides to T cells of the immune system in some (but not all) individuals with the human leucocyte antigens (HLAs) DQ2 or DQ8 (Henderson et al., 2007), expressed by specialized antigen-presenting cells (approximately 95% of CD patients express *HLA-DQ2*, and the remaining patients are usually *HLA-DQ8* positive). Several studies are carried out to reduce gluten toxicity (De Vita et al., 2012; Lamacchia et al., 2014). Moreover, a wide range of wheat grain proteins have been shown to react with immunoglobulin E (IgE) in sera of patients with baker's asthma, including gliadins, glutenins, serpins (serine proteinase inhibitors), thioredoxin, agglutinin, and a number of enzymes (α- and β-amylases, peroxidase, acyl CoA oxidase, glycerinaldehyde-3-phosphate dehydrogenase and triosephosphate isomerase) (Tatham and Shewry, 2008). The durum wheat seed proteome is significantly altered from heat stress that can have a strong effect on grain composition in terms of compounds which are beneficial or detrimental for human health (Dias and Lidon, 2010). Many of the heat-induced polypeptides are considered to be allergenic for sensitive individuals (Thatam and Shewry, 2008). For example, serpins (serine protease inhibitors), also considered wheat grain allergens (Sŏtkovský et al., 2008), increased in response to high temperature (Laino et al., 2010).
Also some housekeeping enzymes, involved in glycolysis and the pentose phosphate pathway glyceraldehyde 3-phosphate dehydrogenase (GAPDH), increased in response to heat stress.

On the contrary the increase of anthocyanin levels in response to stress (Hosseinian et al., 2008) can have a benefiacial effect on human health because it is known that anthocyanins can reduce the risk of coronary heart desease (Mazza et al., 2007; Gosh and Konishi, 2007; de Pasqual-Teresa et al., 2010).

Other molecules with beneficial effects on human health are phenolic compounds, which are commonly found in cereal grains, fruits and vegetables. They are important antioxidants and are suggested to play a preventive role in the development of many chronic human diseases such as cancer and cardiovascular diseases (Middleton et al., 2000; Sobrattee et al., 2005).

Ferulic acid is abundant among phenolic compounds and possesses antibacterial and antioxidant properties (Srinivasan et al., 2007; Ibtissem et al., 2012). Finally, some studies show that also free aromatic amino acids (Nimalaratne et al., 2011) and polyamines can have antioxidant properties (Lagishetty and Naik, 2008; Binh et al., 2011). Polyamines are aliphatic amines that are reported to be essential components of all living cells; in the body, the pool of polyamines derives from endogenous or de novo biosynthesis, intestinal microorganisms and exogenous supply through the diet (Kalac et al., 2005; Binh et al., 2011). Studies on the biological significance of dietary polyamines and on their important role in supporting a normal metabolism and maintaining optimal health have been carried out (Bardocz et al., 1995; Larquè et al., 2007; Nishimura et al., 2006; Ali et al., 2011). Polyamines have a role in prevention from chronic desease (Lagishetty and Naik, 2008; Soda et al., 2010), and in regulation of inflammatory reactions and the differentiation of immune cells (Moinard et al., 2005).In children, high polyamine intake during the first year has been significantly correlated to food allergy prevention (Dandrifosse et al., 2000). In response to abiotic stress conditions, elevated levels of polyamines are one of the most remarkable changes that occur in plants (Simon-Sarkadi et al., 2006; Alcàzar et al., 2010a).

Several studies showed that carotenoids are effective in preventing age-related macular degeneration and certain cancers (Landrum and Bone 2004; Rao and Rao, 2007; Singh and Goyal, 2008; Nishino et al., 2009) and β-carotene is a precursor of vitamin A (Yeum and Russell 2002).

Polyphenols (Liu et al., 2010) but also raffinose, polyamines, free aromatic amino acids and pigment compunds are a good source of antioxidant compounds in cereals (Lagishetty et al., 2008; Nimalaratne et al., 2011; Valluru and Van den Ende, 2011) and environmental stresses affect the production and accumulation of these compounds in cereals, so influencing antioxidant activity and nutritional quality of grain (Chalker- Scott 1999; Mpofu et al., 2006; Jing et al., 2007; Hosseinian et al., 2008; Alcàzar et al., 2010 a).

2. AIM OF THE PROJECT

The aim of the project is to study the transcriptomic and metabolomic variations in response to heat stress in grain of two durum wheat genotypes, and their relationship with variation in grain composition that can affect the nutraceutical quality of the final product. The study was carried out on two durum wheat genotypes: Primadur and T1303 (PI 352395 USDA code); the first is an elite cultivar with high grain yield and yellow index and the second an anthocyanin-rich purple cultivar.

Plants were grown in controlled conditions (growth chamber) and heat shock was imposed at 37°C form 5 up to 10 days after flowering (DAF). Immature seeds (14 DAF) and mature seeds (seeds at physiological maturity stage), collected from heat shocked and control plants (20°C), were analyzed. The response to stress was evaluated with different approaches. A genome–wide gene expression analysis was carried out, using Illumina HiSeq2000, to investigate the changes induced by heat shock in the transcriptome of immature seeds. A metabolomic approach was carried out, by gas chromatography-mass spectrometry (GC-MS), to study the profile of polar metabolites in immature and mature seeds. Qualitative traits were evaluated and the effect of stress on chemical grain composition at maturity was studied. Particular attention was devoted to the main classes of bioactive compounds with nutraceutical and antioxidant properties, including carotenoids, anthocyanins, ferulic acid and polyamines. The antioxidant activity of wheat grains obtained in different environmental growth conditions was assessed. The present study increased the knowledge about the effect of mechanisms of adaptation to stress of wheat plants on nutritional and quality traits of grain, whose final products (flour, semolina, bulgur and couscous) are widely used for human consumption.

3. MATERIALS AND METHODS

3.1 Plant material and heat stress treatment

Two durum wheat genotypes were chosen for this study: Primadur (Blondur//2587-8-6-/Leeds Biancolatte et al., 1992), an élite cultivar with high grain yield and carotenoids content (Digesù et al., 2009; De Simone et al., 2010); and the Ethiopian purple durum wheat genotype T1303 (USDA code PI 352395) with high levels of anthocyanins in the grain (Ficco et al., 2014).

Heat stress was applied following the protocol previously described by Laino et al. (2010). Briefly, the two genotypes were grown up to the third leaf stage in a climate chamber in a medium of soil, sand, and peat (6:3:1) at 10 °C (9 h day)/7 °C (15 h night), with 60% relative humidity and a photon flux of 500 μmol m^{-2} s^{-1}. At this point, the conditions were gradually changed (according to the developmental stage) to 20 °C (13 h day)/17 °C (11 h night), with 55% relative humidity and a photonflux of 500 μmol m^{-2} s^{-1}. The heat stress was applied at 5 days after flowering. While the conditions for the control plants remained unchanged, the heat-stressed plants were transferred to a different growth chamber at 37 °C (13 h day)/17 °C (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 at 20 °C (13 h day)/17 °C (11 h night), with 55% relative humidity and a photon flux of 500 μmol m^{-2} s^{-1}. The first sampling was carried out 4 days after the heat-stress treatment (i.e., immature seeds, at 14 days after flowering) for the metabolic profiling and analysis of the transcriptome. From the milk to maturity stage, both control and stressed plants were maintained at 25 °C (16 h day)/20 °C (8 h night), with 45% relative humidity. Physiologically mature seeds were also collected and used for the analysis of metabolic profile and qualitative/nutraceutical parameters. The analyses were carried out for all of the parameters on the seeds from the principal spike, and were sampled from three biological replicates. The samples, used to analyze the yield-related and qualitative/nutraceutical traits, were stored at 4 °C before processing. The samples used for the metabolic profiling were immediately frozen in liquid nitrogen and freeze dried, and stored at –80 °C until analysis. These analyses were conducted on the ground samples. The analyses were all conducted in triplicate, and the data are expressed on a dry weight (dw) basis.

3.2 Analysis of transcriptome

3.2.1 RNA extraction

RNA was extracted, using Sigma's Spectrum Plant Total RNA Kit, from each of the three biological replicates of control and heat stressed samples. Wheat seeds were ground to a fine powder in liquid nitrogen and lysed in a solution that releases RNA and at the same time inactivates ribonucleases interfering secondary metabolites such as polyphenol compounds. Cellular debris was removed and then RNA was captured onto a binding column using a binding solution which prevents polysaccharides and genomic DNA from clogging the column. Residual impurities and most residual genomic DNA were removed by wash solutions, then purified RNA was eluted using RNase-free water. On-Column DNase I Digest set was used to remove traces of DNA during RNA purification.

RNA concentration was first measured using a NanoDrop ND-1000 Spectrophotometer then with the Quant-iT™ RiboGreen® (invitrogen) protocol on a Tecan Genius spectrofluorimeter. The quality of total RNA was checked with an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Rosa, USA), and samples with an RNA Integrity Number (RIN) value greater than eight were selected for library preparation.

3.2.2 Illumina library production

In collaboration with the National Institute of Agronomic Research (Center of Montpellier), a total of 12 libraries including heat stressed and control samples from three biological replicates and two genotypes were prepared and subjected to RNA-Seq analysis. Libraries for next generation sequencing were prepared using the TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego, U.S.A.) according to the manufacturer's instructions with the following modifications.

In brief, poly-A containing mRNA molecules were purified from 2 µg total RNA using poly-T oligo attached magnetic beads. The purified mRNA was fragmented by addition of the fragmentation buffer and was heated at 94°C in a thermocycler for 4 min. The fragmentation time of 4 min was used to yield library fragments of 250-300 bp. First strand cDNA was synthesized using random primers to eliminate the general bias towards 3' end of the transcript. Second strand cDNA synthesis, end repair, A-tailing, and adapter ligation was done in accordance with the manufacturer supplied protocols. Purified cDNA templates were enriched by 15 cycles of PCR for 10 s at 98°C, 30 s at 65°C, and 30 s at 72°C using PE1.0 and PE2.0 primers and with Phusion DNA polymerase. Each indexed cDNA library was verified and

quantified using a DNA 100 Chip on a Bioanalyzer 2100 then equally mixed. The final library was then quantified by real time PCR with the KAPA Library Quantification Kit for Illumina Sequencing Platforms (Kapa Biosystems Ltd, SA) adjusted to 10 nM in water. Paired-end sequencing was performed by Illumina HiSeq 2000 at the Montpellier Genomix platform for sequencing (France).

3.2.3 Illumina library clustering and sequencing conditions

Final mixed cDNA library was sequenced using the Illumina mRNA-Seq, paired-end protocol on a HiSeq2000 sequencer, for 2 x 100 cycles. Library was diluted to 2 nM with NaOH and 2.5 µL transferred into 497.5 µL HT1 to give a final concentration of 10 pM. An aliquot of 120 µL was then transferred into a 200 µL strip tube and placed on ice before loading onto the Cluster Station, mixed library, from 10 individual indexed libraries, being run on a single lane. Flow cells were clustered using Paired-End Cluster Generation Kit V4, following the Illumina PE_amplification_Linearization_Blocking_PrimerHyb_v7 recipe. Following the clustering procedure, the flow cell was loaded onto the Illumina HiSeq 2000 instrument following the manufacturer's instructions. The sequencing chemistry used was v4 (FC-104-4001, Illumina) using the software SCS 2.6 and RTA 1.6 with the 2 x 100 cycles, paired-end, indexed protocol. Illumina base calling files were processed using the GERALD pipeline to produce paired sequence files containing reads for each sample in Illumina FASTQ format.

3.2.4 Bioinformatic analysis

Illumina runs, performed on each separate normalized cDNA pool, generated approximately 40 million reads per sample with average quality scores > Q20 at each base. The first step of de novo assembly was carried out with ABySS (Simpson et al., 2009; Cahais et al., 2012), a *de Brujin* graph assembler. The combined contigs were assembled with CAP3 (Huang and Madan, 1999) that was used to remove redundancy across ABySS assemblies and to merge contigs into longer sequences. Assembly of AbySS followed by CAP3 was able to yield better contigs (Cahais et al., 2012). The analysis was performed separately on the three control and three heat stressed samples of Primadur and T1303. After data were mapped, normalization was performed and then RPKM (reads per kilobase per million mapped reads) was calculated: [Nbr of reads/(length of the contings × total nbr of mapped reads of the individual)]; RPKM associated the reads number with gene expression levels (Morrissy et al., 2009). To identify differentially expressed genes in heat stressed vs. control samples at each time point, fold-changes with respect to RPKM values were calculated. Those genes with a log2-converted fold

change > 2 and < –1 were considered to be differentially expressed genes (DEGs). Blast2GO (B2G) (Conesa et al., 2005) is a comprehensive bioinformatics tool used for the functional annotation and analysis of gene or protein sequences. The tool was originally developed to provide a user-friendly interface for Gene Ontology (The gene ontology consortium 2008). Recent improvements have considerably increased the annotation functionality of the tool and currently Enzyme code (EC), KEGG Maps and InterPro motifs are also supported (Götz et al., 2008). Blast2GO program was used to perfome a gene ontology (GO) enrichment analysis and to predict genes function.

3.3 Yield-related and qualitative/nutraceutical traits

3.3.1. Yield-Related Traits

Six principal spikes were harvested from three biological replicates, and the individual grain weights and grain yields per spike were measured, at both 14 DAF and at physiological maturity. The individual grain weight was measured in triplicate for each of the three biological replicates, as the mean of 50 grains collected from two spikes and expressed in milligrams. The grain yield per spike was measured for each biological replicate as the mean for two spikes and expressed in milligrams. These data provided an indication of the differences between the two genotypes and the efficiency of the heat-stress treatment.

3.3.2. Protein Content

The total nitrogen content was determined using the micro-Kjeldhal method (AACC method 46-13.01). The grain-protein percentage was calculated by multiplying the Kjeldhal nitrogen by the conversion factor 5.7, with this expressed on a dw basis.

3.3.3. Total Anthocyanin Content Using the pH Differential Method

The extraction and purification of the anthocyanins was performed according to the method of Hosseinian (2008), with some modifications. A mixture of methanol acidified with 1 N HCl (85:15; v/v) (8 mL) was added to the wholemeal sample (0.5 g), and then sonicated for 18 min at room temperature in an ultrasonic bath. After centrifugation at 9,000 × *g* for 15 min at room temperature, the supernatant was recovered. The pellet was extracted with 4 mL acidified methanol, and subjected to further centrifugation. The supernatants was then united and incubated at –20 °C in the dark for 48 h, to facilitate macromolecule precipitation. The sample was then centrifuged at 9,000 × *g* for 15 min, and the supernatant was filtered using 0.45–µm

regenerated cellulose syringe filters. Total anthocyanin content was evaluated using a colorimetric method at different pHs. Two dilutions of the samples were prepared, one for pH 1.0 using potassium chloride buffer (0.03 M KCl), and the other for pH 4.5 using sodium acetate buffer (0.4 M CH_3CO_2 Na $3H_2O$). These samples were incubated for 30 min at room temperature in the dark and then filtered with 0.45–µm regenerated cellulose syringe filters. The absorbance of each sample was measured at 520 nm. The concentration of each anthocyanin was calculated according to the following formula and expressed as Cy-3-glc (cyanidin-3-glucoside) equivalents: $(A\times MW\times DF\times 10^3)/\varepsilon\times 1$ where A is the absorbance = $(A_{\lambda vis\text{-}max})_{pH\ 1.0} - A_{\lambda 520})_{pH4.5}$, MW is the molecular weight (g/mol) = 449.2 g/ mol for Cy-3-glc, DF is the dilution factor, and ε is the extinction coefficient ($L\times cm^{-1}\times mol^{-1}$) = 26,900 for Cy-3-glc, where L (pathlength in cm) = 1. The quantification was performed based on a calibration curve of cyanidin chloride. Analysis was performed in triplicate and the total anthocyanin content was corrected for dw and was expressed as cyanidin-3-glucoside equivalents in µg/g dw.

3.3.4. Total Carotenoid Content

The total carotenoid content was analyzed according to method 14-50 of AACC International , with some modifications (Beleggia et al., 2010). Briefly, in the AACC method 14-50 (AACC, 2000) 8 g of sample were extracted into a glass-stoppered flask with 40 mL of water-saturated 1-butanol for 16–18h. Then, the sample extract was filtered and the absorbance read at 435 nm using a spectro-photometer LAMBDABio20 PerkinElmer. The pigment content of the extract was calculated directly from absorbance using the conversion factor of 1.6632 and expressed as β-carotene (1 mg of this pigment in 100 mL water-saturated 1-butanol has optical density of 1.6632 in 1 cm cuvette at 435 nm wavelength). Beleggia et al. (2010) adopted the following modifications: the sample amount was reduced to a value of 100 mg and the extraction was performed in 2 mL reaction tubes with a 500 µL of water-saturated 1-butanol; the extraction condition was set to 15 min in an ultrasonic bath, at room temperature.

3.3.5. In Vitro Determination of Antioxidant Activity

The antioxidant activity was evaluated using the TEAC, or ABTS-2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), method. This method is based on the reduction of the blue–green cation radical $ABTS^+$ at 734 nm due to antioxidants present in the sample tested. To generate the $ABTS^+$, the ABTS stock solution (7 mM) and potassium persulphate (2.45 mM) in phosphate buffer saline (PBS, pH 7.3) were allowed to stand for 16 h. For the reaction 10 mg of the sample was mixed with 7 ml of diluted $ABTS^+$ (absorbance 0.7 at 734 nm). The decline in

absorbance was measured at 734 nm after 5 min incubation at 25°C. A standard curve was prepared using a series of concentrations of Trolox (0, 2, 4 , 8, 12, 16, 20, 22, 25, 27, 30 µM) with 7 ml of diluted $ABTS^{+}$ solution. The decline in absorbance was expressed as percentage inhibition of $ABTS^{+}$, and was determined as a function of the concentration and the time, which is then calibrated against Trolox as the reference standard (Serpen et al., 2007). These data were expressed as mmol Trolox per kg dw.

3.4. Analysis of Polar Metabolites

Seeds at the physiological maturity stage were freeze-dried to determine the dry weight, and then milled using a mill (Pulverisette® 7 Planetary Micro Mill; Classic Line, Fritsch) with an agate jar and balls, and then stored at –80 °C until analysis. Immature seeds were ground to a fine powder in a mortar with liquid nitrogen, freeze-dried and then stored at –80 °C until analysis. The extraction, derivatization, and analysis of these samples for the profiling of the polar metabolites were performed by gas chromatography linked to mass spectrometry (GC-MS), following protocols described previously (Beleggia et al., 2013). All of the analyses were performed in three technical replicates for each of three biological replicates.

Briefly, 100 mg dw of each sample were extracted using a mixture of methanol (1 mL), ultrapure water (1 mL), and trichloromethane (3 mL), added sequentially. The samples were stored at 4 °C for 30 min, and then centrifuged at 4,000× *g* for 10 min at 4 °C. Aliquots (50 µL) of the polar phase were dried in a Speedvac, for further analysis. The polar fraction was redissolved and derivatised for 90 min at 37 °C in methoxyamine hydrochloridein pyridine (70 µL; 20 mg/mL), followed by incubation with N-methyl-N-(trimethylsilyl) trifluoroacetamide (120 µL) at 37 °C for 30 min. The polar metabolites were analyzed using GC (Agilent 6890N; Agilent Technologies, USA) coupled with quadrupole MS (Agilent 5973, Agilent Technologies, USA). Samples (1 µL) were injected in the splitless mode, with GC separation on an HP-5ms capillary column (60 m, 0.25 mm i.d., 0.25 mm film thickness). Helium was used as the carrier gas, at a constant flow rate of 1 mL/min. For the analysis of polar metabolites, the injection temperature, transfer line, and ion source were set at 280 °C, and the quadrupole was adjusted to 180 °C. The oven was kept at 70 °C for 1 min, then increased at a rate of 5 °C/min to 310 °C, and held for 15 min. Subsequently, the temperature was increased to 340 °C and held for 1 min. The spectrometer was operated in electron-impact mode at 70 eV, the scan range was from 30 amu to 700 amu, and the mass spectra were recorded at 2.21 scan/s. The standards and all of the chemicals used were from Sigma Aldrich Chemical Co. (HPLC grade; Deisenhofen, Germany), and N-methyl-N-(trimethylsilyl) trifluoroacetamide was from Fluka.

The polar metabolites were identified by comparing the MS data with those of the National Institute of Standards and Technology 2008 database, and with a custom library compiled with reference compounds. The GC-MS quantification was performed using the Chemstation software. The absolute concentrations of the polar metabolites were determined by comparisons with standard calibration curves obtained in the range of 0.04 ng to 2.00 ng and expressed as μg/g dw. The samples were randomised, with the instrumental performance monitored by internal standards (Ribitol, 20 μl; 0.2 mg/mL) added after the extractions.

3.5. Statistical Analysis

The analysis of variance (ANOVA) was carried out with respect to each analytical compound detected in whole-grain material during grain filling and at maturity stage; the effect of genotypes was assessed according to a completely randomized design (with 3 repetitions). A standard procedure for the analysis of variance was applied to the data, to separate the effects due to genotype (G), treatment (T), timing (t), and their interactions. Statistically significant differences were determined at the probability level of $P < 0.05$. The magnitude of each factor was determined by components of variance analysis. Due to the differences in seed weight which were observed between the two genotypes, and inside each genotype following the heat stress, ANCOVA analysis was also carried out to correct the data with respect to seed weight. As the results were consistent with those of ANOVA, only ANOVA results are shown in the present study. All statistical analyses were performed using the STATISTICA program (StatSoft, vers. 7.1, 2005).

4. RESULTS

4.1. Genome–Wide Gene Expression Analysis by RNA-seq

4.1.1. De novo assembly

The cDNA libraries were synthesized from the seeds collected from the heat stressed and mock-treated plants of the two durum wheat genotypes. Illumina runs, performed on each separate, normalized, cDNA pool generated approximately 40 million reads per sample with average quality scores > Q20 at each base (Table 4.1).

Table 4.1. Mapped Reads

Genotypes	Timing	Replicates	Produced Reads	Mapped Reads (%)
Primadur	Control	rep 1	37,419,966	72.43
		rep 2	43,986,728	69.53
		rep 3	43,087,889	66.59
	Heat-shock	rep 1	52,783,832	68.91
		rep 2	47,516,836	68.79
		rep 3	48,578,183	69.37
T1303	Control	rep 1	43,118,120	67.06
		rep 2	40,463,551	67.48
		rep 3	55,806,947	68.28
	Heat-shock	rep 1	41,914,854	66.29
		rep 2	40,462,251	68.26
		rep 3	43,030,732	64.32

The reads of Primadur generated 67,242 contigs from control conditions and 81,238 contigs from heat shocked conditions; the reads of T1303 generated 68,755 contigs from control conditions and 67,774 contigs from heat shocked conditions. A consensus assembly generated 68,615 contigs on which an average of 67.94% of reads were mapped.

4.1.2. Identification of Differentially Expressed Genes (DEGs) in response to Heat Stress

Gene expression repeatability across replicates was evaluated calculating the coefficient of determination (r^2) that ranged from 0.838 to 0.991 (Figures 4.1–4.4). This analysis revealed that the repeatability was very good for seeds from Primadur in stressed condition (r^2 values higher than 0.98). For the other three samples (Primadur control, T1303 control and T1303 heat stressed), repeats 1 and 2 were very consistent with $r^2 > 0.98$, while the repetition 3 showed a lower r^2 value, but in all cases r^2 values were higher than 0.84.

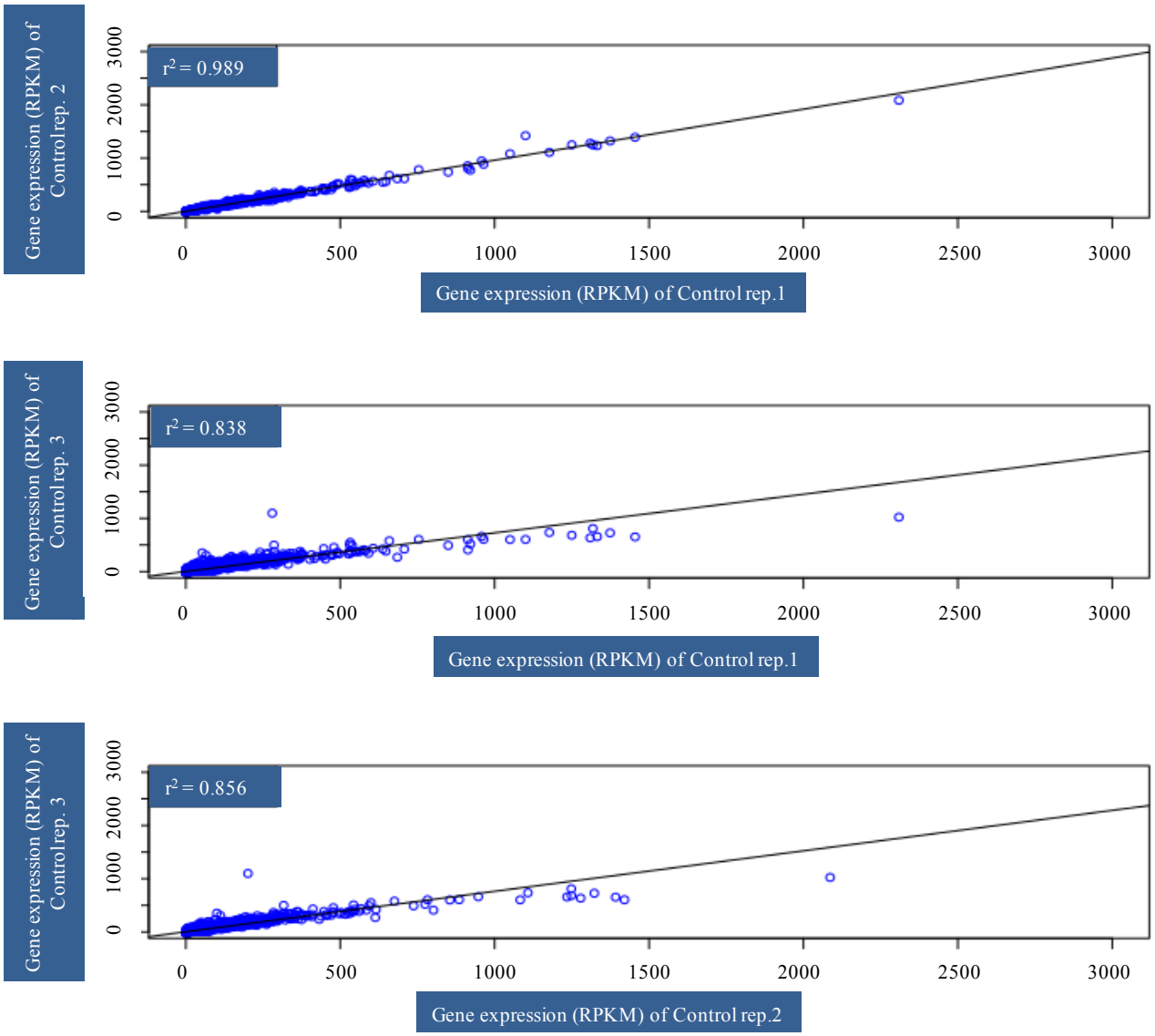


Fig. 4.1 Gene Expression Repeatability across Primadur seeds from control condition

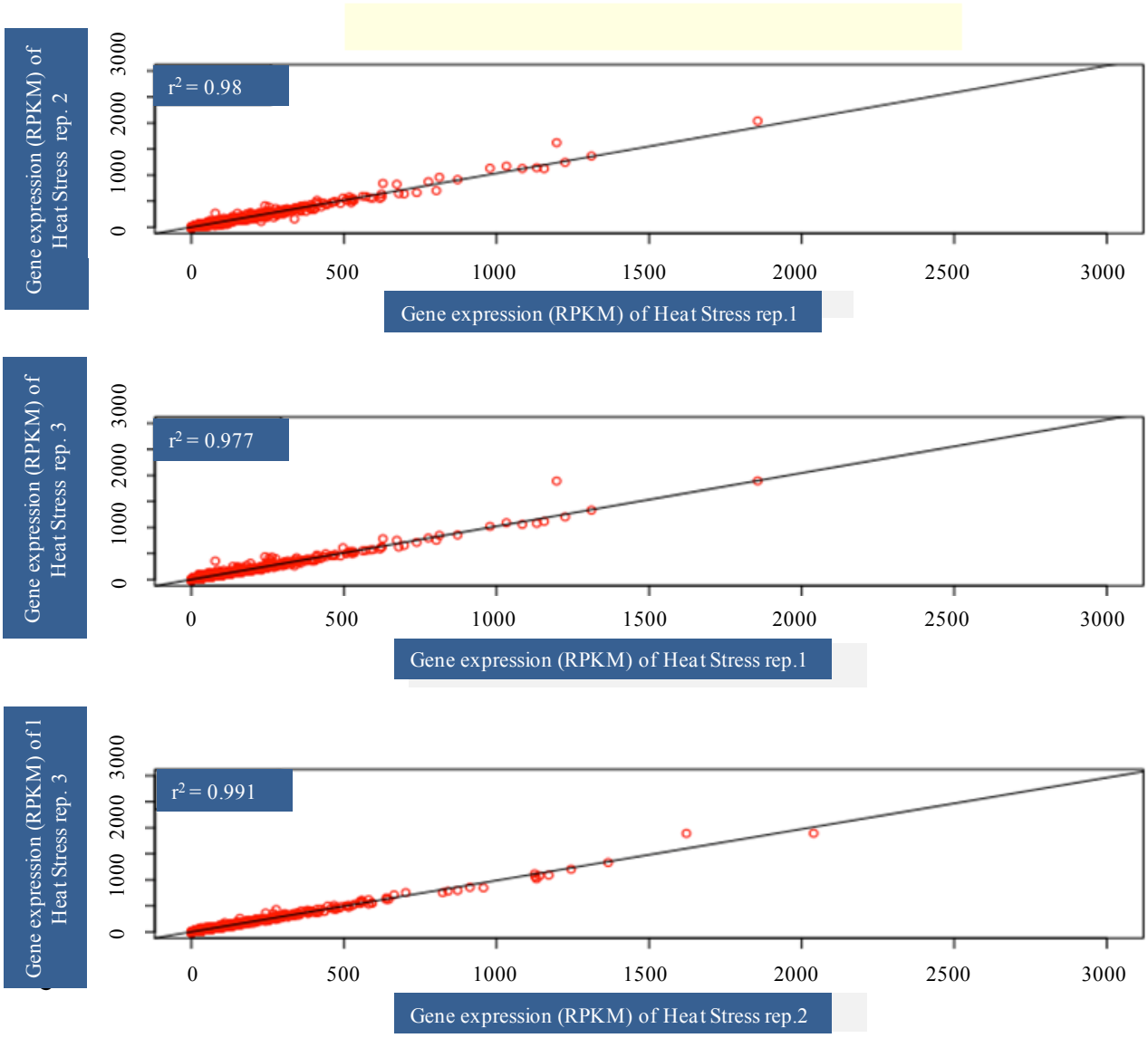


Fig. 4.2. Gene Expression Repeatability across Primadur seeds from heat stress condition

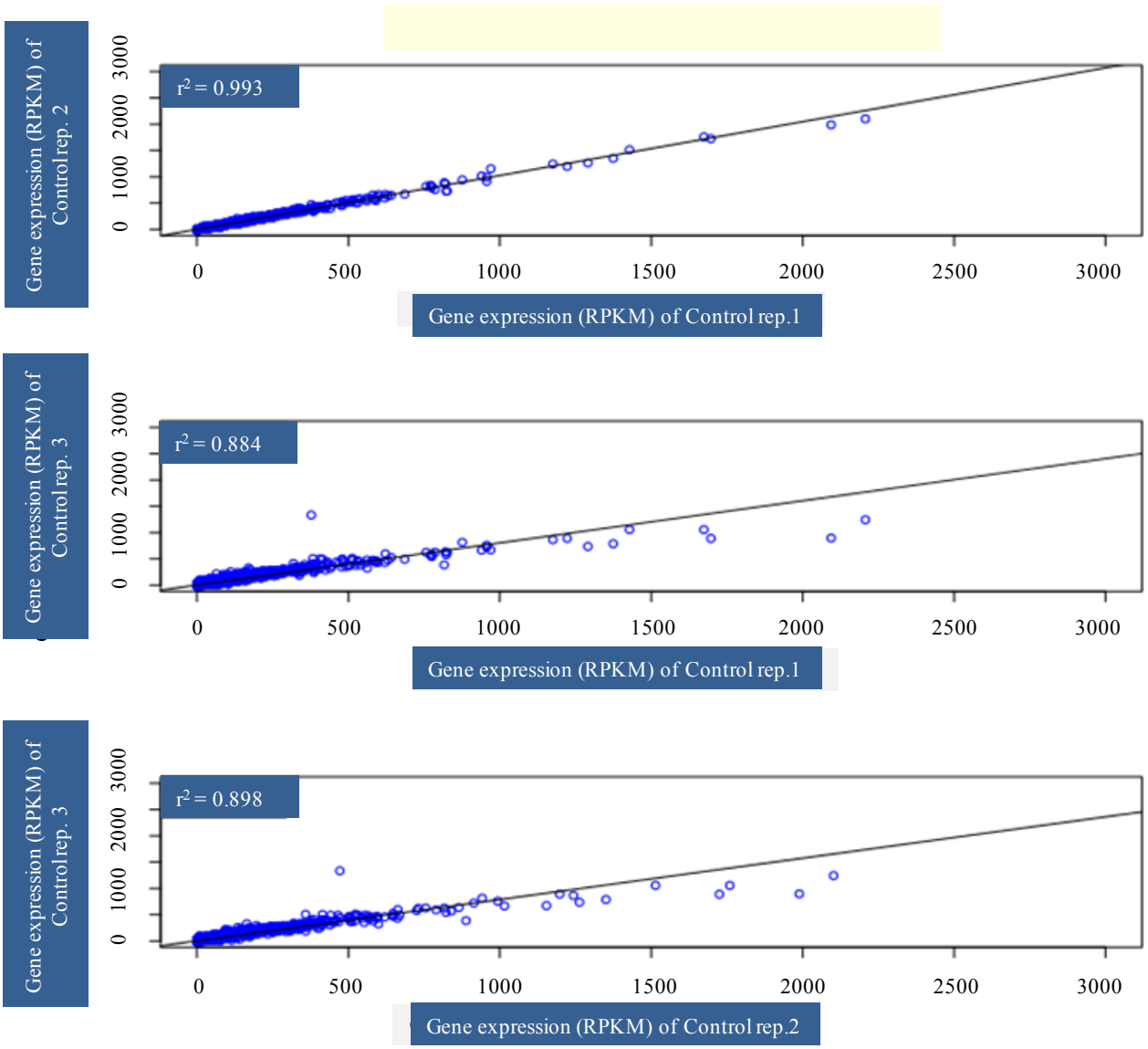


Fig. 4.3. Gene Expression Repeatability across T1303 seeds from control condition

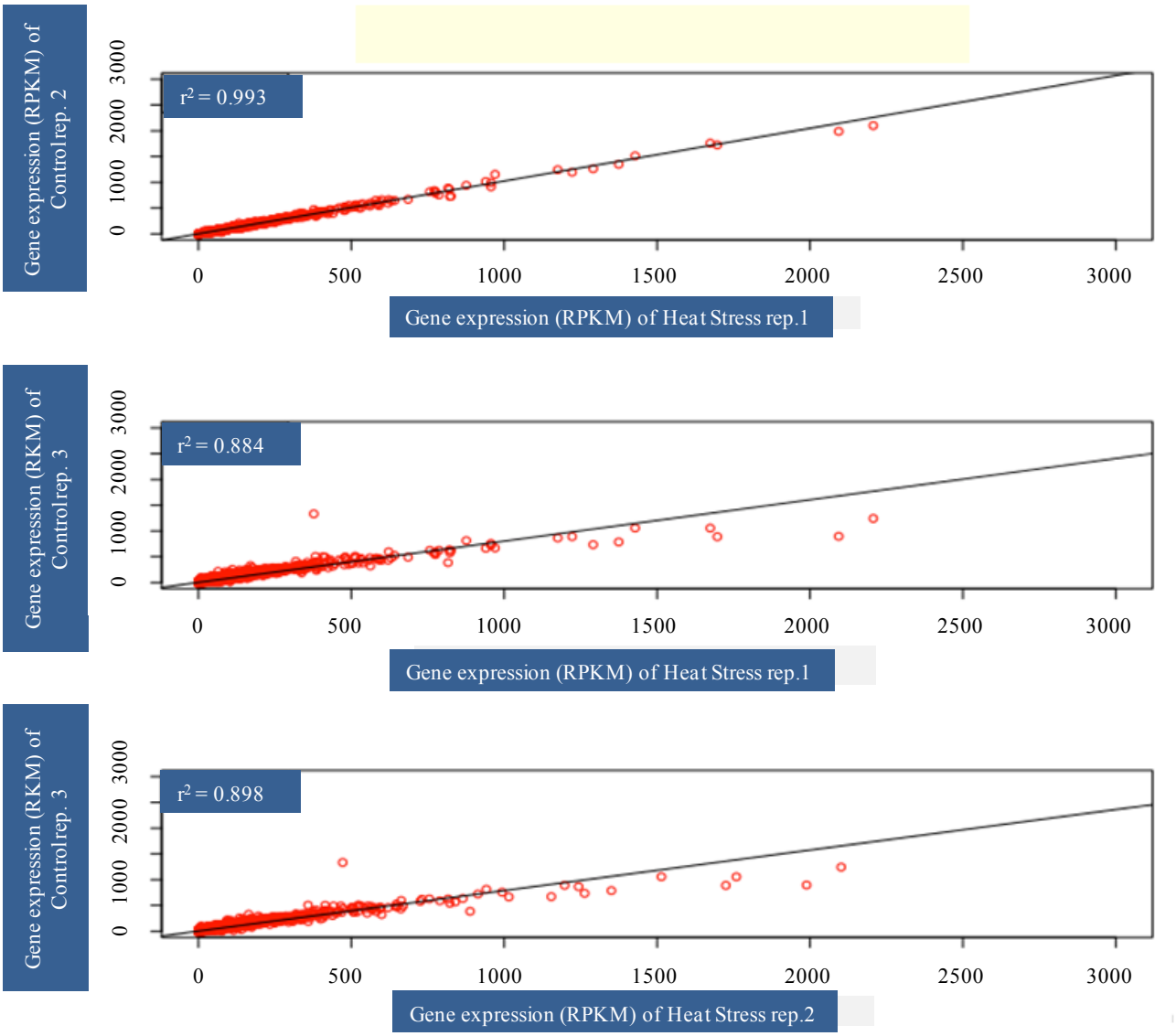


Fig. 4.4. Gene Expression Repeatability across T1303 seeds from heat stress condition

Out of the 11,970 DEGs, 1,201 were differentially expressed between control and heat stress conditions. The largest differences in terms of gene expression were observed between the two genotypes; the number of genes differentially expressed ranged from 2,369 to 2,955. More limited were the differences between the control and the stressed conditions for the same genotype, with 648 DEGs for T1303 and 553 DEGs for Primadur. In Primadur, 214 genes were down-regulated and 339 genes were up-regulated; in T1303, 308 genes were down-regulated and 340 genes were up-regulated (Table 4.2). Out of these 50 and 38 were the genes commonly up-regulated and down-regulated in both genotypes, respectively.

Table 4.2. Number of Differentially Regulated Genes

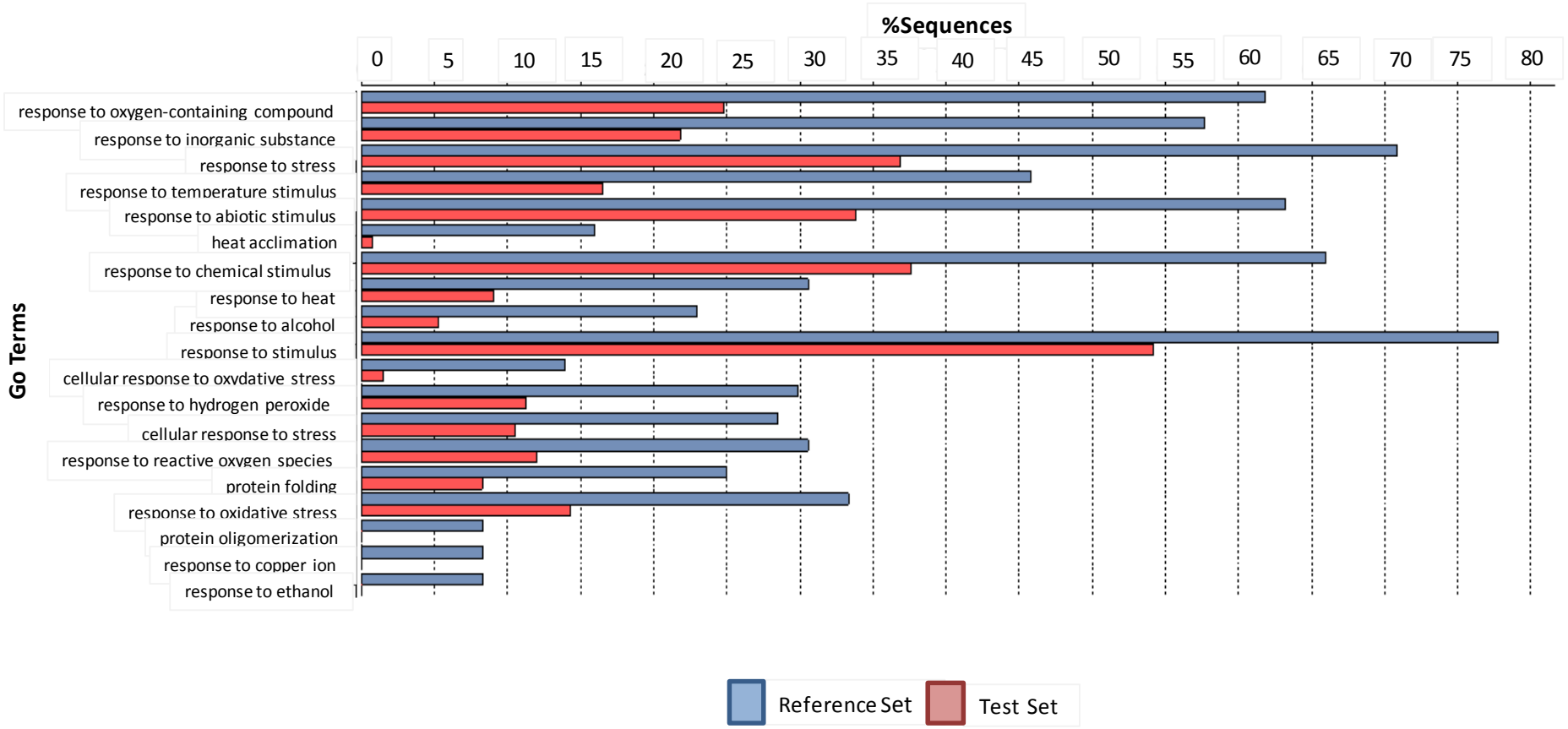
Comparision of samples	DEGs
control /primadur vs T1303/up-regulated in T1303	2,816
control /primadur vs T1303/up-regulated in primadur	2,369
heat stress/primadur vs T1303/up-regulated in T1303	2,955
heat stress/primadur vs T1303/up-regulatedd in primadur	2,629
primadur/control vs heat stress/up-regulated in control	214
primadur/control vs heat stress/up-regulated in heat stress	339
T1303/control vs heat stress/up-regulated in control	308
T1303/control vs heat stress/up-regulated in heat stress	340

DEG, Differentially Regulated Genes.

4.1.3. Functional annotation of the Differentially Expressed Genes

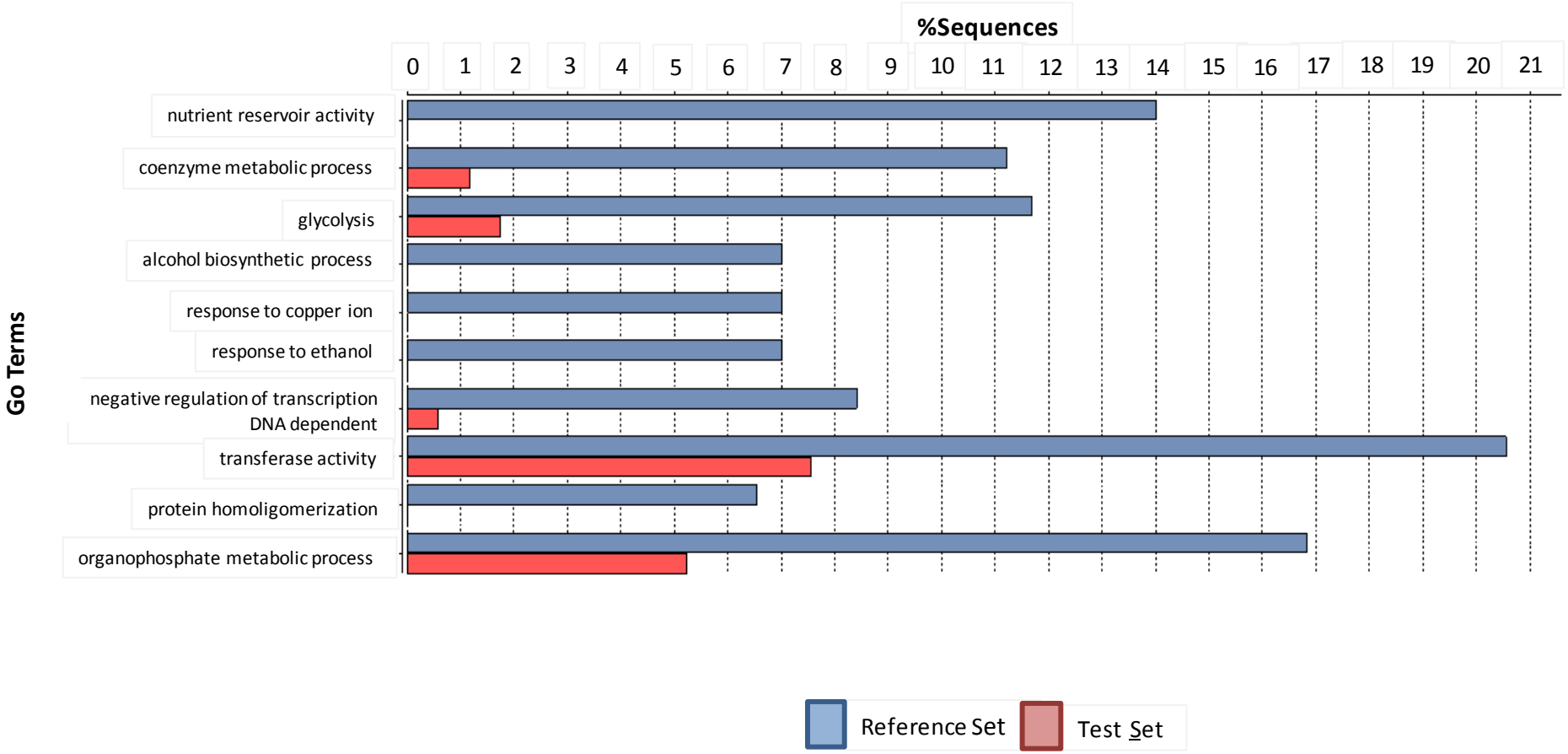
The functional roles of the 1,201 genes, differentially regulated between control and heat stress condition in the two genotypes, was further examined. The Gossip (Blüthgen et al., 2005) package, integrated in the Blast2GO program, was used for statistical assessment of annotation differences between two sets of sequences. Enrichment analysis was performed separately on 553 genes differentially regulated in response to stress in Primadur (Figure 4.5) and the 648 genes differentially regulated in response to stress in T1303 (Figure 4.6). This analysis identified gene categories that were statistically more represented in immature seeds from stressed plants (test set) with respect to seeds from plants grown in controlled conditions (reference set).

Figure 4.5. Enrichment Analysis on Primadur transcripts



Test Set, Genes up-regulated in heat stress condition; Reference Set, Genes up-regulated in Control condition; GO, Gene Onthology.

Figure 4.6. Enrichment Analysis on T1303 transcripts



Test Set, Genes up-regulated in heat stress condition; Reference Set, Genes up-regulated in Control condition; GO, Gene Onthology.

For the 553 differentally regulated genes in Primadur, stress related categories including categories related to heat and oxidative stresses were enriched in the test set (genes up-regulated following heat stress) with respect to the reference set (genes up-regulated in control condition).

The enrichment analysis carried out on T1303 differentially regulated sequences produced different results: abiotic stress related categories were not enriched in statistically significant manner; genes involved in glycolysis and in negative regulation of transcription, and genes coding for proteins with nutrient reservoir activity were over-represented in the test set (genes up-regulated following heat stress) (Figure 4.6). To better understand these results, the gene onthology categories were analysed to attribute a putative function to genes up-regulated in response to stress (Table 4.3).

Table 4.3 Functional annotation of genes up-regulated in response to heat stress

T1303		**Primadur**	
Biological process	**Genes**	**Genes**	**Biological process**
nutrient reservoir activity	w8qn15_bradi brachypodium distachyion farinin protein	gl19_orysj-oryza s.19 kda globulin	nutrient reservoir activity
nutrient reservoir activity	vclb_goshi- gossypium vicilin c72	m8c814_aegta- aegylops tauchii vicilin-like	nutrient reservoir activity
nutrient reservoir activity	ave3_avesa -avena sativa avenin-3	axs_orysj- oryza udp-d-apiose udp-d-xylose synthase	UDP-glucuronate decarboxylase carbohydrate
nutrient reservoir activity	avee_avesa- avena sativa avenin-e	bgl31_orysj oryza beta-glucosidase	metabolism
nutrient reservoir activity	avla2_wheat avenin-like a2	glgs_orysj- oryza s. glucose-1-phosphate adenylyltransferase	response to sucrose stimulus
nutrient reservoir activity	avla4_wheat avenin-like a4	M8BQJ5_AEGA protein	amino acid biosynthetic process
nutrient reservoir activity	avla6_wheat avenin-like a6	prf1_sollc- solanum lycopersicum proline-rich protein	amino acid transport transport
nutrient reservoir activity	avla7_wheat avenin-like a7	tocc_orysj- oryza s. tocopherol	involved in the synthesis of vit.E
nutrient reservoir activity	avlb1_wheat avenin-like b1	m7ym99_triua- wheat subtilisin-like protease	peptidase activity
nutrient reservoir activity	avlb4_wheat avenin-like b4	mob1_arath- arabidopsis thaliana mob kinase activator-like	apoptotic process
nutrient reservoir activity	avlb5_wheat avenin-like b5	pxg_orysj- oryza s. peroxygenase	carotenoid biosynthetic process
nutrient reservoir activity	avlb8_wheat avenin-like b8	ago1b_orysj- oryza s.protein argonaute 1b	miRNA metabolic process
nutrient reservoir activity	avlba_wheat avenin-like b10	elof1_orysj- oryza s. transcription elongation factor 1	transcription elongation factor 1
nutrient reservoir activity	gda3_wheat alpha beta-gliadin	f2cym8_hordeum transcript. factor	molecular function function

Table 4.3 Cont. Functional annotation of genes up-regulated in response to heat stress

T1303		Primadur	
Biological process	**Genes**	**Genes**	**Biological process**
nutrient reservoir activity	gda9_wheat alpha beta-gliadin	f2eba5_hordeum transcript. factor	molecular function
nutrient reservoir activity	gdb1_wheat gamma-gliadin b-i	grf11_orysj- oryza sativa growth regulating factor	regulation of transcription
nutrient reservoir activity	gdbx_wheat gamma-gliadin	nfyb_maize- nuclear transcription factor y subunit b	regulation of transcription
nutrient reservoir activity	glt0_wheat HMW subunit dy 10	rcaa_horvu- hordeum vulgare ribulose bisphosphate carboxylase /oxygenase activase	activation of rubisco
nutrient reservoir activity	glt3_wheat HMWsubunit 12	spt16_orysj- oryza sativa fact complex subunit spt 16	DNA repair, replication and regulation of transcription
nutrient reservoir activity	glt5_wheat HMW subunit dx5	sui1_salba- salix bakko translation factor	translation initiation factor
nutrient reservoir activity	glta_wheat LMW subunit	pip13_orysj- oryza sativa aquaporin	stress response and transport
nutrient reservoir activity	gltc_wheat LMW subunit	pip21_maize aquaporin	stress response and transport
polysaccharide catabolic process	amyb_secce- secale cereale beta-amylase	pip25_maize aquaporin	stress response and transport
carbohydrate metabolic process	m8cw07_aegilops tauchii atp-citrate synthase	ccr2_arath- arabidopsis thaliana cinnamoyl- reductase 2	pathway of phenylpropanoid
carbohydrate metabolic process	mdhc_orysj- oryza sativa malate cytoplasmic enzyme	drpe_crapl - ctraterostigma plantagineum desiccation-related protein PCC 13-62.	dessication related proteins
tryptophan catabolic process	ppa1_arath - arabidopsis thaliana inactive purple acid phosphatase 1	hsp7c_pethy heat shock 70 Kda	response to heat
		hsp7h_arath – arabidopsis thaliana heat shock 70 kda protein	response to heat
tryptophan catabolic process	ppa3_arath- arabidopsis thaliana purple acid phosphatase 3		
		hsp7m_phavu-phaseolus vulgaris heat shock protein70 kda	response to heat
negative regulator of peptidase	iaac2_wheat alpha-amylase trypsin inhibitor		
		hsp81_orysj- oryza sativa heat shock protein 81	response to stress
negative regulator of peptidase	ici2_horvu- hordeum vulgare subtilisin- chymotrypsin inhibitor-2a		
		hsp82_maize heat shock protein	response to stress
negative regulator of peptidase	icib_horvu - hordeum vulgare subtilisin- chymotrypsin		
	chymotrypsin inhibitor ci-1b	lea3_wheat late embryogenesis	response to stress

Table 4.3 Cont. Functional annotation of genes up-regulated in response to heat stress

T1303		Primadur	
Biological process	**Genes**	**Genes**	**Biological process**
negative regulator of peptidase	iciw_wheat subtilisin-chymotrypsin inhibitor	y531_metja – methanococcus janneshii universal stress protein	response to stress
flavonoid biosynthetic process	g6pi_maize glucose-6-phosphate cytosolic	nlt41_horvu – hordeum vulgare lipid-transfer protein	response to stress
replicative polymerase	dpola_orysj- oryza sativa dna polymerase alpha catalytic subunit	p2c54_orysj- oryza sativa protein phosphatase 2c	response to stress
translation elongation factor	ef2_betvu- beta vulgaris elongation factor 2	per4_vitvi- vitis vinifera peroxidase 4	hydrogen peroxide catabolic process
RNA-dependent DNA replication	m7yf97_triua- triticum urartu galacturonosyl transferase 11	hsp16b_wheat 16.9 kda class I HSP2	heat acclimatation
exonuclease activity	m7zvd2_triua- triticum urartu exosome complex component rrp45	hsp21_maize 21kda class II HSP	heat acclimatation
transcription regulation	m8cs47_aegta aegilops thauschii protein cup- shaped cotyledon 2	hsp20/alpha crystallin family protein	heat acclimatation
dna mismatch repair protein	msh5_arath- arabidopsis thaliana dna mismatch repair protein	hs21.9_orysj- oryza sativa 21.9 kda HSP	heat acclimatation
molecular function	f2cym8_hordeum vulgare protein		
DNA binding	3h11_orysj zinc finger ccch domain-containing protein 11		
response to oxidative stress	odo2a_arath- arabidopsis thaliana dihydrolipoyl lysine-residue succinyltransferase		
defense response	puib_wheat puroindoline-b		
heat acclimatation	skp2a_arath f-box protein skp2a		
defense response	thn5_wheat type-5 thionin		
defense response	W5G4V0_WHEAT protein		
defense response	hina_horvu hordoindoline-a		
response to oxidative response to stress	kpk2_arath serine threonine-protein kinase		
glycolytic process	eno_orysj- oryza sativa enolase		
heat acclimation	f2dm71_hordeum vulgare protein		
heat acclimation	hsp232_orysj- oryza sativaa HSP		

Genes with nutrient reservoir activity are represented in orange, genes involved in metabolic processes are represented in grey, genes with a regulatory role are represented in blu, genes with a role in defense response are represented in red.

Even if it was not possible to identify a putative function for all of the differentially expressed genes, the results of the enrichment analysis were confirmed. The number of genes with nutrient reservoir activity was higher in T1303 (22) with respect to Primadur (2). The number of genes with a role in metabolic process (carbohydrate metabolic process, response to sucrose stimulus, polysaccharide catabolic process) were the same in both genotypes but as expected some genes involved in carotenoid biosynthetic process and flavonoid biosynthetic process were up-regulated in Primadur and T1303, respectively. (Table 4.3). The number of up-regulated genes with a role in gene regulation and mismatch repair mechanisms was the same in the two genotypes, but some of them were up-regulated in T1303 compared to Primadur, as a gene with high similarity to the rice gene OsC3H11 (Os01g61830). This gene belongs to the CCCH zinc finger protein family, whose members are involved in the response to different biotic and abiotic stresses (D'Orso et al., 2015). The number of up- regulated genes, involved in defense response mechanisms, was higher in Primadur (19) with respect to T1303 (10) and included genes involved in heat acclimation (Table 4,3). In T1303 wheat alpha-amylase trypsin inhibitor genes were up-regulated in response to stress (Table 4.3) and for some genes involved in trypsin inhibitor activity (i.e. iaa2 gene) the level of transcription observed in T1303 was three fold than the level observed in Primadur in control condition (Table 4.4). Moreover, some heat shock proteine (HSP7C, HSP81, HSP83, HSP7I, HSP dnjh2) and genes coding for proteins involved in chlorophyll biosynthetic process showed higher levels of transcripts in T1303 with respect to Primadur, in control condition (Table 4.4). These results suggest that tolerance to stress is higher for T1303 than for Primadur, and that it may be related to a higher expression level of stress-related genes in control conditions.

Even if it was not possible to identify a putative function for all of the differentially expressed genes, the results of the enrichment analysis were confirmed. The number of genes with nutrient reservoir activity was higher in T1303 (22) with respect to Primadur (2). The number of genes with a role in metabolic process (carbohydrate metabolic process, response to sucrose stimulus, polysaccharide catabolic process) were the same in both genotypes but as expected some genes involved in carotenoid biosynthetic process and flavonoid biosynthetic process were up-regulated in Primadur and T1303, respectively (Table 4.3). The number of up-regulated genes with a role in gene regulation and mismatch repair mechanisms was the same in both genotypes. The number of up- regulated genes, involved in defense response mechanisms, was higher in Primadur (19) with respect to T1303 (10) and included genes involved in heat acclimation (Table 4,3). In T1303 wheat alpha-amylase trypsin inhibitor genes were up-regulated in response

to stress (Table 4.3) and for some of genes involved in trypsin inhibitor activity (i.e. iaa2 gene) the level of transcription observed in T1303 was three fold that observed in Primadur, in control condition (Table 4.4). Also some heat shock proteins (HSP7C, HSP81, HSP83, HSP7I, HSP dnjh2) and genes coding for proteins involved in chlorophyll biosynthetic process showed higher levels of transcripts in T1303 with respect to Primadur, in control condition (Table 4.4). These results suggest higher tolerance to stress for T1303 compared to Primadur.

Table 4.4 RPKM values in Control conditions

Stress Response Genes	**Primadur**	**T1303**
HSP7C	6.4 ± 0.05	12.6 ± 3.06
HSP81	24.9 ± 0.26	41.6 ± 2.11
HSP83	93.2 ± 1.5	189.3 ± 9.2
HSP DNJH2	13.19 ± 0.9	33.14 ± 0.17
HSP7i	7.8 ± 0.26	13.4 ± 6.51
CBP gene	2.4 ± 1.3	33.55 ± 3.9
iaac2 trypsin inhinibitor	32.4 ± 8.1	142.1 ± 15.3

CBP gene = gene involved in chlorophyll biosynthetic process
RPKM = reads per kilobase per million mapped reads

4.1.4. Enzyme code annotation by KEGG

For genes corresponding to enzymes, the enzyme code annotation was performed with KEGG (Götz et al., 2008) map.

In Primadur, this analysis showed the stress induced up-regulation of genes involved in myo-inositol phosphate metabolism (e.c. 1.13.99.1–inositol oxigenase; 5.5.1.4 – inositol synthase), in aromatic amino acids synthesis (e.c. 2.5.1.54–7P-2dehydro-3-deoxy-D-arabino-heptonate synthase) and phenylpropanoid biosynthesis (e.c. 1.2.1.44–reductase and e.c. 1.11.1.7 – lactoperoxidase), in valine, leucine and isoleucine metabolism (e.c. 1.2.1.44–3-methyl-2-oxobutanoate), in alanine, aspartate and glutamate metabolism (e.c. 2.6.1.2 – alanine transaminase and e.c. 2.6.1.44–alanine-pyruvate transaminase), in glycine metabolism (e.c. 2.6.1.4. – glycine transaminase and e.c.2.6.1.44– glycine-glyoxilate transaminase), in sucrose and glucose metabolism (e.c. 2.4.1.13– sucrose synthase and e.c. 3.2.1.21– glucose synthase), in amino sugar and nucleotide sugar metabolism (e.c. 4.1.1.35–UDP-D-glucuronate decarboxylase

involved in the sinthesis of UDP-D- xylose). The decrease of the transcripts of the glutamine-hydrolysing enzyme (e.c. 6.3.5.7.- synthase - involved in the phatways of aminoacyl-tRNA biosynthesis) and citrate synthase (e.c. 2.3.3.8.- synthase- involved in TCA cycle) was observed in response to heat stress. An example of KEGG results is shown in Figure 4.7, in which the genes up-regualted by heat stress in Primadur and belonging to the pathways of starch and sucrose metabolism are represented in blu.

In T1303, enzyme code annotation showed the stress dependent up-regulation of inositol oxigenase (e.c. 1.13.99.1), and of genes involved in glycine metabolism (e.c. 2.6.1.4. – glycine transaminase and e.c. 2.6.1.44– glycine-glyoxilate transaminase), in phenylalanine, tyrosine and tryptophan synthesis (e.c. 4.2.3.5–5-0-(1carboxyvinil)-3-phosphoshimate-chorismate synthase), in alanine, aspartate and glutamate metabolism (e.c. 2.6.1.2 – alanine transaminase and e.c. 2.6.1.44–alanine-pyruvate transaminase), in sucrose and glucose metabolism (e.c. 2.4.1.13– sucrose synthase; e.c. 5.3.1.9– D-Glucose-6P synthase; e.c. 2.7.7.9–UDP-glucose synthase; e.c. 3.2.1.2–maltoxtrin synthase; e.c. 2.4.1.18–glycogen synthase; all involved in starch-glycogen biosynthesis) (Figure 4.8).

The transcript of phosphoenolpyruvate carboxylase (ec:4.1.1.31 – carboxylase), the enzyme replenishing oxalacetate in the tricarboxylic acid cycle when operating in the reverse direction, decreased in response to heat stress. The down-regulation of the gene coding for e.c. 1.11.1.7-lactoperoxidase, involved in phenylpropanoid biosynthesis, was observed in T1303.

Some pathways, such as those involved in myo-inositol phosphate, phenylpropanoid and sucrose metabolism, showed differences between the two genotypes, whereas others, such as those involved in alanine and aspartate metabolism and glycine metabolism, showed a similar response in the two genotypes (Table 4.5).

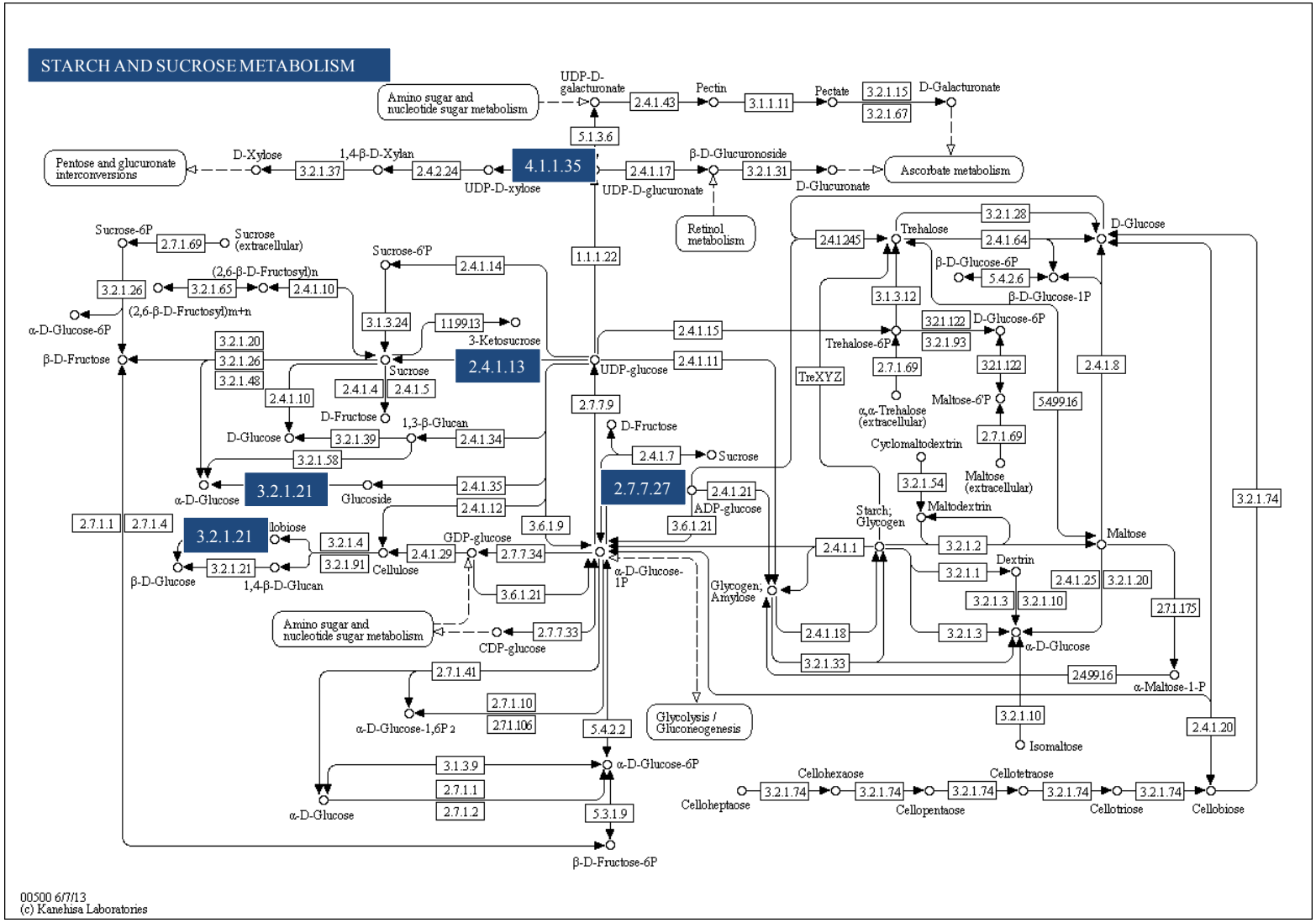


Fig.4.7 Results of KEGG analysis for starch and sucrose metabolism in Primadur
Enzyme code of up regulated genes coding for enzyme proteins are represented in blu. Enzyme code (e.c.) 3.2.1.21 glucose-synthase; e.c. 2.4.1.13 sucrose synthase; e.c. 2.7.7.27 Adenylyltransferase; e.c. 4.1.1.35 UDP-D-glucuronate decarboxylase.

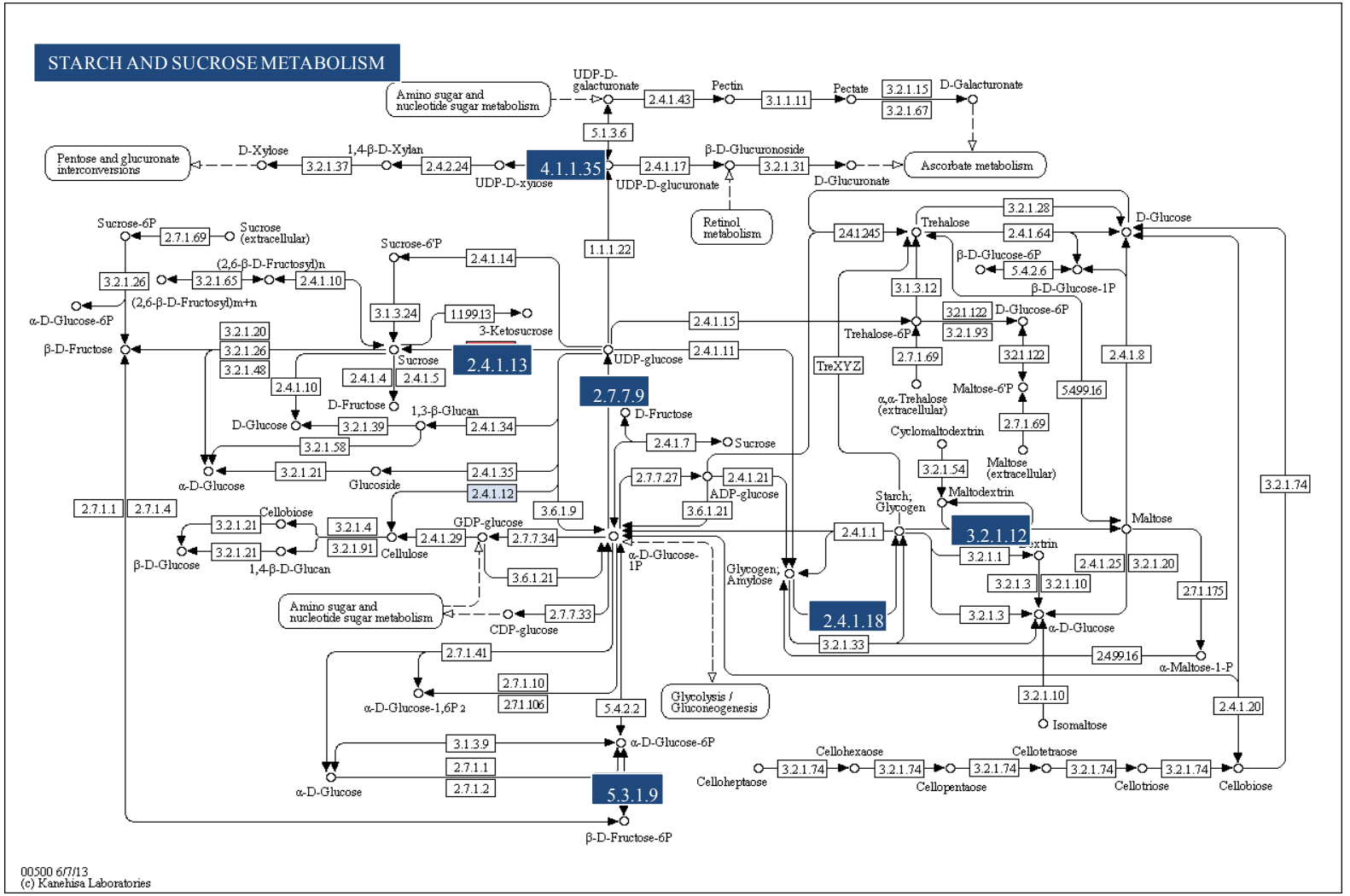


Fig. 4.8 Results of KEGG analysis for starch and sucrose metabolism in T1303

Enzyme code of up regulated genes coding for enzyme protein are represented in blu. e.c. 2.4.1.13 = sucrose sunthase; e.c. 2.7.7.9 = UDP-glucose synthase; e.c. 2.4.1.18 = glycogen synthase; 4.1.1.35 = UDP-D-glucuronate decarboxylase; e.c.3.2.1.2 = maltoxtrin synthase; e.c.5.3.1.9 = D-Glucose-6P synthase.

Table 4.5 Enzyme Code Annotation of some transcripts up-regulated in response to heat shock

Phatways	Primadur	T1303
myo-inositol phosphate metabolism	e.c. 1.13.99.1-inositol oxigenase e.c. 5.5.1.4 – inositol synthase	e.c. 1.13.99.1-inositol oxigenase
aromatic amino acids synthesis	e.c.2.5.1.54 7P-2dehydro-3-deoxy-D-arabino-heptonate synthase	e.c. 4.2.3.5 5-0-(1carboxyvinil)-3-phosphoshimate-chorismate synthase
phenylpropanoid biosynthesis	e.c. 1.2.1.44-reductase and e.c. 1.11.1.7-lactoperoxidase	e.c. 1.4.3.21-oxidase
alanine and aspartate metabolism	e.c. 2.6.1.2 – alanine transaminase e.c. 2.6.1.44 alanine-pyruvate transaminase	e.c. 2.6.1.2 – alanine transaminase e.c. 2.6.1.44 alanine-pyruvate transaminase
glycine metabolism	e.c. 2.6.1.4. glycine transaminase e.c.2.6.1.44 glycine-glyoxilate transaminase	e.c. 2.6.1.4. glycine transaminase e.c.2.6.1.44 glycine-glyoxilate transaminase
sucrose and glucose metabolism	e.c. 2.4.1.13 sucrose synthase e.c. 3.2.1.21 glucose synthase	e.c. 2.4.1.13 sucrose synthase e.c. 2.4.1.18-glycogen synthase
amino sugar and nucleotide sugar metabolism	e.c.4.1.1.35 UDP-D-glucuronate decarboxylase	e.c.4.1.1.35 UDP-D-glucuronate decarboxylase

e.c., enzyme code

4.2. Effects of Heat Stress on Yield-Related and Qualitative/Nutraceutical Traits

Two-way ANOVA analysis showed that the effects of Genotype, Treatment and the Genotype × Treatment interactions were highly significant for the protein content of these grain (Table 4.6). However, only the effects of Genotype were significant for carotenoid content, while for antioxidant activity, the effects of Treatment and the Genotype × Treatment interaction were highly significant (Table 4.6).

Higher protein content was seen for T1303 compared to Primadur in both the control and the heat-stressed plants, and the protein content increased significantly in response to the heat stress only for T1303. Primadur showed higher carotenoid content with respect to T1303 (i.e., about 3-fold greater), although no significant variations in carotenoids were observed in response to the heat stress for either of these genotypes. Anthocyanins were measured as cyanidin-3-glucoside equivalents, and these were detected only for T1303, as expected, with a significant increase in response to the heat stress (Table 4.6). The antioxidant activity showed very similar levels for the Primadur control and heat-stressed conditions, and for the T1303 heat-stressed wholemeal samples. These values were significantly higher with respect to those observed for the T1303 control, and therefore for the T1303 alone, the heat stress induced an increase in antioxidant activity.

For the yield-related parameters, the effects of both Genotype and Treatment were highly significant for the individual grain weight and grain yield per spike for the mature grains. However, only the effect of Genotype was highly significant for these two traits for the immature grain.

The heat stress resulted in significant changes for the individual grain weight and grain yield per spike for the mature seeds of both of these durum-wheat genotypes, but not for the immature seeds. Statistically significant weight losses of 11.0% (from 28.2 ±0.1 to 25.4 ±1.05 mg) and 8.4% (from 52.2 ±0.32 to 47.8 ±1.3 mg) were observed for Primadur and T1303, respectively, in response to the heat stress. However, the grain yield per spike was not statistically different across the T1303 control and the heat-stressed Primadur and T1303.

Table 4.6. ANOVA analysis of the yield-related and qualitative/nutraceutical traits.

Seeds	Trait	G	T	G×T	Primadur		T1303	
					Control	Heat shocked	Control	Heat shocked
Mature	Protein content (%)	****	***	**	14.94 ±0.52 ^{c}	15.04 ±0.25 ^{c}	16.74 ±0.42 ^{b}	19.00 ±0.33 ^{a}
	Carotenoids (µg/g dw)	***	ns	ns	9.26 ±0.54 ^{a}	8.67±0.23 ^{a}	3.5 ±0.49 ^{b}	3.56 ±0.04 ^{b}
	Anthocyanins (µg/g dw)	–	–	–	nd	nd	9.3 ±0.54 ^{b}	16 ±0.73 ^{a}
	Antioxidant activity (mM Trolox/kg dw)	ns	***	***	13.64 ±0.02 ^{b}	13.85 ±0.13 ^{b}	12.95 ±0.13 ^{b}	14.2 ±0.28 ^{a}
	Individual grain weight (mg dw)	****	****	ns	28.2 ±0.1 ^{c}	25.4 ±1 ^{d}	52.2 ±0.32 ^{a}	47.8 ±1.3 ^{b}
	Grain yield per spike (mg dw)	***	***	ns	1410 ±38 ^{a}	1184 ±89 ^{b}	1210 ±13 ^{b}	1093 ±5,8 ^{b}
	Grain number per spike	****	ns	ns	50 ±2 ^{a}	47 ±5 ^{a}	23 ±1 ^{b}	23 ±1 ^{b}
Immature	Individual grain weight (mg dw)	****	ns	ns	10.8 ±0.97 ^{b}	11.2 ±0.81 ^{b}	15.9 ±0.41 ^{a}	16.3 ±0.24 ^{a}
	Grain yield per spike (mg dw)	****	ns	ns	513 ±20.1 ^{a}	536 ±22.3 ^{a}	354 ±6.5 ^{b}	370 ±19.1 ^{b}
	Grain number per spike	****	ns	ns	49 ±2 ^{a}	48 ±4 ^{a}	23 ±1 ^{b}	22 ±2 ^{b}

ns, not significant; **, P <0.01; ***, P <0.001; ****, P <0.0001; nd, not detected; –, not evaluated
Values in the same row followed by different superscript letters are significantly different (P <0.05). dw, dry weight; G, Genotype; T, Treatment; G×T, Genotype×Treatment interaction

4.3. Analysis of Polar Metabolites

4.3.1. Analysis of Variance for Classes of Polar Metabolites

Metabolic profiling of the whole-grain samples led to the identification of 42 metabolites that were included in five classes of compounds: amino acids, *N*-compounds, organic acids, sugars, and sugar alcohols. ANOVA analysis assuming a random model was carried out for the different classes of compounds to determine the contribution of each variance component, as Genotype, Treatment, and timing, and their interactions (Table 4.7).

Table 4.7. ANOVA analysis for each class of polar metabolite.

Class of metabolite	**G**	**T**	**t**	**G×T**	**G×t**	**T×t**	**G×T×t**
Amino acids	***	***	***	***	***	***	***
N-compounds	***	**	***	***	***	ns	ns
Organic acids	***	**	***	***	***	*	***
Sugars	***	*	***	***	***	ns	**
Sugar alcohols	***	*	***	***	***	ns	**

ns, not significant; *, P <0.05; **, P <0.01; ***, P <0.001; ****, P <0.0001.

G, Genotype; T, Treatment; t, timing; and their interactions

The effects of Genotype, timing and the Genotype × Treatment and Genotype × timing interactions were highly significant for all classes of compound, and for the total amino acids the effect of Treatment was also highly significant.

The levels of each class of metabolite were higher for Primadur than T1303, and the immature seeds showed higher levels of these metabolites with respect to the mature seeds (Table 4.8). All of the classes of compounds analyzed showed general increases in response to the heat stress (Table 4.8). Analysis of the relative variance showed that the largest part of the variance was represented by timing for the amino acids, sugar, and sugar alcohols, whereas Genotype represented the largest part of the variance for the *N*-compounds and organic acids (Fig. 4.9). These data suggested the need to perform the statistical analysis for the individual metabolites separately for the immature and mature seeds, and to evaluate the individual responses of the two genotypes to heat stress.

Table 4.8. ANOVA analysis of Genotype, timing and Treatment for each class of metabolite.

Variance component		Amino acids	*N*-compounds	Organic acids	Sugars	Sugar alcohols
Genotype	Primadur	6189 [a]	48.01 [a]	8738 [a]	228679 [a]	1226 [a]
	T1303	3895 [b]	6.86 [b]	3668 [b]	142187 [b]	745 [b]
	Significance	***	***	***	***	***
timing	Immature	7757 [a]	43.72 [a]	8075 [a]	260274 [a]	1745 [a]
	Mature	2327 [b]	11.15 [b]	4331 [b]	110592 [b]	226 [b]
	Significance	***	***	***	***	***
Treatment	Control	4551 [b]	22.34 [b]	5807 [b]	177697 [b]	952 [b]
	Heat-shocked	5534 [a]	32.54 [a]	6599 [a]	193169 [a]	1019 [a]
	Significance	***	**	**	*	*

*, P <0.05; **, P <0.01; ***, P <0.001

Values in the same column followed by different letters are significantly different (P <0.05)

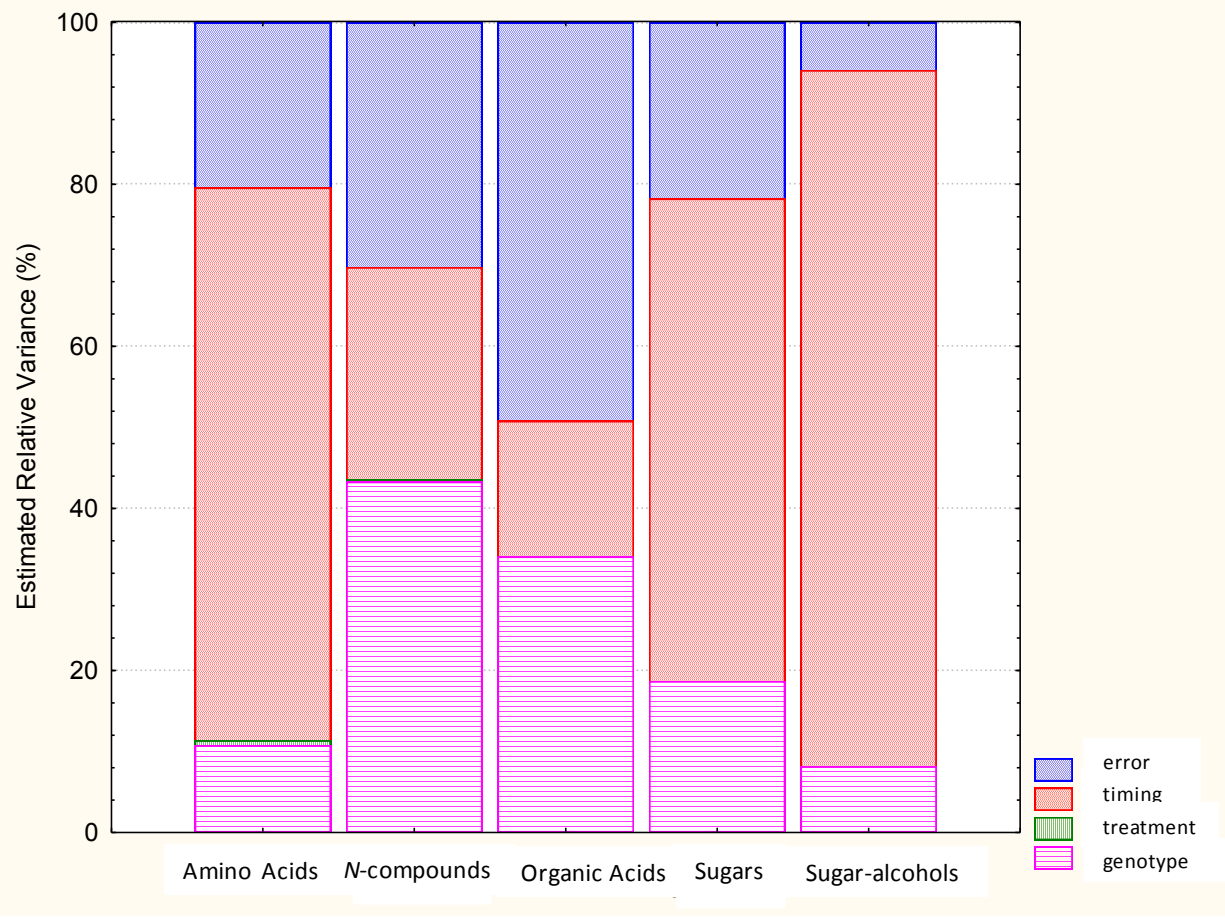


Fig. 4.9 Analysis of the relative variance for each class of metabolite

4.3.2. Analysis of Variance for Individual Metabolites

Sixty-two percent and thirty-six percent of the compounds evaluated here showed statistically significant differences between the control and heat-stress conditions for the immature and mature seeds, respectively. The Genotype effect was significant for all of the free amino acids assessed for the immature seeds, with the exception of leucine; on the contrary, only the levels of valine, isoleucine, and asparagine were significantly different between the two genotypes for the mature seeds (Table 4.9).

Table 4.9. ANOVA analysis performed separately for the immature and mature seeds.

Metabolite	**ANOVA significance**					
	Immature seeds			**Mature seeds**		
	G	**T**	**G×T**	**G**	**T**	**G×T**
Amino acids	****	***	***	ns	ns	****
Valine	****	ns	ns	*	****	****
Alanine	****	****	*	ns	ns	****
Asparagine	****	**	***	**	ns	****
Aspartic acids	****	***	****	ns	ns	****
Glutamic acid	****	ns	ns	ns	ns	****
Isoleucine	***	**	ns	**	ns	****
Serine	**	ns	ns	ns	ns	****
Glycine	****	**	**	ns	**	****
Threonine	****	ns	ns	ns	ns	****
Leucine	ns	ns	ns	ns	ns	*
β-Alanine	****	ns	ns	ns	ns	*
Phenylalanine	****	****	****	ns	ns	****
Tryptophan	****	**	ns	ns	ns	***
Tyrosine	****	**	*	ns	ns	****
GABA	****	**	**	ns	ns	****
***N*-compounds**	***	*	*	*	**	****
Putrescine	****	**	**	*	*	*
Cadaverine	****	***	***	*	***	**
Spermidine	****	ns	ns	ns	ns	****

Table 4.9 Cont. ANOVA analysis performed separately for the immature and mature seeds.

Metabolite	ANOVA significance					
	Immature seeds			Mature seeds		
	G	T	G×T	G	T	G×T
Organic acids	****	**	***	ns	ns	****
Citric acid	****	ns	***	ns	ns	****
Ferulic acid	****	****	****	*	*	****
Fumaric acid	****	**	****	**	*	****
Malic acid	****	*	****	**	**	****
Nicotinic acid	****	ns	ns	ns	ns	***
Oxalic acid	****	ns	****	ns	ns	***
Quinic acid	****	ns	ns	ns	ns	****
Saccharic acid	****	*	*	*	**	****
Shikimic acid	****	ns	ns	*	ns	ns
3PGA	****	****	****	****	****	****
Sugars	****	ns	***	*	*	****
Raffinose	****	ns	**	ns	ns	****
Sucrose	ns	***	**	ns	ns	****
Glucose	****	****	****	**	**	****
Glucose 6-phosphate	****	ns	ns	*	*	**
Fructose	****	***	****	ns	ns	****
Fructose 6-phosphate	****	ns	ns	ns	ns	***
Ribose	****	***	***	ns	ns	****
Mannose	****	**	**	ns	ns	****
Palatinose and maltitol	****	****	****	****	****	****
Xylose and lyxose	****	****	****	ns	ns	****
Maltose and turanose	****	***	***	**	*	****
Sugar alcohols	****	ns	ns	ns	**	****
Mannitol	****	*	**	**	****	****
myo-Inositol	****	*	**	ns	ns	****
Glycerol	****	ns	ns	***	****	****

ns, not significant; *, P <0.05; **, P <0.01; ***, P <0.001; ****, P <0.0001.
GABA, γ-4-aminobutyric acids; 3PGA, 3-phosphoglyceric acid.
G, Genotype; T, Treatment; G×T, Genotype × Treatment interaction.

The effects of Treatment were significant for the free amino acids except valine, glutamic acid, serine, threonine, leucine and β-alanine for the immature seeds, whereas Treatment was significant only for valine and glycine for the mature seeds, in which a very strong effect of the interaction was seen. The effects of Genotype on the levels of *N*-compounds was highly significant for the immature seeds, while the effects of the Genotype × Treatment interaction were highly significant for the mature seeds. For putrescine and cadaverine, the effects of Genotype, Treatment, and the Genotype × Treatment interaction were significant for both the immature and mature seeds, whereas for spermidine only the effects of Genotype and the Genotype × Treatment interaction were highly significant for the immature and mature seeds, respectively. The highest levels of significance were for Genotype and the Genotype × Treatment interaction for organic acids for the immature seeds. For ferulic acid, the effects of Genotype, Treatment and the Genotype × Treatment interaction were highly significant.

The effects of the Genotype × Treatment interaction were highly significant for all of the organic acids for the mature seeds, with the exception of shikimic acid, for which only the effect of Genotype was significant (Table 4.9; $P < 0.05$). Treatment affected the accumulation of some of the organic acids (i.e., malic, fumaric, ferulic, 3PGA, saccharic) for both the immature and mature seeds. For the immature seeds, the effects of Genotype were highly significant for the sugars, with the exception of sucrose, whereas the effects of Treatment and the Genotype × Treatment interaction were not significant for any of the sugars. On the contrary, for the mature seeds, the effects of the Genotype × Treatment interaction was highly significant for the sugars, whereas the effects of Genotype and Treatment were significant for glucose, glucose 6-phosphate, palatinose, maltitol, maltose, and turanose. The effects of Genotype on the variability of all of the sugar alcohols were higly significant for the immature seeds, whereas Treatment and the Genotype × Treatment interaction had significant effects on the variability of the sugar alcohols for the mature seeds.

4.3.3. Metabolite Changes in Response to Heat Stress in the Two Durum Wheat Genotypes

Ninety-five percent and thirty-eight percent of the compounds evaluated here showed statistically significant differences between the two genotypes for the immature and mature seeds, respectively (Table 4.9). Following the heat stress, all of the classes of compounds analyzed, except for the *N*-compounds, showed general increases in their levels for Primadur and general decreases for T1303 (Table 4.10).

Table 4.10. ANOVA analysis of Genotype × Treatment interactions for each class of metabolite.

Genotype × Treatment		**Amino acids**	***N*-compounds**	**Organic acids**	**Sugars**	**Sugar alcohols**
Primadur	Control	4424 ^{b}	34.5 ^{b}	5945 ^{b}	177865 ^{b}	1098 ^{b}
	Heat shocked	7955 ^{a}	61.5 ^{a}	11531 ^{a}	279494 ^{a}	1355 ^{a}
T1303	Control	4677 ^{b}	10.1 ^{c}	5668 ^{b}	177529 ^{b}	807 ^{c}
	Heat shocked	3113 ^{c}	3.5 ^{c}	1668 ^{c}	106845 ^{c}	684 ^{d}
	Significance	***	***	***	***	***

***, P <0.001
Values in the same column followed by different letters are significantly different (P <0.05)

The analytical data for each metabolite, the analysis of variance, and the least significant difference test among the means of all of the detected compounds are reported in Table 4.11. In general, the most abundant metabolites were sugars, and among these, raffinose had the highest levels, followed by sucrose.

Table 4.11 Metabolite composition of the seeds.

Metabolite class	Metabolite	Metabolite content according to seeds, genotype and heat stress (µg/g DW)							
		Immature seeds				Mature seeds			
		Primadur		T1303		Primadur		T1303	
		Control	Heat shocked	Control	Heat shocked	Control	Heat shocked	Control	Heat shocked
Amino acids	Valine	270.70 ±21.41^{b}	267.00 ±7.2^{b}	472.61 ±33.09^{a}	417.28 ±31.48^{a}	4.61 ±0.36^{c}	35.71 ±1.9^{a}	18.64 ±0.78^{b}	12.41 ±4.65^{b}
	Alanine	564.62 ±141.78^{c}	872.74 ±163.77^{b}	1101.31 ±64.53^{b}	1755.42 ±33.55^{a}	2.30 ±1.51^{b}	52.33 ±10.07^{a}	46.00 ±13.9^{a}	2.54 ±0.64^{a}
	Asparagine	1288.58 ±240.59^{b}	2937.24 ±463.81^{a}	762.11 ±108.55^{c}	399.26 ±28.08^{c}	94.80 ±22.3^{d}	785.64 ±153.1^{b}	1130.97 ±198.7^{a}	251.81 ±17.4^{c}
	Aspartic acid	905.30 ±99.02^{b}	1594.59 ±91.14^{a}	234.81 ±36.14^{c}	109.90 ±13.69^{c}	12.18 ±6.4^{b}	731.34 ±128.6^{a}	651.2 ±134.8^{a}	19.89 ±2^{b}
	Glutamic acid	1783.16 ±38.36^{b}	2044.83 ±282.1^{a}	225.47 ±12.81^{c}	170.25 ±11.73^{c}	48.8 ±10.14^{b}	922.88 ±188.47^{a}	843.14 ±71.3^{a}	58.70 ±4.02^{b}
	Isoleucine	102.73 ±5.5^{a}	79.90 ±12.47^{b}	145.72 ±14.61^{a}	122.15 ±9.02^{a}	1.82 ±1.36^{b}	7.56 ±1.25^{a}	8.91 ±0.85^{a}	5.53 ±0.55^{a}
	Serine	1288.70 ±275.98^{a}	1239.40 ±200.18^{a}	816.27 ±40.28^{b}	871.37 ±50.38^{b}	11.49 ±0.37^{b}	507.24 ±111.58^{a}	452.58 ±102.7^{a}	43.95 ±35.5^{b}
	Glycine	388.48 ±30.45^{b}	477.34 ±4.29^{a}	364.77 ±15.54^{c}	349.93±12.33^{c}	0.93 ±0.04^{c}	11.46 ±1.9^{a}	7.08 ±1.45^{b}	2.22 ±1.53^{c}
	Threonine	377.24 ±26.29^{a}	370.0 ±18.08^{a}	114.34 ±12.16^{b}	111.11 ±4.75^{b}	24.90 ±1.46^{b}	188.53 ±36.85^{a}	193.26 ±9.17^{a}	24.96 ±18.12^{b}
	Leucine	54.16 ±12.83	44.22 ±10.61	66.34 ±9.04	57.12 ±8.96	1.76 ±0.51	3.87 ±1.54	4.11 ±0.8	2.73 ±0.56
	β-Alanine	36.84 ±0.58^{a}	41.59 ±4.86^{a}	3.25 ±0.3^{b}	2.31 ±0.06^{b}	1.29 ±1.8^{b}	24.36 ±4.9^{a}	20.39 ±1.8^{a}	0.15 ±0.04^{b}
	Phenyl-alanine	122.70 ±5.13^{b}	184.59±2.87^{a}	17.56 ±1.47^{c}	15.45 ±0.53^{c}	27.73 ±0.4^{b}	74.41 ±10.9^{a}	75.69 ±9^{a}	33.67 ±0.4^{b}
	Tryptophan	374.54 ±20.03^{c}	495.99 ±76.84^{c}	885.13 ±119.2^{b}	1174.36 ±76.73^{a}	217.38 ±15.8^{b}	328.26 ±14.5^{a}	288.04 ±51.8^{a}	177.44 ±32.7^{c}
	Tyrosine	42.84 ±2.79^{b}	59.27 ±7.69^{a}	7.09 ±1.32^{c}	8.64 ±1.16^{c}	2.27 ±1.2^{b}	23.78 ±7.4^{a}	25.42 ±4.8^{a}	4.30 ±2.8^{b}
	GABA	787.32 ±48.17^{b}	1101.48 ±114.79^{a}	37.75 ±6.45^{c}	14.06 ±4.3^{c}	8.44 ±0.99^{b}	403.25 ±102.8^{a}	335.83 ±37.7^{a}	7.54 ±0.84^{b}
N-compounds	Putrescine	22.93 ±3.8^{b}	39.68 ±7.3^{a}	2.35 ±0.2^{c}	2.46 ±0.4^{c}	0.21 ±0.05^{b}	11.22 ±6.7^{a}	0.72 ±0.56^{b}	0.73 ±0.3^{b}
	Cadaverine	7.13 ±0.8^{b}	10.49 ±0.7^{a}	0.52 ±0.07^{c}	0.43 ±0.03^{c}	0.52 ±0.07^{b}	3.48 ±0.6^{a}	0.82 ±0.7^{b}	1.59 ±0.06^{b}
	Spermidine	38.22 ±1.7^{a}	47.45 ±16.4^{a}	1.34 ±0.2^{b}	1.88 ±1^{b}	0.21 ±0.05^{b}	10.68 ±5.1^{a}	14.57 ±2.8^{a}	0.21 ±0.05^{b}
Organic acids	Citric acid	459.28 ±64.1^{b}	634.92 ±41.6^{a}	177.07 ±12.5^{c}	81.50 ±16.1^{d}	1.74 ±0.18^{b}	287.19 ±94.4^{a}	274.99 ±28^{a}	4.99 ±0.42^{b}
	Ferulic acid	10.32 ±0.7^{b}	19.34 ±0.3^{a}	0.71 ±0.08^{c}	0.50 ±0.15^{c}	0.24 ±0.3^{c}	8.70 ±1.5^{a}	5.85 ±1.1^{b}	0.08 ±0.01^{c}
	Fumaric acid	177.35 ±2.9^{b}	217.43 ±7.4^{a}	97.94 ±11.2^{c}	18.34 ±1.4^{d}	27.47 ±0.2^{c}	153 ±16^{a}	113.33 ±5.1^{b}	23.34 ±10.8^{c}
	Malic acid	3644.35 ±217.6^{b}	4643.75 ±122.3^{a}	1423.95 ±82.4^{c}	998.79 ±156.1^{d}	388.73 ±11.8^{c}	1714.39 ±371^{b}	2485.56 ±113.5^{a}	384.11 ±13.7^{c}
	Nicotinic acid	12.5 ±1.6^{a}	13.61 ±0.2^{a}	7.70 ±0.3^{b}	7.05 ±0.7^{b}	3.70 ±1.1^{c}	5.97 ±0.22^{bc}	7.23 ±1.5^{ab}	3.50 ±0.8^{cd}

Table 4.11 Cont. Metabolite composition of the seeds

Organic acids	Oxalic acid	195.75 ±4.2^{c}	278.72 ±15.9^{a}	227.04 ±6.7^{b}	133.30 ±10.7^{d}	190.53 ±58.6^{b}	403.1 ±44.6^{a}	355.3 ±56.7^{ac}	172.87 ±60.9^{bc}
	Quinic acid	159.48 ±52.6^{a}	160.95 ±20^{a}	4.63 ±0.24^{b}	3.65 ±0.05^{b}	1.1 ±0.61^{b}	33.52 ±14.1^{a}	40.93 ±10.1^{a}	0.24 ±0.01^{b}
	Saccharic acid	6478.77 ±828.1^{b}	9090.44 ±1296.2^{a}	1573.27 ±22.5^{c}	1299.86 ±71.3^{c}	108.98 ±8.9^{c}	5359.32 ±277.3^{a}	4525.53 ±335.5^{b}	188.93 ±5.7^{c}
	Shikimic acid	22.21 ±4.4^{a}	26.66 ±10.4^{a}	1.46 ±0.3^{b}	0.97 ±0.1^{b}	8.86 ±0.26	11.59 ±2.7	15.36 ±4.3	14.40 ±1.8
	3PGA	5.67 ±0.2^{a}	3.7 ±0.1^{b}	0.38 ±0.07^{c}	0.38 ±0.04^{c}	26.19 ±0.7^{b}	4.17 ±0.6^{c}	4.49 ±1.5^{c}	35.63 ±0.8^{a}
Sugars	Raffinose	171×10^{3} ±23837^{a}	194.2×10^{3} ±17216^{a}	109.9×10^{3} ±12833^{b}	51.1×10^{3} ±3581^{c}	23.6×10^{3} ±488^{b}	79.3×10^{3} ±17194^{a}	60×10^{3} ±11498^{a}	29.1×10^{3} ±1801^{b}
	Sucrose	74.1×10^{3} ±4287^{b}	93.6×10^{3} ±6549^{a}	79.9×10^{3} ±1056^{b}	83.7×10^{3} ±958^{ab}	22.8×10^{3} ±367^{b}	54.7×10^{3} ±7948^{a}	50.5×10^{3} ±4090^{a}	23.4×10^{3} ±546^{b}
	Glucose	6333.51 ±183^{b}	8777.81 ±208^{a}	609.21 ±38^{c}	453.68 ±123^{c}	5.26 ±0.4^{c}	5626.15 ±517^{a}	3179.70 ±763^{b}	8.00 ±0.5^{c}
	Glucose 6-phosphate	48.37 ±9.3^{a}	52.1 ±11.1^{a}	0.32 ±0.04^{b}	0.18 ±0.07^{b}	0.18 ±0.07^{c}	41.67 ±12^{b}	141.7 ±71^{a}	0.32 ±0.03^{c}
	Fructose	15186.55 ±1824^{b}	22969.97 ±188^{a}	1178,03 ±395^{c}	808.44 ±100^{c}	502.52 ±376^{b}	13476.94 ±2103^{a}	8658.34 ±3269^{a}	284.88 ±54^{b}
	Fructose 6-phosphate	919.3 ±139^{a}	736.7 ±307^{a}	3.8 ±0.8^{b}	2.63 ±0.5^{b}	0.86 ±0.56^{b}	345.09 ±135^{a}	316.75 ±105^{a}	2.8 ±0.71^{b}
	Ribose	257.68 ±28.6^{b}	367.70 ±17.2^{a}	15.72 ±1.2^{c}	17.09 ±0.4^{c}	4.33 ±0.2^{b}	127.98 ±15^{a}	140.92 ±32.5^{a}	2.48 ±0.08^{b}
	Mannose	1749.64 ±223^{b}	2780.72 ±294^{a}	124.04 ±37^{c}	117.94 ±11^{c}	27.44 ±1.3^{b}	1596.85 ±34.3^{a}	1461.67 ±284^{a}	31.77 ±8.8^{b}
	Palatinose and maltitol	23.19 ±2.5^{b}	74.29 ±8.7^{a}	9.53 ±2.1^{c}	15.14 ±1.9^{c}	20.30 ±9.5^{c}	22.91 ±9.5^{bc}	134.31 ±17^{ab}	21.15 ±5.4^{cd}
	Xylose and lyxose	236.62 ±17.8^{b}	914.38 ±51^{a}	42.55 ±2.8^{c}	35.72 ±3^{c}	6.80 ±0.6^{c}	229.21 ±38.6^{a}	155.01 ±37.5^{b}	17.26 ±2.4^{c}
	Maltose and turanose	32.6×10^{3} ±2528^{b}	50×10^{3} ±4071^{a}	11.8×10^{3} ±348^{c}	20.1×10^{3} ±3258^{c}	7176.69 ±132^{c}	30.1×10^{3} ±4058^{a}	21.3×10^{3} ±1875^{b}	4382.71 ±399^{d}
Sugar alcohols	Mannitol	133.43 ±12.33^{a}	140.58 ±6^{a}	41.39 ±3.7^{b}	11.96 ±1.1^{c}	2.90 ±0.5^{d}	87.08 ±5.5^{a}	62.05 ±3.3^{b}	10.37 ±2.1^{c}
	myo-Inositol	335.32 ±12.9^{b}	364.13 ±6.5^{a}	61.86 ±3^{c}	52.99 ±1.3^{c}	10.96 ±0.88^{b}	256.76 ±48.8^{a}	227.33 ±43.1^{a}	14.98 ±0.45^{b}
	Glycerol	1698.32 ±73^{a}	1733.06 ±128^{a}	1181.24 ±60^{b}	1229.17 ±79^{b}	16.06 ±0.96^{c}	128.94 ±14.9^{a}	40.61 ±4.6^{b}	49.77 ±2.9^{b}

Data are means ± standard deviation. Values in the same row followed by different superscript letters are significantly different ($P < 0.05$; Tukey's tests). No letters are given when differences are not statistically different. ANOVA analysis was performed independently on immature and mature seeds. GABA, γ-4-aminobutyric acids; 3PGA, 3-phosphoglyceric acid.

Based on the large differences between the two genotypes, we decided to use Student's test to evaluate the significance of variation in response to heat stress for each individual metabolite at both stages of seed development (Table 4.12).

Table 4.12. Significance levels in the comparisons of the differences in the levels of the metabolites under control conditions with respect to heat-shocked conditions (Student's t-tests).

Metabolite class	**Metabolite**	**Control *versus* heat shocked**			
		Significance level according to seeds and genotype			
		Immature seeds		**Mature seeds**	
		Primadur	**T1303**	**Primadur**	**T1303**
Amino acids	Glutamic acid	0.12	0.030	0.002	0.0012
	β-Alanine	0.2	0.02	0.0003	0.001
	Asparagine	0.02	0.01	0.008	0.0008
	Aspartic acid	0.001	0.005	0.0003	0.0006
	GABA	0.013	0.004	0.001	0.0001
	Threonine	0.40	0.33	0.0001	0.00006
	Serine	0.19	0.16	0.001	0.018
	Glycine	0.02	0.052	0.013	0.0054
	Isoleucine	0.07	0.08	0.016	0.0026
	Leucine	0.27	0.28	0.001	0.042
	Valine	0.80	0.10	0.001	0.056
	Alanine	0.58	0.0001	0.001	0.016
	Phenylalanine	0.001	0.078	0.000004	0.008
	Tyrosine	0.04	0.20	0.004	0.02
	Tryptophan	0.04	0.062	0.009	0.017
N-compounds	Putrescine	0.025	0.67	0.024	0.48
	Cadaverine	0.006	0.11	0.001	0.071
	Spermidine	0.39	0.44	0.011	0.0004
Organic acids	Oxalic acid	0.001	0.0002	0.023	0.048
	Fumaric acid	0.001	0.0003	0.0001	0.0001
	Ferulic acid	0.00003	0.104	0.0003	0.0004
	Quinic acid	0.97	0.002	0.008	0.0011
	Citric acid	0.016	0.001	0.017	0.002
	Shikimic acid	0.53	0.051	0.08	0.37
	Malic acid	0.002	0.014	0.002	0.0006
	Saccaric acid	0.042	0.003	0.0004	0.001
	Nicotinic acid	0.310	0.236	0.0236	0.011
	3PGA	0.001	0.500	0.0003	0.0008

Table 4.12.Cont. Significance levels in the comparisons of the differences in the levels of the metabolites under control conditions with respect to heat-shocked conditions (Student's t-tests).

Metabolite class	Metabolite	Control *versus* heat shocked Significance level according to seeds and genotype			
		Immature seeds		Mature seeds	
		Primadur	T1303	Primadur	T1303
Sugars	Raffinose	0.245	0.002	0.014	0.028
	Sucrose	0.012	0.010	0.0096	0.0044
	Glucose	0.0001	0.104	0.0014	0.009
	Glucose 6-phosphate	0.305	0.075	0.014	0.037
	Fructose	0.002	0.191	0.0054	0.023
	Fructose 6-phosphate	0.128	0.114	0.024	0.017
	Ribose	0.005	0.134	0.0024	0.0089
	Mannose	0.008	0.80	0.0001	0.0066
	Palatinose and maltitol	0.001	0.028	0.42	0.0037
	Xylose and lixilose	0.00003	0.045	0.0003	0.016
	Maltose and turanose	0.003	0.250	0.0003	0.00005
Sugar alcohols	Mannitol	0.42	0.0002	0.0007	0.0009
	myo-Inositol	0.026	0.009	0.0005	0.0005
	Glycerol	0.704	0.451	0.026	0.0084

GABA, γ-4-aminobutyrric acids; 3PGA , 3-phosphoglyceric acid.

Although very different behaviors were seen for the two genotypes, some of the metabolites (i.e., sucrose, glycerol, alanine) increased in response to the heat stress for both Primadur and T1303 (Figures 4.10, 4.11).

Differences in the timing of the responses to the heat stress for each individual metabolite were observed. In Primadur some of the amino acids (i.e., asparagine, aspartic acid, γ-4-aminobutyrric acid [GABA], phenylalanine, tyrosine, glycine, tryptophan) increased in response to heat stress for both stages of seed development, whereas other amino acids increased only for the mature seeds. For the immature seeds of T1303, alanine increased in response to the heat stress, whereas the other amino acids did not change or decreased (i.e., glutamic acid, β-Alanine, asparagine, aspartic acid, GABA); for the mature seeds, all of the amino acids decreased, with the exception of valine, which did not change. For the *N*-compounds, the levels of putrescine and cadaverine increased for Primadur, whereas these did not change for T1303, while spermidine increased and decreased for the mature seeds of Primadur and T1303, respectively.

Among the organic acids, the levels of shikimic acid did not change in either durum-wheat genotype, while the other organic acids increased and decreased for the mature seeds of Primadur and T1303, respectively. Among the sugars and sugar alcohols, the levels of sucrose for the immature seeds and glycerol for the mature seeds increased in response to the heat stress for both of these genotypes.

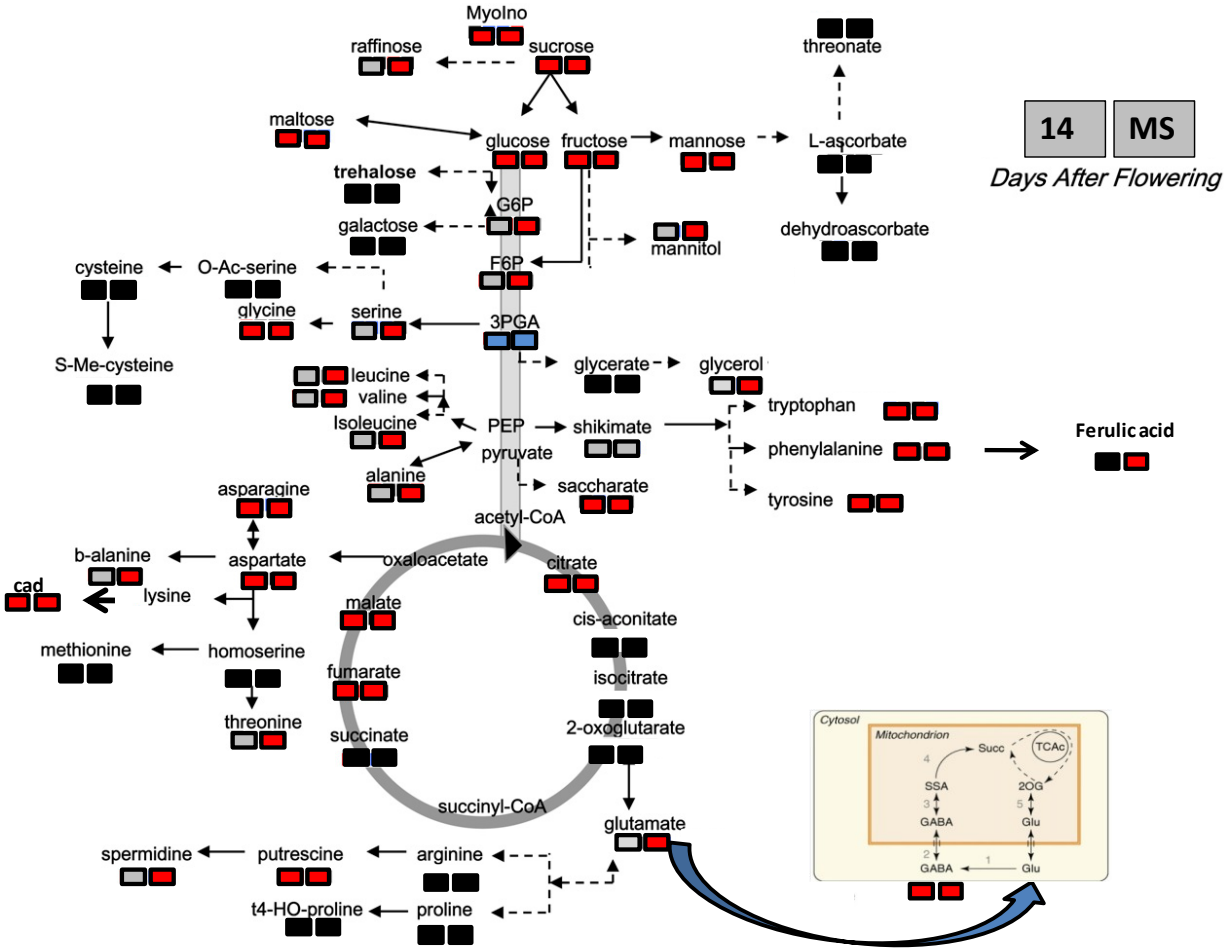


Fig. 4.10. Schematic picture of primary metabolism for Primadur

Changes in the metabolite profiles for the Primadur seeds, for the control compared to the heat-stressed conditions, for the immature (14 DAF) and mature (MS) stages of seed development. Changes in the metabolite levels were calculated as the ratios between the levels for the heat stress and the control. Anthocyanins were not detected. To visualize the changes, significant increases and decreases are indicated in red and blue, respectively, within a metabolic scheme ($p < 0.05$; Student's t tests). cad, cadaverine; b-alanine, β-Alanine; GABA, γ-4-aminobutyric acids. Solid line arrows: single step reactions; dash line arrows: pathways composed of more than one reaction; light gray shape: compounds which are not significantly different in seeds from heat stressed plants and control; red shape: compounds whose level is higher in seeds from heat stressed plants compared to control; blue shape: compounds whose level is lower in seeds from heat stressed plants compared to control; black shape: compounds not evaluated in the present study.

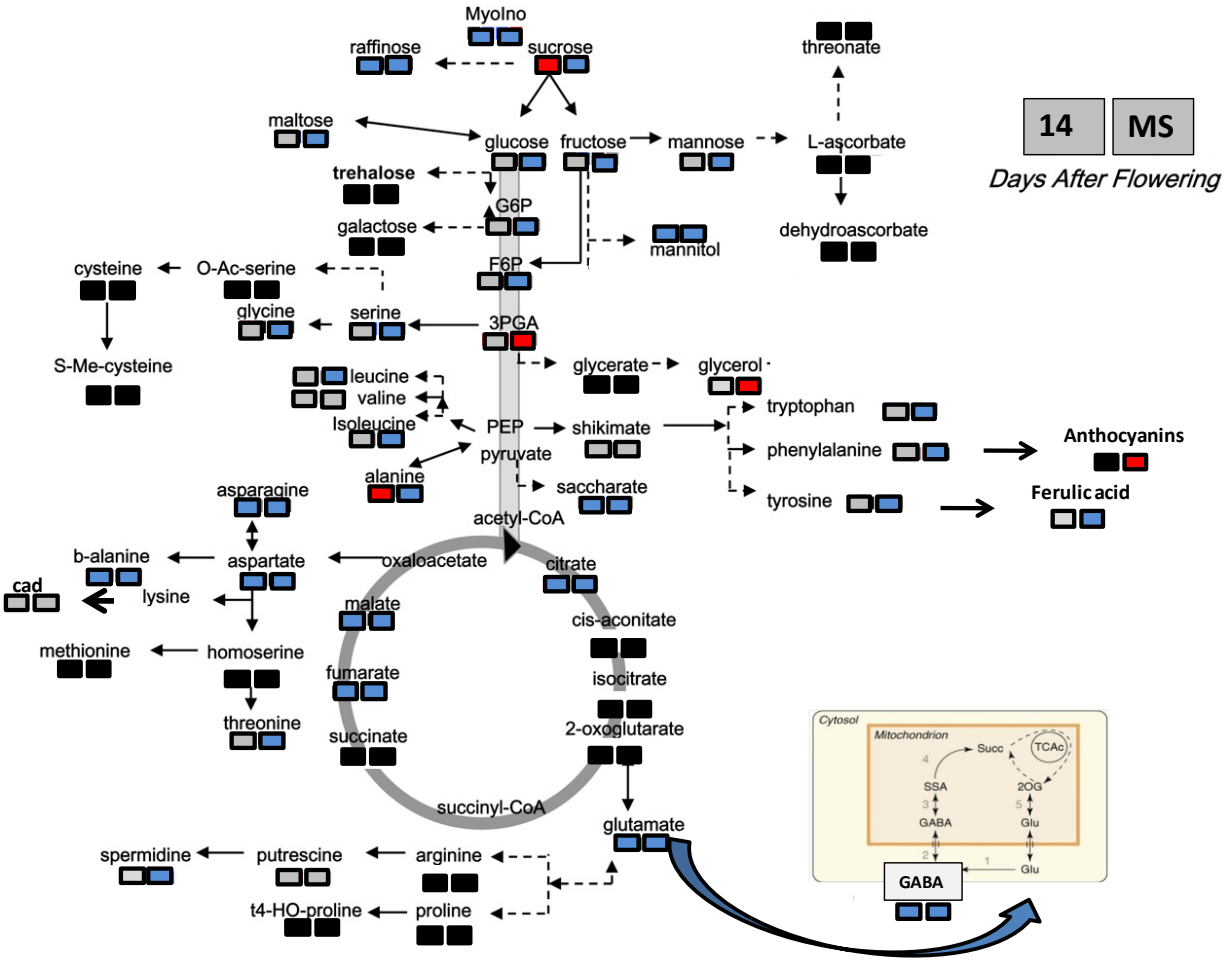


Fig. 4.11. Schematic picture of primary metabolism for T1303

Changes in the metabolite profiles for the T1303 seeds, for the control compared to the heat-stressed conditions, for the immature (14 DAF) and mature (MS) stages of seed development. Changes in the metabolite levels were calculated as the ratios between the levels for the heat stress and the control. Anthocyanins were not detected. To visualize the changes, significant increases and decreases are indicated in red and blue, respectively, within a metabolic scheme ($p < 0.05$; Student's t tests).cad, cadaverine; b-alanine, β-Alanine; GABA, γ-4-aminobutyric acids. Solid line arrows: single step reactions; dash line arrows: pathways composed of more than one reaction; light gray shape: compounds which are not significantly different in seeds from heat stressed plants and control; red shape: compounds whose level is higher in seeds from heat stressed plants compared to control; blue shape: compounds whose level is lower in seeds from heat stressed plants compared to control; black shape: compounds not evaluated in the present study.

5. DISCUSSION

5.1. Heat Shock Applied Early during Seed Development Produces Different Long-Term Effects in Two Durum Wheat Genotypes

A significant reduction in the grain weight was induced by the heat stress for both durum wheat genotypes used in the present study. The decrease in the grain weight was greater for Primadur than for T1303, while the grain numbers per spike did not change significantly in response to the heat stress for either of the genotypes. These data indicate that heat shock was effective in producing a clear effect on the developing seeds, and they suggest that Primadur was more susceptible to the heat stress than T1303. The effects of heat stress were evident not only in terms of the grain weight, but also in terms of the protein content, changes in gene expression and metabolite accumulation. The protein content increased in response to the heat stress only for T1303.

A total of 1,201 genes were differentially expressed following heat stress in the two durum wheat genotypes, indicating that the effect of the applied stress was mantained up to some days after the stress application. Enrichment analysis, performed to analyze the abundance of genes belonging to various functional classes across differentially expressed genes, showed that most of differentially expressed genes in Primadur belong to stress-related categories, which were not represented for differentially expressed genes in T1303. This different response between the two genotypes could be due to the fact that only one time-point was considered for transcriptome analysis. So, it is possible that stress related genes could also be regulated by heat shock in T1303 during a developmental stage that was not analyzed in our experiment. Another possible explanation is that some genes with an important role in heat stress response are already expressed at high levels in control conditions. Indeed, our analysis identified some genes coding for heat shock proteins whose transcript levels were higher for T1303 than Primadur seeds from control plants, and this can support the hypothesis of the greater susceptibility to heat stress of Primadur with respect to T1303.

These results are according to a previous study carried out by Aprile et al. (2013) in which two durum wheat genotypes were subjected to heat stress; this study showed that a large set of well-known stress-related genes were up-regulated in one of the two genotypes, while a constitutive expression of these genes was observed in the other genotype. Moreover, some studies showed that the number of stress related genes that were up-regulated by a stress treatment was higher in genotypes more susceptible with respect to genotypes less susceptible to stress (Qin et al, 2008).

The greater susceptibility to heat stress of Primadur with respect to T1303 is also suggested by the accumulation of polyamines, observed in response to heat stress only for Primadur. This result is in agreement with previous studies showing stress-induced selective accumulation of polyamines in sensitive plant species and wheat varieties (Kuzetsow et al., 2006; Galiba et al., 1989, 1993). Similarly, the level of GABA increased in response to the heat stress only for Primadur, whereas decreased for T1303. Increase in GABA level after heat stress is well documented in the literature, as following high temperatures, the increase in the cytosolic levels of Ca^{2+} can lead to calmodulin-mediated activation of glutamate decarboxylase, the enzyme that catalyzes the synthesis of GABA from glutamate (Kinnersley and Turano, 2000; Yamakawa and Hakata 2010). Furthermore, under oxidative stress conditions, degradation of GABA can also limit the accumulation of reactive oxygen intermediates (Breitkreuz et al., 1999), and therefore the differences observed in the present study might be related to different ability to reduce the accumulation of reactive oxygen species in T1303 with respect to Primadur. The accumulation of GABA and myo-inositol was previously observed in particular in genotypes with high susceptibility to stress (Taiz and Zeiger, 2006; Valluru and Van den Ende, 2011). In the present study an higher accumulation of polyamines, GABA and myo-inositol was observed for Primadur than T1303. Furthermore, at trascriptional level we also found the stress-dependent up-regulation of the gene coding for the inositol synthase enzyme (e.c. 5.5.1.4.) only in Primadur.

For metabolite accumulation, 62% and 36% of the compounds evaluated here showed statistically significant differences between the control and stress conditions for the immature and mature seeds, respectively. Similar findings were reported for mature maize grains, in which from 17% to 37% of the compounds included showed statistically significant differences that depended on the growing season (Rőhling et al., 2009) . These data underline that the effects of heat stress are stronger for the immature seeds, but, interestingly, most of the changes observed a few days after the stress imposition were maintained up to grain maturity.

Indeed, three types of behaviors in response to the heat stress were observed: for the total amino acids and organic acids, the effects of the heat treatment were significant only for the immature seeds, for the sugar and sugar alcohols the effects of the heat treatment were significant only for the mature seeds, whereas for the *N*-compounds, the effects of the heat treatment were significant for both the immature and mature seeds. The strong effects of the heat treatment at both stages of seed development for the polyamines (i.e., *N*-compounds) can be explained according to their roles in plant hormone signal transduction pathways in response to

abiotic stress (Liu et al., 2007; Kusano et al., 2008; Alcàazar et al., 2012; Shi and Chan, 2014). There is evidence that putrescine and abscissic acid are part of a positive feedback loop, in which each one induces the biosynthesis of the other following abiotic stress treatments (Alcázar et al. 2010a, 2010b). Abscissic acid also enhanced the increase of polyamine (i.e., putrescine, spermidine, spermime) amount, and induced the polyamine oxidation pathway in grapevine in response to drought stress, which resulted in secondary protective effects, such as stomata closure (Toumi et al., 2010). When we consider the effects of Genotype and the Genotype × Treatment interaction, the first was highly significant for the immature seeds (95% of the analyzed compounds), and the second was highly significant for the mature seeds (98% of the analyzed compounds). These data are compatible with the different responses to stress of these two genotypes; Primadur and T1303 showed general increases and decreases, respectively, for all of the classes of polar metabolites in response to the heat stress.

Moreover, the content of 62% of analyzed metabolites increased for the immature seeds of Primadur, and the same response to stress was observed for 95% of the metabolites analyzed for the mature seeds. The content of eight amino acids and polyamines increased for the immature seeds, with the same behavior maintained for the mature seeds. According to this result, we observed at transcriptional level the heat stress driven up-regulation of genes involved in the amino acid biosynthetic process in Primadur. The increase in levels of metabolites related to the amino acids biosynthetic process following abiotic stress was also reported as associated to the up-regulation of genes involved in amino acids biosynthesis by Espinoza et al. (2010). The increased levels of amino acids accumulation might be attributed to the heat-stable translocation of amino acids into the caryopsis and/or to the repression of protein synthesis, especially the tRNA charging step under high temperatures, as previously suggested in a study carried out on rice (Yamakawa and Hakata, 2010). In the present study, the decreased level of the transcript of the glutamine-hydrolysing enzyme (e.c. 6.3.5.7.), involved in the phatways of aminoacyl-tRNA biosynthesis, suggests that the increased levels of amino acids accumulation could be due to the down-regulation of the protein synthesis. Moreover, the heat-stress induced down-regulation of citrate synthase (e.c. 2.3.3.8.) was observed in Primadur, this suggested a reduced synthesis of amino acids from carbohydrates (Deucorcelle et al., 2015).

An opposite behavior was observed in T1303, in which a general decrease for all of the classes of polar metabolites was observed in response to stress. In this case the increase of protein content in response to stress is according to the up-regulation of genes coding for negative regulators of peptidase activity. This can explain the lower amount of free amino acids

observed in this genotype following the stress exposure, together with the down-regulation of the gene coding for phosphoenolpyruvate carboxylase (e.c. 4.1.1.31 – carboxylase), the enzyme replenishing oxalacetate in the tricarboxylic acid cycle when operating in the reverse direction. The decreased expression of phosphoenolpyruvate carboxylase at the point branching from the central pathway of glycolysis/gluconeogenesis suggests a decreased synthesis of amino acids from carbohydrates (Deucorcelle et al., 2015).

For the sugars, the levels of raffinose, glucose 6-phosphate and fructose 6-phosphate did not change in response to the heat stress for the immature seeds and increased for the mature seeds, whereas palatinose and maltitol increased only for the immature seeds in Primadur.

In response to the heat stress, for T1303, the content of 40% of the analyzed metabolites decreased for the immature seeds, and the same response was observed for the mature seeds, while the content of 55% of the analyzed metabolites, including ferulic acid and the polyamines, did no change for the immature seeds and decreased for the mature seeds. Whereas alanine, palatinose and maltitol increased for the immature seeds and decreased for the mature seeds.
The different responses to heat stress, observed between T1303 and Primadur genotypes, might be due to differences in the timing of the metabolite induction; for T1303, some metabolites might be induced by the heat stress at a stage not considered in this analysis, or the two genotypes might show differences in stress perception and tolerance.

Finally, some of the differences observed in the metabolite levels for the Primadur and T1303 grain can be related to the accumulation of anthocyanins in T1303. Sugar metabolism can be affected by the synthesis of anthocyanins, which are present in plants in their glycosylated forms, usually bound to sugars as glucose, galactose, arabinose, rhamnose, xylose, and fructose (Mazza et al., 2004; Choia et al., 2007; Hosseinian and Beta, 2007). If we consider the control conditions, for the mature seeds, T1303 was characterized by high levels of sugars compared to Primadur, and this confirmed previous studies carried out in other species. A higher concentration of glucose, galactose and myo-inositol was observed in the flesh of mature fruit of the red ‘Anjou’ pear, with respect to the green fruit (Li et al., 2014). Moreover red and purple genotypes of potato can accumulate up to 30% and 60% higher sucrose and glucose levels with respect to the white and yellow genotypes (Payyavula et al. 2013). Also in black rice, the content of sugars, sugar alcohols and protein were higher with respect to noncolored rice (Lee et al., 2006; Frank et al., 2012). Similar results were found for raffinose in pears (Li et al., 2014).

Glucose 6-phosphate isomerase is an enzyme of the carbohydrate biosynthesis pathway that can be differently regulated during the flavonoid biosynthetic process (Li et al., 2014;

Payyavula et al., 2013); it was up-regulated in response to stress in T1303 according to the increase of anthocyanins content observed in mature seeds from stressed plants. For the mature seeds derived from T1303 when heat stressed, with respect to the control plants, the relationships between sugar and anthocyanins metabolisms might explain the decrease of many sugars and some related compounds, such as myo-inositol, which regulates the production of galactinol, a precursor of raffinose (Smith et al., 1991; Hitz et al., 2002). Sugars, like the raffinose family, oligosaccharides, and sugar alcohols, have antioxidant properties and protect plant cells from oxidative stress through maintenance of the redox homeostasis (Nishizawa-Yokoi et al., 2008). The decrease of free sugars, observed in response to stress in T1303, was according to the up-regulation of glycogen synthase (e.c. 2.4.1.18), while the decrease of myo-inositol levels was according to the up-regulation of the gene coding for the inositol oxigenase (e.c. 1.13.99.1), observed in T1303 in absence of up-regulation of inositol synthase (e.c. 5.5.1.4). Moreover, we also observed differences in the expression levels of genes of the phenylpropanoid biosynthesis pathway, involved in anthocyanin synthesis, between the two genotypes. In particular, the amount of the transcript of the gene coding for lactoperoxidase (e.c. 1.11.1.7), one of the enzymes of the phenylpropanoid biosynthesis pathway, increased in Primadur and decreased in T1303 following the stress treatment.

The increase of anthocyanins and decrease of tryptophan amino acid were observed in T1303 seeds from plants subjected to heat stress. The decrease of tryptophan was according to the up-regulation of transcripts involved in tryptophan catabolic process (ppa3_arat purple acid phosphatase3), observed in response to stress only in T1303. Moreover, the up-regulation of the gene coding for 5-0-(1carboxyvinil)-3-phosphoshimate-chorismate synthase (e.c. 4.2.3.5), involved in phenylalanine, tyrosine and tryptophan biosynthesis, was also observed. The reduction of aromatic amino acids accumulation could be due to their use for the anthocyanins biosynthesis.

Despite the large differences in the metabolite changes in response to heat stress between these two genotypes, where 95% of the metabolites increased for the Primadur mature seeds in contrast to 81% of the metabolites that decreased for the T1303 mature seeds (except for 3PGA and glycerol, which increased), it is interesting to observe that some metabolites showed the same behavior for both genotypes.

These included some amino acids (i.e., threonine, serine, leucine, valine), spermidine and nicotinic acid, which did not change for immature seeds, shikimic acid, which did not change for both immature and mature seeds, and glycerol and 3PGA, which did not change for immature

seeds and increased for mature seeds. For alanine an up-regulation of genes involved in its metabolism (e.c. 2.6.1.2 – alanine transaminase and e.c. 2.6.1.44 – alanine-pyruvate transaminase) was observed for both genotypes and was associated with increased levels of alanine in immature and mature seeds of T1303 and Primadur, respectively.

Some of the responses in terms of the metabolite amounts were clearly related to the heat-stress response, and both genotypes were indeed characterized by an increase in sucrose for immature seeds and in glycerol for mature seeds from heat-stressed plants, with respect to the controls. In Primadur the greater sucrose amount observed in seeds from heat stressed plants was according to the up-regulation of glucose synthase (e.c. 2.4.1.18), and it was observed together with an increase of carotenoids level. A similar behavior was described for transgenic maize engineered for enhanced carotenoid synthesis (Deucorcelle et al., 2015). Sucrose can act as a signaling molecule, and in Arabidopsis leaves, it was shown that sucrose increased very rapidly in response to temperature shock and mantained high levels for the duration of the stress treatment (Kaplan et al, 2004). While most studies describe the effect of heat stress on vegetative tissues of plants (Hasanuzzaman et al., 2013), in the present study, we show that heat-induced accumulation of sucrose and glycerol is part of the mechanism of response to heat stress also for seeds. In agreement with these data, heat stress applied a few days after flowering induced the accumulation of sucrose in the developing caryopsis of rice (Yamakawa and Hakata 2010).

5.2. Effects of Heat Stress on Antioxidant Activity and Nutritional Properties of Durum Wheat Grain

Cereals are a good source of antioxidant compounds, which include polyphenols in particular (Liu et al., 2010), and also raffinose, *N*-compounds, free aromatic amino acids, and pigments (Lagishetty et al., 2008; Nishizawa-Yokoi et al., 2008; Mueller and Boehm, 2011; Nimalaratne et al., 2011). The levels of the carotenoids were not affected by the heat stress in the present study, in agreement with the strong Genotype component and the low Genotype × Environment interaction of this trait (Van Hung and Hatcher 2011). Otherwise, the higher carotenoid content observed for Primadur with respect to T1303 is in agreement with studies that have indicated that carotenoid levels are higher in modern durum-wheat varieties compared to older durum-wheat varieties, and wild populations and landraces; this is due to the breeding activities where an increasing yellow color became important over the last few decades (Digesù et al. 2009). Similar data have been reported for other species, such as peach, where there is evidence that the carotenoid content is higher in yellow flesh than in white and red-flesh peach genotypes (Vizzotto et al., 2006).

Anthocyanins derive from the metabolism of aromatic amino acids, and for T1303, the anthocyanin levels increased significantly in response to the heat stress, in agreement with a previous study carried out in wheat (Hosseinian et al., 2008). In mature seeds under control conditions, the levels of aromatic amino acids were higher for T1303 (purple grain) than for Primadur (yellow grain), in agreement with data from red and purple potato genotypes with respect to white and yellow ones (Payyavula et al., 2013). Furthermore, the levels of aromatic amino acids and a product of their metabolism, ferulic acid, were higher for the mature grain of Primadur from the heat-stressed plants, compared to the mature grain of Primadur plants grown under control conditions, with the opposite effect for T1303, where the heat stress induced decreases in the levels of these compounds.

The significant increase in the anthocyanins induced by heat stress for the mature seeds of T1303 can explain these differences, as aromatic amino acids are used in the biosynthetic pathway of the anthocyanins. A similar hypothesis can be proposed in relation to the different accumulation of the sugars (which include raffinose), which were more abundant for the T1303 mature seeds from the control plants, and lower for the T1303 mature seeds from the heat-stressed plants, compared to Primadur. Similar data were reported in a previous study in potato in which Payyavula et al. (2013) suggested that the regulatory loop of expression of the anthocyanin 1 transcription factor gene *AN1* can liberate hexoses that are used in the phenylpropanoid pathway.

For the antioxidant activity, it is known that no single method is adequate to evaluate its value in complex samples, as many individual compounds are known to contribute to the antioxidant capacity of foods or biological samples (Nimalaratne et al., 2011; Huang et al., 2005). The Trolox equivalent antioxidant capacity (TEAC) method considers most of the compounds with antioxidant properties analyzed in the present study, including phenolic compounds (Ficco et al., 2014), free aromatic amino acids (i.e., Tyr, Trp), polyamines, β-carotene metabolites, and the different β-carotene isomers (Mueller and Bohem, 2011; Nimalaratne et al., 2011; Kanna et al., 2011; Toro-Funes et al., 2013). This method does not measure the antioxidant capacity of cyanidin 3-glucoside, the most abundant anthocyanin metabolite in wheat, but instead it includes the phenolic compounds that are highly correlated to the kernel pigmentation (Ichikawa et al., 2001; Finocchiaro et al., 2007, Shen et al., 2009). Therefore, the increase in the antioxidant activity seen for the T1303 seeds derived from the heat-stressed plants is probably due to the activity of the bulk polyphenolic compounds, which were not measured in the present study, as these correlate to the increase in the kernel

pigmentation (i.e., the anthocyanin content). For Primadur, the antioxidant activity will be mainly related to the carotenoid pigment content (or the related phenolic compounds), the levels of which did not change here following the heat stress. At the same time, the increased levels of polyamines, ferulic acid, and aromatic amino acids observed for Primadur did not increase the antioxidant capacity of this grain.

Although an increase in the antioxidant capacity for Primadur in response to the heat stress was not seen in the present study, the accumulation of polyamines in response to the heat stress can have beneficial effects on human health. The major roles of polyamines in prevention of chronic diseases (Lagishetty and Naik, 2008; Soda, 2010) and in the regulation of inflammatory reactions and differentiation of immune cells (Moinard et al., 2005) are well known. In plants, elevated levels of polyamines are one of the most remarkable changes that occurs in response to abiotic stress conditions (Alcàzar et al., 2010; Simon-Sarkadi et al., 2006); however, in response to heat stress, there was only an increase for Primadur. Raffinose also increased in response to the heat stress for Primadur, and it is known to have antioxidant and antinutritional properties because it cannot be digested by monogastric animals (Zhawar et al., 2011; Valluru and Van den Ende, 2011). Therefore, our data indicate that heat stress can affect the accumulation of these compounds in different ways, which can result in either beneficial or detrimental effects on human health in relation to the wheat genotype and the environmental conditions that occur during the plant growth.

6. CONCLUSIONS

Twelve different wheat mega-environments have been defined worldwide based on cropping systems (e.g., rain fed *versus* irrigated, spring *versus* winter) together with biotic and abiotic constraints (Braun et al., 2010; Hansen et al. 2012). Most of these constraints are expected to increase in the framework of climate change. Therefore, new genotypes that are well adapted to these environments have to be selected, particularly when considering the trends towards increasing temperatures. Improved genotypes have to be selected not only in terms of their productive capacity, but also on the basis of their qualitative and nutritional parameters, which are strongly influenced by heat stress. The present study shows that heat stress applied to wheat plants early during seed development can have strong effects on wheat grain in terms of the transcriptome changes and metabolite accumulation, and that these responses are strictly dependent upon the genotype. For the antioxidant activity in particular, the Trolox equivalent antioxidant capacity (TEAC) method revealed an increase in antioxidant activity of ground grains only for T1303. The level of anthocyanins increased significantly in response to the heat stress whereas the level of carotenoids was not affected. In light of this, even if the genetic background of two durum wheat genotypes is different, it cannot be excluded that some of the differences observed in the responses to heat stress are due to anthocyanin accumulation, which can be considered a trait of interest for future breeding activities with wheat. Further studies are needed to better clarify the effect of anthocyanin accumulation on the response to heat stress. The analysis of a large number of yellow and red-grain genotypes and the development of near-isogenic lines, with the background of a yellow-grain cultivar and the locus/loci for anthocyanin synthesis, may be suitable approaches to study this aspect.

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