CHAPTER 2

REVIEW OF LITERATURE

2.1 Wheat Overview

2.1.1. Origin and Domestication

Wheat (*Triticum* spp.) is the most important food crop of the world in terms of the harvested area, trade value, and human nutrition. It is one of the oldest domesticated grain crops and for 8000 years has been the basic staple food of different civilizations of Europe, West Asia and North Africa **(Anonymous, 2010)**. Wheat was a key factor enabling the emergence of city-based societies at the start of civilization because it was one of the first crops that could be easily cultivated on a large scale, and had the additional advantage of yielding a harvest that provides long-term storage of food **(Cauvain *et al*., 2003)**.

Bread wheat is known to have been grown in the Nile Valley by 5000 BC and it is believed that the Mediterranean region was the centre of domestication. The archaeological record suggests that this first occurred in the regions known as the Fertile Crescent, and the Nile Delta About 35 percent of the world's population are engaged in wheat cultivation **(Lumpkin, 2011)**.

2.1.2. Botany, Morphology, and Growth of Wheat

Wheat is an annual grass with inflorescence called spike. Wheat plant development can be classified into three broad phases: seed germination and seedling establishment phase, vegetative phase, and reproductive phase followed by maturity and ripening **(Large, 1954).** When wheat plant switches to reproductive phase from vegetative phase, the shoot apical meristems elongate and differentiated into inflorescence meristems, on which spikelet meristems are directly formed as lateral branches. On these spikelet meristems, floret meristems are developed that gives rise to flowers or florets **(Shitsukawa *et al*., 2006)**. Thus, a wheat inflorescence (spike) consists of a main axis, rachis, on which spikelets are arranged alternately on opposite sides, and the spikelets are composed of florets joined at the axis (rachilla) as two opposite rows. Each floret has a pistil (female organ), three stamens (male organs) and two lodicules enclosed within lemma and palae. A hexaploid wheat spikelet may have four to six fertile florets, and all the florets are encompassed within two small bract leaves called glumes **(Shitsukawa *et al*., 2009)**.

Wheat plants consist of root and shoot systems. Root system comprises of the seminal roots and the crown roots, which arise from the lower nodes of the shoot **(Kirby, 2002).** The shoot comprises of a series of phytomers, each having a node, a leaf, an extended internodes, and a bud in the axil of the leaf. A leaf consists of a leaf-sheath and a leaf blade (lamina), with a membranous structure, the ligule, and a pair of small hairy organs, auricles at their junction. The shoot is terminated by spike as explained above. Wheat has a tendency of tillering. A tiller has the same basic structures like that of the main stem, and it arises from the axil of the basal leaves, i.e., from the points of attachment of the coleoptiles and the basal leaves on the main shoot. Each tiller has potential to develop a spike; and number of fertile tillers (spike bearing tiller) in a plant is one of the important yield components.

2.1.3. Genetics and Taxonomy

Wheat (*Triticum* spp.) is a monocot and belongs to tribe Triticeae of family Poaceae (previously called Gramineae). Other important crops like rice (*Oryza sativa* L.), maize (*Zea mays* L.) and bamboo also belong to this family.

Triticum aestivum L. em Thell. is recorded in the National Center for Biotechnology Information Taxonomy Browser as belonging to the family Poaceae, subfamily Pooideae and tribe Triticeae. It has the recorded synonyms *Triticum aestivum* L., *Triticum vulgare, Triticum aestivum* subsp. *aestivum* and the common names, wheat, bread wheat and common wheat (NCBI Taxonomy Browser).

Bread wheat is an allohexaploid (6x), Hexaploid wheat (*Triticum aestivum*) is a segmented hexaploid with 2n = 6x = 42 chromosomes that generally forms 21 pairs of chromosomes during meiosis **(Kimber and Sears, 1987**) Hexaploid wheat is believed to have originated from the wild form of einkorn (*Triticum monococcum L. sensu lato*, AA) in the Fertile Crescent **(Feldman., 1976**) through two hybridization and spontaneous chromosome doubling events. First, T. monococcum combined with the donor of the B genome (T. searsii Feldman and Kislev) to form a tetraploid (2n=4x=28, AABB) **(Kimber, and Sears, 1987; Anonymous. 2005)**, followed by hybridization of the tetraploid and T. tauschii (Coss.) the progenitor of the D genome **(Kumar *et al*., 2012).**

The genome of hexaploid wheat is large (AABBDD), estimated at 16 billion base pairs per haploid nucleus **(Bennett, 1972)**. Most of this (75%) contains repeated DNA sequences varying in length and the amount of repetition, while about 25% is comprised of unique sequences **(Smith and Flavell, 1975)**. Actual coding regions comprise only about 1% of the genome **(Kimber, and Sears, 1987**).

2.1.4. Major Cultivated Species

Most of the wild and cultivated wheats are one of three species, *T. compactum* Host. (club wheats), *T. turgidum* L. ssp. durum (Desf.) Husn. (Durum wheats), or *T. aestivum* (hexaploid wheat) or (bread wheat), The latter being the most commonly cultivated species worldwide. Bread wheat (*Triticum aestivum* L., 2n = 6x = 42 and genomes AABBDD) occupies 90% of world wheat area because it has an extremely buffered genotype due to polyploid **(Faris *et al*., 2002)**; and three divergent alleles may be harbored at each locus. These genetic attributes enable bread wheat to display arrays of phenological responses to wide ranges of photoperiod and temperature regimes, including vernalization **(Slafer and Rawson, 1994)**. Thus, wheat is grown from the tropical to temperate climates and from a few meters to more than 3600 meters above sea level **(Aase *et al*., 2010)**.

Two types of bread wheat varieties are in cultivation. First called winter wheat plant type varieties are grown where young crop faces extended period of frosty weather conditions or undergoes vernalization. Winter wheat crops develop flowering competence in response to vernalization. Second called spring wheat plant varieties are cultivated in the rest of the cold agro-climates. Spring wheat varieties have been bred to adapt enormous variation in crop season length experienced in diverse areas of cultivation. This has become possible because of the flowering time of spring wheat is much shorter than that of winter wheat.

2.1.5. Cultivated Species in Egypt

Boulos (2005) mentioned that there are five species of *Triticum* to be distinguished in Egypt, namely: *Triticum aestivum*, *Triticum dicoccum*, *Triticum turgidum*, *Triticum durum*, *Triticum pyramidale*.

2.1.6. The Nutrition of Wheat

Wheat is grown on more land area than any other commercial crop and is the most important staple food for humans. It is a common source of energy and proteins for the world population. It is the second main source of world's food energy and nutrition. Globally, wheat is the leading source of vegetable protein in human food, having higher protein content than either maize (corn) or rice, the other major cereals. Wheat provides 21% of food calories and 20% of protein to more than 4.5 billion people worldwide. In terms of total production tonnages used for food, it is currently second to rice as the main human food crop and ahead of maize, after allowing for maize's more extensive use in animal feeds. Approximately 17–20% of global wheat production is used for feeding animals and poultry. Although wheat is not the main feed ingredient worldwide, increasing demand for meat, especially in the Asia-Pacific region, has led to an increasing need for proteins to feed poultry, pigs and cattle. Accordingly, an increase in the livestock population requires an increase in the production of feed grain (**FAO, 2009**).

Wheat grain is a staple food has special gluten proteins that made it possible to make flour for leavened, flat and steamed breads, biscuits, cookies, cakes, breakfast cereal, pasta, noodles, and couscous and for fermentation to make beer, other alcoholic beverages, or biofuel.

According to **FAO-OECD** estimates, approximately 93 million tons of wheat and coarse grains were used for ethanol production in 2007, double the amount used in 2005 **(OECD-FAO, 2008).** This represents more than half of the total growth in wheat and coarse grain usage. Wheat, among other cereal crops, yields less ethanol per 1 ton of feedstock used or 1 ha of land cultivated .While in the United States maize is the primary feedstock for bio-ethanol plants, in the EU the main feedstock is wheat. Wheat is planted to a limited extent as a forage crop for livestock, and its straw can be used as a construction material for roofing thatch.

2.1.7. Wheat Production, Consumption and Scale of Cultivation

Wheat is grown on about 221.70 million hectares in a range of environments, with annual production of about 655.8 million tons **(FAO, 2010)**. In human history, a most rapid increase in the population took place in 20th century Although, the world population increased from 6.16 to 6.92 billion (12.34% increase) during the period of 2001-2011 **(USCB, 2012)** and although the global wheat production fluctuated during the same period and lacked behind the population growth, it increased from 589.3 to 694.5 million tonnes (17.84% increase) **(FAO, 2011**).

Although wheat is trade internationally and developing countries are major importers (43% of food imports), the reality is that 81% of wheat consumed in the developing world and utilized within the same country, if not the same community

(CIMMYT, 2005). In these circumstances, many poor households depend on increased wheat production on their own farms for improved household food security. In the period leading up to 2020, demand for wheat for human consumption in developing countries is expected to grow at 1.6% per annum, and for feed at 2.6% per annum **(Tubiello *et al*., 2000)**. And, globally, forecasted around 950 million tonnes to meet future demands imposed by population growth **(Prasad and Nagarajan, 2004)**.

The food demand for the exploding human population was met by the technological revolution that began in 1960's, popularly known as Green Revolution‖. During the green revolution, architectures of wheat and rice plant were modified through dwarfing genes (wheat, Rht1 and Rht2; rice Sd-1), followed by the availability and the heavy application of chemical fertilizers, irrigation, and pesticides. The sustained increase in cereal yield has then been realized by the development of location specific high yielding varieties with resistance/tolerance to diseases and pests **(Evenson, 2003)**. Now, in order to meet the global food-demand in 2050, another technical revolution encompassing effective disease and insect pest control and tolerance to abiotic stress for cereal crops, especially for bread wheat, will be needed.

Bread wheat (*Triticum aestivum* L.) is one of the most important crops in Egypt and cultivated area is about 1.2 million hectares (2.85 million Fadden) **(FAO 2009)**. It occupies about 32.6 percent of the total winter land area and is mostly used to make bread; a very important component of the Egyptian diet, Average consumption of wheat and wheat products is about 200 kilograms (kg) per capita per year, one of the highest levels in the world **(Kherallah *et al*. 1999)**.

2.1.8. Wheat Problem Statement in Egypt:

Gain (2012) reported that, Wheat is viewed as a strategic commodity and considered a main ingredient in the Egyptian diet, it is mainly consumed in the form of bread; therefore the consumers have no other choice except consuming the bread since it is still the cheapest food. Consumption of wheat is increasing as a result of the annual population increase approaches 2.0 million/year. Egypt continues to have one of the highest wheat per capita consumption levels in the world. Post forecasts that wheat consumption in MY 2011/12 is estimated to be 18.8 MMT. The local production is about 8 million tons however; it covers less than 60% of local consumption.

Egypt remains the world's largest wheat importer. Accordingly, cereal import requirements in the current marketing year 2013/14 (July/June) are put at about 15.4 million tonnes, about 16 percent higher than last year but some 4 percent lower than the five-year average **(FAO 2013).** Efforts to increase food production, in particular wheat, have received top priority in the agricultural development programs starting since 1983. In the context of Egypt's food security policy, wheat policy has **two main dimensions**: First, the food availability dimension, where the main focus is to increase the self-sufficiency ratio of wheat production from the current level (55 percent) to full self-sufficiency **(Siam 2006)**. Increasing wheat productivity is a national target in Egypt to fill the gap between wheat consumption and production **(FAO, 2009)**. The second is the accessibility aspect to ensure that the low-income households are able to acquire food **(Siam 2006)**. Global warming as a result of climate change affect negatively at wheat grain yields potentially increasing food insecurity and poverty **(Tubiello *et al*., 2000)**.

2.2. The challenge of multiple Biotic and Abiotic stress on plants

According to **Taiz and Zeiger (2006)** stress is usually defined as an external factor that exerts a disadvantageous influence on the plant and is measured in relation to plant survival, crop yield, growth (biomass accumulation), or the primary assimilation processes, which are related to overall growth. The survival and growth of plants under a stress depend on both stress and plant characteristics.

Most crop plants grow in environments that are suboptimal, which prevents the plants from attaining their full genetic potential for growth and reproduction **(Bray *et al.*, 2000; Rockstrom and Falkenmark, 2000)**. This is highlighted by analyzing the difference between maximum crop yields and the average yield for that crop. For example, US wheat yields in a record year can be up to eight times as great as the average yield **(Boyer, 1982)**. The yield difference can largely be explained by unfavorable environmental conditions, which, when creating potentially damaging physiological changes within plants, are known as stresses **(Shao *et al.*, 2008)**.

Boyer (1982), calculating crop insurance payments, reported about 87% yield decrease in US wheat, and pointed out that only 6% yield loss was due to biotic factors, whereas environmental factors were responsible for 94% yield loss.

Abiotic stress factors such as heat, cold, drought, salinity, and nutrient stress have a huge impact on world agriculture, and it has been suggested that they reduce average yields by >50% for most major crop plants **(Wang *et al.*, 2003)**. Further to this, plants must defend themselves from attack by a vast range of pests and pathogens, including fungi, bacteria, viruses, nematodes, and herbivorous insects **(Hammond-Kosack and Jones, 2000)**. Each stress elicits a complex cellular and molecular response system implemented by the plant in order to prevent damage and ensure survival, but often at the detriment of growth and yield **(Herms and Mattson, 1992)**.

2.2.1. Global Warming

Gaseous emissions due to human activities are substantially adding to the existing concentrations of greenhouse gases Emission of green house gases (GHG) such as carbon dioxide (CO2), methane (CH4) and nitrous oxide (N2O) from agricultural systems is one of the major sources contributing to this global increase of temperature **(Maraseni *et al.* 2009; Smith & Olesen 2010)**. Studies have shown that the annual mean maximum and minimum temperatures have increased by 0·35 and 1·13 °C, respectively, for the period 1979–2003 at the International Rice Research Institute, Manila, Philippines **(Peng *et al.* 2004)**. Current climate prediction models indicate that average surface temperatures will rise by 3–5 °C in the next 50–100 years, drastically affecting global agricultural systems **(IPCC, 2007)**. Rising temperatures may lead to altered geographical distribution and growing season of agricultural crops by allowing the threshold temperature for the start of the season and crop maturity to reach earlier **(Porter, 2005)**. Rising temperatures also will be concurrent with an increased frequency of drought, flood, and heat waves **(IPCC, 2008; Mittler and Blumwald, 2010)**, In particular, warmer, drier summers in mid-continental regions such as central Europe and central Africa are predicted, along with a reduction in growing season in many regions, extensive salinization as sea levels rise, and a decrease in land suitable for agriculture **(Easterling *et al.*, 2000; IPCC, 2007, 2008; Morison *et al.*, 2008)**. A change in variability of rainfall and temperature may itself

affect yields as well as adversely affecting nutritional quality of crops (**Porter and Semenov, 2005**).

Global warming, accompanied by an increased frequency of periods with high temperatures, is one of the most important characteristics of accelerated climatic changes. Nowadays greenhouse gases emission and growth of population increase the mean surface air temperatures **(Shakun *et al*., 2012)** and the frequency of periods of high temperatures **(Wheeler *et al*., 2000)**. Additionally, climate models predict that there will be a relatively greater increase in nighttime temperatures as compared to daytime temperatures which can have some effects on crops. Over the past century global daily minimum temperatures increased more than twice compared to increases in daily maximum temperatures. Many dry environments exist in the world, which suffer from severe heat stress during grain filling. Recent studies have shown that the yields of maize **(Grassini and Cassman, 2012)**, sunflower **(Chaki *et al*., 2011)** and wheat (**Prasad *et al*., 2008; Pradhan *et al*., 2012)** were strongly correlated with minimum (nighttime) temperatures. Crop development and growth rates and duration of critical phases can be differently sensitive to minimum temperatures and maximum temperatures **(Lobell and Ortiz-Monasterio, 2007).** The **CIMMYT-ICARDA (2011)**, **CGIAR (2009)** and **OECD (2003)** reported that world wheat production will decrease due to global warming and developing countries will be highly affected by the negative effects on wheat and other crops production.

Developing countries will be affected most for three reasons: (i) climate change will have its most negative effects in tropical and subtropical regions; (ii) most of the predicted population growth to 2030 will occur in the developing world **(United Nations Population Division DoEaSA, 2009)**; and (iii) more than half of the overall work force in the developing world is involved in agriculture **(FAO, 2005)**. Climate change will also influence the habitat range of pests and pathogens, with increasing temperature facilitating pathogen spread **(Bale *et al.*, 2002; Luck *et al.*, 2011; Madgwick *et al.*, 2011; Nicol *et al.*, 2011)**. Crop plants are therefore likely to encounter a greater range and number of environmental stresses, which when occurring simultaneously can have severe consequences. The changing climatic conditions, combined with an increasing pressure on global food productivity due to population increase, result in a demand for stress-tolerant crop varieties that has never been greater **(Takeda and Matsuoka, 2008; Newton *et al.*, 2011)**. Understanding the mechanisms of plant responses to multiple simultaneous stresses is therefore crucial in providing opportunities for the development of broad-spectrum stress-tolerant crops.

2.3. Abiotic Stress, Causes and Plant Responses

Abiotic stress is the primary cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50%. Basic Stresses such as drought, salinity, temperature, and chemical pollutants are simultaneously acting on the plants causing cell injury and producing secondary stresses such as osmotic and oxidative ones **(Wang *et al*., 2003)**.

A number of abiotic stress lead to over production of reactive oxygen species (ROS) including H_2O_2 causing extensive cellular damage and inhibition of photosynthesis **(Wang *et al*., 2006).** Plants could not change their sites to avoid such stresses, but have different ways to tolerate these stresses **(Shao *et al*., 2007a)**. In the natural environment,

adverse situations are always a combination of several stress factors (e.g., water limitation, high temperature or irradiation and high osmolality). This is the reason why it is always difficult to determine which stress factor (if not all) is behind the elicitation of a particular physiological response.

It is well known that abiotic stresses in general, through regulation of both gene expression and protein turnover, alter the abundance of many transcripts and proteins **(Yang *et al*., 2006).** Physiological responses of plants to environmental cues involve changes not only at the transcriptional level but also in post-translational protein modifications and metabolite alteration and/or accumulation, leading to a particular physiological response or phenotype **(Verslues *et al*., 2006)**. Plant physiological responses to stress are oriented towards tolerance, sensitivity or avoidance of the stressful conditions (**Cattivelli *et al*., 2008**).

2.3.1. Drought

The most important stress factors limiting plant growth, reproductive development and, ultimately survival, is drought. This stress factor is related to water supply limitation, not only understood as the strict cease in water supply but also as continuous water deficit throughout growth, reproductive or developmental stages **(Flexas, J., 2002)**. One of the most important physiological parameters being affected by drought or water shortage is photosynthesis; in this sense both water and salt stress are quite similar causing a progressive and severe reduction in the CO2 assimilation capacity. This decrease in net photosynthetic rate is first associated to a stomata closure induced by a decline in leaf cell turgor that limits diffusion of CO2 into the substomatal chamber. Under these conditions that diminish CO2 diffusion through the mesophyll, photo-inhibition, a process that reduces quantum yield of PSII and induces photorespiration and H2O2 production **(Hossain., 2009)** is likely to occur. Hence, the production of Reactive Oxygen Species (ROS) is one of the primary responses to stress following the decline in photosynthesis, causing cell damage but also a signal to be transmitted **(Kirakosyan *et al*., 2004; Djoukeng *et al*., 2008)**.

Besides this, drought stress induces as a general response the accumulation of several amino-acids such as valine, leucine, isoleucine and agmatine (as a precursor of polyamines) along with carbohydrates and carbohydrate alcohols which, in combination with proline (Pro), could have an osmoprotective role.

2.3.2. Salinity

Another major factor limiting plant growth and production is salinity. This stress factor is derived from the massive accumulation of salts near the root zone and causes an osmotic effect followed by a specific toxicity, derived from the accumulation of saline ions in plant tissues **(Visser *et al*., 2005)**. The most studied effect is the salinity associated to the accumulation of NaCl due to overexploitation of freshwater resources and the subsequent marine intrusion, known as primary salinization **(Bailey *et al*., 2008)**. In natural environments, osmotic and ionic effects co-occur and usually the symptoms of ion toxicity precede leaf drop. Under these conditions, non-tolerant plants exhibit succulence, arrest in growth and reproductive development, continuous organ abscission and, if the saline

conditions persist, death. However, under artificial stress conditions, plants are suddenly exposed to high saline concentrations (e.g., 100 or 200 mM NaCl) or in increasing steps (25, 50, 75, 100 mM NaCl). This triggers the massive accumulation of saline ions and compatible osmolyte biosynthesis to counterbalance the severe osmotic effect **(Visser *et al*., 2005)**.

2.3.3. Soil Flooding

Water stress is either associated to a deficit in water availability or to an excess irrigation that impairs water uptake. In particular, soil water logging constitutes a seasonal stress factor whose incidence on crops is difficult to predict. When the soil water content raises above field capacity a fast depletion of O2 occurs due to the low diffusion rate of this gas in water together with the consumption made by plants roots. This O2 depletion can occur in less than 24 h, depending on the root/micro biota biomass present in soil **(Arbona *et al*., 2009 ; Allakhverdiev *et al*., 2008)**.

2.3.4. Heat stress

According to **Wahid *et al*., 2007;** high temperature stress may be defined as the increase in air temperature well above a threshold level for a period of time sufficient to cause irreversible damage to plant organ, growth and/or development. In general, a transient elevation in temperature, usually 10–15 ◦C above ambient, is considered heat shock or heat stress. Heat stress affects the stability of proteins, nucleic acids, the cytoskeleton structure and the efficiency of enzymatic reactions, causing a severe metabolic imbalance. The sensing of heat stress takes place at the plasma membrane of cells which is physically altered, acting as a real thermometer (**Scholz *et al*., 2004)**. Heat also causes several metabolic changes associated to impairment in electron transport chains and production of ROS such as the membrane bound NADPH oxidase **(Theocharis *et al*., 2012)**. In addition, another primary target of this stress is the photosynthetic system, especially the PSII and the oxygen-evolving complex, the ATP generating system and the carbon assimilation process (**Scholz *et al*., 2004)**. Heat stress due to high ambient temperatures is a serious threat to crop production worldwide **(Hall, 2001)**. Plants must face temperature stress, at present increasing year by year, because of greenhouse gas emission.

Worldwide, extensive agricultural losses are attributed to heat, often in combination with drought or other stresses (**peng S. *et al*., 2004)**. Moreover, in the field, most of the time high temperatures follow the drought, i.e., drought and high temperature occur simultaneously causing significant yield loss **(Mittler, 2006; Lott *et al*., 2011).** The combined effects of drought and high temperature on physiology, growth, water relations, and yield were significantly higher than the individual effects **(Grigorova *et al*., 2011)**.

At moderately high temperatures, injuries or death may occur only after long-term exposure. Direct injuries due to high temperatures include protein denaturation and aggregation, and increased fluidity of membrane lipids. Indirect or slower heat injuries include inactivation of enzymes in chloroplast and mitochondria, inhibition of protein synthesis, protein degradation and loss of membrane integrity **(Howarth, 2005)**. These injuries eventually lead to starvation, inhibition of growth, reduced ion flux, production of

toxic compounds and reactive oxygen species (ROS) **(Sch¨offl *et al*., 1999; Howarth, 2005)**. However, while some researchers believe that night temperatures are major limiting factors, others have argued that day and night temperatures do not affect the plant independently and that the diurnal means temperature is a better predictor of plant response to high temperature with day temperature having a secondary role (**Peet and Willits, 1998)**.

2.3.4.1. Heat Tolerance Mechanisms

Heat tolerance is generally defined as the ability of the plant to grow and produce economic yield under high temperatures. Plants manifest different mechanisms for surviving under elevated temperatures, including long-term evolutionary phonological and morphological adaptations and short-term avoidance or acclimation mechanisms such as changing leaf orientation, transpirational cooling, or alteration of membrane lipid compositions, Elucidating the various mechanisms of plant response to stress and their roles in acquired stress tolerance is of great practical and basic importance. Some major tolerance mechanisms, including ion transporters, osmoprotectants, free-radical scavengers, late embryogenesis abundant proteins and factors involved in signaling cascades and transcriptional control are essentially significant to counteract the stress effects **(Wang *et al*., 2004)**.

Series of changes and mechanisms, beginning with the perception of heat and signaling and production of metabolites that enable plants to cope with adversaries of heat stress, have been proposed. Heat stress effects are notable at various levels, including plasma membrane and biochemical pathways operative in the cytosol or cytoplasmic organelles **(Sung *et al*., 2003)**. Initial effects of heat stress, however, are on plasmalemma, which shows more fluidity of lipid bilayer under stress. This leads to the induction of Ca2+ influx and cytoskeletal reorganization, resulting in the upregulation of mitogen activated protein kinases (MAPK) and calcium dependent protein kinase (CDPK). Signaling of these cascades at nuclear level leads to the production of antioxidants and compatible osmolytes for cell water balance and osmotic adjustment.

Production of ROS in the organelles (e.g., chloroplast and mitochondria) is of great significance for signaling as well as production of antioxidants **(Bohnert *et al*., 2006)**. The antioxidant defense mechanism is a part of heat-stress adaptation, and its strength is correlated with acquisition of thermotolerance **(Maestri *et al*., 2002). Iba,(2002)** reported that after exposure to high temperatures and perception of signals, changes occur at the molecular level altering the expression of genes and accumulation of transcripts, thereby leading to the synthesis of stress-related proteins as a stress tolerance mechanisms. One of the most closely studied mechanisms of thermotolerance is the induction of HSPs, which, as described in above, comprise several evolutionarily conserved protein families. However, each major HSP family has a unique mechanism of action with chaperonic activity. The protective effects of HSPs can be attributed to the network of the chaperone machinery, in which many chaperones act in concert. An increasing number of studies suggest that the HSPs/chaperones interact with other stress-response mechanisms **(Wang *et al*., 2004)**.

2.4. Impact of Heat Stress on Plants Physiology and Biochemically

2.4.1. Membrane thermostability (MTS) and relative cell injury (%)

MTS has been widely accepted as a suitable technique for estimating the cellular thermotolerance of plants. It is a quick tool of screening against heat stress **(Shanahan *et al*., 1990).** Cellular membranes are dynamic structures formed essentially by lipids and proteins, supporting many biophysical and biochemical traits, with emphasis in regulation and transport of ions and enzymatic activity. These permeable selective barriers allow the development of many biological responses, but they also are a main target of environmental stresses, therefore having an essential role in the adaptation to adverse conditions **(Routaboul *et al*., 2000).**

Heat shock increases cell membrane permeability, thereby inhibiting cellular function, as a result of the denaturation of proteins and increments of unsaturated fatty acids that disrupt water, ion, and organic solute movement across membranes. Heat shock accelerates the kinetic energy and movement of molecules across membranes thereby loosening chemical bonds within molecules of biological membranes. This makes the lipid bilayer of biological membranes more fluid by either denaturation of proteins or an increase in unsaturated fatty acids **(Savchenko *et al*., 2002).** The integrity and functions of biological membranes are sensitive to high temperature, as heat stress alters the tertiary and quaternary structures of membrane proteins. Such alterations enhance the permeability of membranes, as evident from increased loss of electrolytes.

The increased solute leakage, as an indication of decreased cell membrane thermostability (CMT), has long been used as an indirect measure of heat-stress tolerance in diverse plant species, including soybean **(Martineau *et al*., 1979)**, potato and tomato **(Chen *et al*., 1982)**, cotton **(Ashraf *et al*., 1994)**, sorghum **(Marcum, 1998)**, cowpea **(Ismail and Hall, 1999)** wheat **(Blum *et al*., 2001),** and barley **(Wahid and Shabbir, 2005)**. Cellular membrane stability is one of the effective methods of screening against heat tolerance on a physiological basis **(Ibrahim and Quick, 2001).** MTS has a positive correlation with yield performance. Membrane stability at high temperature is determined by conductivity of electrolyte leakage from leaves membrane stability **(Saadalla *et al*., 1990a).**

The primary sensing event of heat stress in the moss Physcomitrella patens occurs at the plasma membrane **(Saidi, Y. *et al*., 2009 and 2010)**.

Efeoglu and Terzioglu (2007) reported that high temperatures at seedling growth decreased MTS in wheat. Membrane thermo stability is heritable **(Fokar *et al*., 1998)** and it is highly correlated to yield. Cell membrane thermostability (CMT) method on flag leaves in field grown plants resulted in thermo tolerant lines which results in significant increase of yield of spring wheat **(Shanahan *et al*., 1990)**. Genetic variation in thermo stability of membranes in various crops including wheat was found using conductive metric measurements in field grown plants **(Blum and Ebercon, 1981).**

Such improvements make plant growth and development possible under heat stress. However, not all plant species or genotypes within species have similar capabilities in coping with the heat stress. There exists tremendous variation within and between species, providing opportunities to improve crop heat-stress tolerance through genetic means. Some attempts to develop heat-tolerant genotypes via conventional plant breeding protocols have been successful **(Ehlers and Hall, 1998; Camejo *et al*., 2005)**.

Shanahan *et al.* (1990) used electrolyte leakage to study the phenomena of heat tolerance in spring wheat (*T. aestivum* L.) based on MT values genotypes were grouped as heat tolerant (HT) and heat sensitive (HS). Based on the results they concluded that the MT test is a useful screening procedure for selecting HT genotypes of spring wheat. Similarly **(Saadalla *et al.* 1990a and 1990b)** also advocated that the technique is a reliable method for exploring heat tolerant germplasm in wheat. Some studies regarding heat tolerance with respect to cell injury (%) revealed that the genotypes with less injury to plasma membranes are tolerant as compared to the genotypes with more injury to cell membrane **(Renu *et al.*, 2004).**

2.4.2. Photosynthesis

Photosynthesis is the most sensitive physiological process to elevated temperature **(Wahid *et al.*, 2007).** High temperature has a greater influence on the photosynthetic capacity of plants especially of C3 plants than C4 plants **(Yang, X. *et al.*, 2007)**. High temperature decreases photosynthesis in wheat **(Reynolds *et al.*, 2000; Yang *et al.*, 2002)** and any reduction in photosynthesis affects growth and grain yield of wheat **(Al-Khatib and Paulsen, 1999)**. High temperatures damage photosynthetic membranes and cause chlorophyll loss **(Díaz-Almeyda *et al.*, 2011).**Increase in high nighttime temperature from 14°C to 23°C decreased leaf photosynthesis rate by about 15 µ mol m-2 s-1 in spring wheat **(Prasad *et al.*, 2008b)**. Photosynthesis is a temperature-dependent process, and damages due to high temperature include a wide range of changes in structures or functions of the photosystem apparatus, including enzymes and suggested photochemical reaction in thylakoid lamellae and carbon metabolism in the stroma of chloroplast are the primary sites of damage under high temperature **(Georgieva., 1999; Wise *et al.*, 2004).**

Prasad *et al.* (2008) reported that the most important reasons for PSII sensitivity to high temperature are heat-induced increase in thylakoid membrane fluidity and electron-transport dependent integrity of PSII. The inhibition of PSII electron transport under heat stress is often indicated by a sharp increase in the basal level of chlorophyll fluorescence that corresponds to photosynthetic inhibition **(Ristic *et al.*, 2007)**. Heat-stressinduced damage and disruption of the integrity of thylakoid membranes also causes the photophosphorylation to cease **(Dias and Lidon, 2009)**. Alterations in various photosynthetic attributes under heat stress are good indicators of thermotolerance of the plant as they show correlations with growth. Any constraint in photosynthesis can limit plant growth at high temperatures. Photochemical reactions in thylakoid lamellae and carbon metabolism in the stroma of chloroplast have been suggested as the primary sites of injury at high temperatures (**Wise *et al.*, 2004; Salvucci and Crafts- Brandner, 2004; Marchand *et al.*, 2005**).

High temperature decreases leaf chlorophyll. Chlorophyll is harbored in the thylakoid membranes, and loss of chlorophyll may be due to high temperature-induced electrolytic leakage of thylakoid membrane **(Ristic *et al.*, 2007)** and/or lipid peroxidation of chloroplast membranes **(Djanaguiraman *et al.*, 2010)**.Contents of chlorophylls and carotenoids and the ratios of chl *a*/*b* and chl/car are good indicators for revealing the stress and level of tolerance in plants **(Zhang *et al.*, 2008).** Increasing leaf temperatures and photosynthetic photon flux density influence thermotolerance adjustments of PSII, indicating their potential to optimize photosynthesis under varying environmental conditions as long as the upper thermal limits do not exceed.

In tomato genotypes differing in their capacity for thermotolerance as well as in sugarcane, an increased chlorophyll a:b ratio and a decreased chlorophyll:carotenoids ratio were observed in the tolerant genotypes under high temperatures, indicating that these changes were related to thermotolerance of tomato (**Camejo *et al*., 2005; Wahid and Ghazanfar, 2006**). Furthermore, under high temperatures, degradation of chlorophyll a and b was more pronounced in developed compared to developing leaves (**Karim *et al*., 1997, 1999**). Such effects on chlorophyll or photosynthetic apparatus were suggested to be associated with the production of active oxygen species **(Camejo *et al*., 2006; Guo *et al*., 2006).**

Baker, (2008); Baker & Rosenqvist, (2004); Chaerle & Van Der Straeten, (2001); Woo *et al*. (2008) defined Chlorophyll fluorescence as a tool which is widely used to examine photosynthetic performance in algae and plants.

Chlorophyll fluorescence analysis is widely used to estimate photosystem II (PSII) activity, which is an important target of abiotic stresses **(Balachandran *et al*., 1994; Baker *et al*., 1983; Briantais *et al*., 1996; Calatayud *et al*., 2008; Chaerle & Van Der Straeten, 2000; Ehlert & Hincha, 2008; Gilmore & Govindjee, 1999; Guidi *et al*., 2007; Guidi & Degl'Innocenti, 2008; Hogewoning & Harbinson, 2007; Krause, 1988; Lichtenthaler *et al*., 2007; Massacci *et al*., 2008; Osmond *et al*., 1999; Scholes & Rolfe, 1996; Strand & Oquist, 1985).**

2.4.3. Accumulation of compatible osmolytes

A key adaptive mechanism in many plants grown under abiotic stresses, including salinity, water deficit and extreme temperatures, is accumulation of certain organic compounds of low molecular mass, generally referred to as compatible osmolytes **(Hare *et al*., 1998; Sakamoto and Murata, 2002)**.

Under stress, different plant species may accumulate a variety of osmolytes such as sugars and sugar alcohols (polyols), proline, tertiary and quaternary ammonium compounds, and tertiary sulphonium compounds (**Sairam and Tyagi, 2004**).

Glycinebetaine (GB), an amphoteric quaternary amine, plays an important role as a compatible solute in plants under various stresses, such as salinity or high temperature **(Sakamoto and Murata, 2002)**. Capacity to synthesize GB under stress conditions differs from species to species (**Ashraf and Foolad, 2007**).For example, high level of GB accumulation was reported in maize **(Quan *et al*., 2004)** and sugarcane (**Wahid and Close, 2007**) due to desiccating conditions of water deficit or high temperature.

In contrast, plant species such as rice (*Oryza sativa*), mustard (*Brassica* spp.), Arabidopsis (*Arabidopsis thaliana*) and tobacco (*Nicotiana tabacum*) naturally do not produce GB under stress conditions. However, genetic engineering has allowed the introduction of GB-biosynthetic pathways into GB-deficit species (**Sakamoto and Murata, 2002; Quan *et al*., 2004**).

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 buffer cellular redox potential under heat and other

environmental stresses. Similarly, accumulation of soluble sugars under heat stress has been reported in sugarcane, which entails great implications for heat tolerance (**Wahid and Close, 2007**). Under high temperatures, fruit set in tomato plants failed due to the disruption of sugar metabolism and proline transport during the narrow window of male reproductive development **(Sato *et al*., 2006)**.

Among other osmolytes, _-4-aminobutyric acid (GABA), a non-protein amino acid, is widely distributed throughout the biological world to act as a compatible solute.GABAis synthesized from the glutamic acid by a single step reaction catalyzed by glutamate decarboxylase (GAD). An acidic pH activates GAD, a key enzyme in the biosynthesis of GABA. Episodes of high tempeartures increase the cytosolic level of Ca, which leads to calmodulin-mediated activation of GAD **(Taiz and Zeiger, 2006)**. Several other studies show that various environmental stresses increase GABA accumulation through metabolic or mechanical disruptions, thus leading to cytosolic acidification. Kinetics of GABA in plants shows a stress-specific pattern of accumulation, which is consistent with its physiological role in the mitigation of stress effects. Rapid accumulation of GABA in stressed tissues may provide a critical link in the chain of events stemming from perception of environmental stresses to timely physiological responses **(Kinnersley and Turano, 2000**).

Because of significant roles of osmolytes in response to environmental stresses in plants, crop stress (e.g., heat) tolerance might be enhanced by increased accumulation of compatible solutes through traditional plant breeding, marker-assisted selection (MAS) or genetic engineering (GE) approaches **(Ashraf and Foolad, 2007**).

2.4.3.1. Osmoprotectants Aminoacids: Proline

Proline is an amino acid and compatible solute commonly accumulates in many plants exposed to various stress conditions such as water **(Barnett and Naylor 1966, Blum and Ebercon 1976)**, salinity **(Stewart and Lee 1974, Treichel 1975)**, air pollution **(Godzic and Linskens 1974)** and unfavorable temperature **(Chu *et al*., 1974, 1978)**. Under stress condition, proline is synthesized from glutamate due to loss of feedback regulation in the proline biosynthetic pathway **(Boggess and Stewart 1980)**. This biosynthesis might be an adaptive mechanism to reduce the accumulation of NADPH, which increased as a result of the decrease in photosynthetic CO2 reduction rate of the plant **(Berry and Bjorkman 1980)**. Proline has also been found to serve as a substrate for respiration **(Britikov *et al*., 1965)** and as a source of nitrogen and other metabolites **(Stewart and Boggess 1978).**

Accumulation of proline could be due to *de novo* synthesis or decreased degradation, or both, and it is synthesized from glutamate and ornithine. In plants, the main pathway is from glutamate, which is converted to proline by two successive reductions catalyzed by pyrroline-5-carboxylate synthases (P5CS) and pyrroline-5-carboxylate reductases (P5CR), respectively. Ornithine is the alternative precursor for proline, which can be transaminated to P5C by Orn-d-aminotransferase (OAT), a mitochondrial located enzyme (**Kishor *et al*., 2005, Verbruggen and Hermans, 2008**).

Genotypic variations in proline accumulation have been observed in many studies and attempts were made to correlate its accumulation with tolerance of plants to stress, this apparent correlation between proline accumulations. **Paleg *et al*., (1981)** demonstrated that a number of solutes, including proline, protected enzymes, isolated from various tissues,

from inactivation by heat. Rapid catabolism of proline upon relief of stress may provide reducing equivalents that support mitochondrial oxidative phosphorylation and the generation of ATP for recovery from stress and repair of stress induced damage **(Hare and Cress 1997)**.

Proline accumulation in high temperature has been reported in barley and radish leaves **(Chu *et al*. 1974)**, in tomato floral buds and leaves **(Kou *et al*., 1986)**, 1.5-fold high in mulberry leaves **(Chaitanya *et al*., 2001)**, in the leaves of *Brassica* vegetables **(Takeda *et al*., 1999)**, in cotton leaves **(Ronde *et al*., 2001)**, in cabbage and Chinese cabbage **(Hossain *et al*., 1995)**, in apple **(Park *et al*., 2001)** and in flag leaves of wheat **(Hasan *et al*., 2007)**. Under supra-optimal temperature genotypic difference in proline accumulation pattern has also been reported in six cotton cultivars **(Ronde *et al*. 2001)** and in different cabbage and Chinese cabbage varieties **(Hossain *et al*., 1995)**.

Hasan *et al*., (2007) reported genotypic difference in change in flag leaf and kernel proline level in heat tolerant and heat sensitive wheat genotypes due to post anthesis heat stress condition.

Ju Ahmed and MA Hasan (2011) indicate that the seedling of all wheat genotypes grown at 35^{o} C maintained a higher proline level than those grown at 25o C. The increment in proline level was higher in HT than that in HS. The increased seedling proline level due to high temperature can be used to screen HT wheat genotypes, which is comparable to cell membrane thermostability test.

2. 5. An overview of HSPs

Historically, the observation of the Italian Scientist R. Ritossa on gene expression of the puffing in the chromosomes of Drosophila melanogaster after exposure to heat was the start of discovering the heat-shock proteins. The result was an increase in protein synthesis that occurred also by the use of other stress factors such as azide, 2, 4-dinitrophenol, and salicylate **(Ritossa, 1962)**. After that report, these proteins were identified and named as heat-shock protein (Hsp) **(Tissieres *et al*., 1974)**. Hsps in higher plants was discovered in tobacco and soybean using cell culture technique **(Barnett *et al*., 1980)**.

Al-Whaibi (2011) indicated that HSPs protect cells from injury and facilitate recovery and survival after a return to normal growth conditions. HSPs may confer thermotolerance by protection of nucleus, ribosome, protein synthesis, thylakoid membranes of chloroplast and electron transport chain of mitochondria and chloroplasts under heat stress. When soybean was subjected to 40 °C for four hours, ten new proteins were found, but disappeared after 3 h treatment at 28 °C. Researchers identified many types of Hsps in almost all organisms **(Bharti and Nover, 2002)**. Hsps are located in both the cytoplasm and organelles, such as the nucleus, mitochondria, chloroplasts and endoplasmatic reticulum. Heat-shock proteins having molecular weights ranging from 10 to 200 KD are characterized as chaperones where they participate in the induction of the signal during heat stress **(Scho¨ ffl *et al*. 1999)**. Some researchers concluded that although there are some evidences for the genetic expression phenomenon in some specific cases, there are no final and conclusive evidence that this is what is happening in natural environment **(Feder and Hofmann, 1999)**.

The heat stress response is a highly conserved reaction caused by exposure of an organism tissue or cells to sudden high temperature stress, and it is characterized by rapid induction and transient expression of Hsps. The tolerance conferred by HSPs results in improved physiological phenomena such as photosynthesis, assimilate partitioning, water and nutrient use efficiency, and membrane stability **(Camejo *et al*., 2005; Ahn and Zimmerman, 2006; Momcilovic and Ristic, 2007).**

As many molecular chaperones are stress proteins and many of them were originally identified as heat-shock proteins, the names of these molecular chaperones follow their early nomenclatures and are referred here as Hsps **(Wang *et al*., 2004)**, So, these proteins primarily function as molecular chaperones to control the proper folding and conformation of both structural (*i.e.*, cell membrane) and functional (*i.e.*, enzyme) proteins, ensuring the correct function of many cellular proteins under conditions of elevated temperature. Overall, the chaperone system is a necessary and conserved method responsible for the reactivation of aggregated proteins. They are an essential aid to many plant species as well **(Tyedmers *et al*., 2010)**.

2.5.1. Correlations between Hsps synthesis and stress tolerance

Positive correlations between the expression levels of several Hsps and stress tolerance have been described extensively by functional genomics and proteomics in different plant species. Comparison of expression data under variable conditions, for example from different tissue types, developmental stages, growth conditions, or applications and durations of stress treatments, shows related patterns of transcript accumulation, with the expression of about 2% of the genome being affected **(Vinocur *et al*., 2005, Kotak *et al*., 2007)**. In the tomato, for instance, several Hsps are induced by heat stress **(Frank *et al*., 2009)**. Sometimes a second abiotic stress, when combined with heat stress, increases the Hsp expression. In wheat, the highest Hsp expression was established under the combined drought and heat stress **(Grigorova *et al*., 2011)**. These results corroborated the hypothesis of **Miller (2006)** that simultaneous exposure to different abiotic stresses results in the activation of several stress response pathways. On the other way, some Hsps can be down-regulated under specific stress, such as two Hsps from rice that were down-regulated undercold stress **(Ye, S.F *et al*., 2012)**.

A study with *Arabidopsis* revealed there are some Hsps induced by different stresses, with changes in expression under a number of environmental conditions **(Swindell *et al*., 2007)**. Salt stress induced the expression of six different Hsps in rice **(Ye, S.F *et al*., 2012)**. It has been suggested that Hsps general role is to act as molecular chaperones regulating the folding and accumulation of proteins as well as localization and degradation in all plants and animal species **(Panaretou and Zhai, 2008; Hu *et al*., 2009; Gupta *et al*., 2010**). These proteins, as chaperones, prevent the irreversible aggregation of other proteins and participate in refolding proteins during heat stress conditions **(Tripp *et al*., 2009)**. Each group of these Hsps has role of each is briefed.

2.5.2. Plant heat-shock proteins families

Plants vary greatly in the amount of expressed Hsps as well as their type. The most studied species of plants is *Arabidopsis thaliana* where the response to heat shock

treatment occurs through the participation of a number of different Hsps:13 (Hsp20), 8 (Hsp70), 7 (Hsp90), 8 (Hsp100) and 21 transcription factors (Hsfs) **(Swindell *et al*., 2007)**, but in tomato there are at least 15 Hsfs **(von Koskull-Do¨ ring *et al*., 2007)**. sHsps, which are usually undetectable in plant cells under physiological conditions, are induced upon stress and plant tolerance to stress, including drought, salinity, oxidized species, and low temperatures **(Zhang *et al*., 2008)**. Expression of HSPs in cereal species was first revealed in some early works in 1980s. In a study **A. Necchi *et al*., (1987)** examining HSP metabolism in seedlings of five cereal species (common, drurm wheat, barley, rye, and triticale) responding to heat shock at 40◦C, inductions of 13 HSPs (14-15, 35–69, 83– 99 kDa) were detected. It was also reported that distinct levels of acquired thermal tolerance between wheat varieties were associated with significant quantitative differences in the synthesis of multiple HSPs (16, 17, 22, 26, 33, and 42 kDa) **(M. Krishnan *et al*., 1989)**. More thorough characterization of heat-responsive proteins including HSPs benefits from successful application of proteomic-based techniques, particularly two-dimensional gel electrophoresis coupled with mass spectrometry. **Lee *et al*. (2005)** identified 18 HSPs in a study investigating rice leaf proteome in response to heat stress, including seven HSP70s, three HSP100s, one HSP60, and seven newly induced or highly upregulated sHSPs.

Kotak *et al*., (2007) suggested five principal classes of Hsps characterized in plants by their activities as molecular chaperones according to their approximate molecular weight: (1) Hsp100, (2) Hsp90, (3) Hsp70, (4) Hsp60, and (5) small heat-shockproteins (sHsps). Recently, heat-shock proteins put into families according to their molecular weight, amino acid sequence homologies and functions: Hsp100 family, Hsp90 family, Hsp70 family, Hsp60 family, and the small Hsp family **(Gupt *et al*., 2010)**.

2.5.2.1. Class: Hsp70

In almost all organisms, the Hsp70 functions as chaperones for newly synthesized proteins to prevent their accumulations as aggregates and folds in a proper way during their transfer to their final location ((**Sung *et al*., 2003; Su and Li, 2008)**. Furthermore, Hsp70 and sHsps primarily act as molecular chaperone and play a crucial role in protecting plant cell from the detrimental effects of heat stress **(Rouch *et al*., 2004)** and Hsp70 and sHsp17.6 might play a crucial role in the development of cross-adaptation to temperature stress induced by heat acclimation (HA)- or cold acclimation (CA) pretreatment in grape plants **(Zhang *et al*., 2008)**. There is some indication that Hsp70B found in the stroma of chloroplasts participate in photo protection and the repairing of photosystem II during and after the photoinhibition **(Schroda *et al*., 1999)**. A more recent study on *A. thaliana* indicated the necessity of Hsp70 found in the stroma of chloroplast for the differentiation of germinating seeds and its tolerance of heat **(Su and Li, 2008)**.

In *Porphyra seriata*, PsHsp70 enhanced heat stress tolerance **(Park *et al*., 2012)**. **Sung & Guy (2003)** also related an altered expression in these proteins in *Arabidopsis*. They noticed an increased expression of some Hsp70 under heat stress. In rice, there was an increased expression of Hsp70 under different kinds of stress **(Goswami *et al*., 2010)**.

2.5.2.2. Family: HSP100/Clp

The HSP100 family or caseinolytic protease (CLP) chaperones are members of the large AAA ATPase super-family with a broad spectrum of diverse functional properties **(Agarwal, M. *et al*. 2001)**.Interestingly, rather than the regular chaperone function of preventing protein aggregation and misfolding, the Hsp100/Clp family functions in protein disaggregation and/or protein degradation. The removal of non-functional but potentially harmful polypeptides arising from misfolding, denaturation or aggregation is important for the maintenance of cellular homeostasis **(Burton and Baker 2005).**

Members of the Hsp100 family were first described as components of the two-subunit bacterial Clp protease system, which consists of regulatory ATPase/chaperones (such as ClpA and ClpX) and proteolytic (ClpP) subunits. The family is further divided into two major classes and eight distinct subfamilies within these classes. Members of the first class (A–D) contain two nucleotidebinding domains (also called ATP-binding domains), whereas those in the second class (M, N, X, Y) have only one nucleotide-binding domain **(Schirmer *et al*., 1996)**. Hsp100/Clp proteins are typically hexameric rings.

Hsp100/Clp proteins have been reported in many plant species, such as Arabidopsis, soybean, tobacco, rice, maize (Zea mays), Lima bean (Phaseolus lunatus) and wheat **(Adam *et al*., 2001; Agarwal *et al*., 2001)**. Like many other Hsps/chaperones, Hsp100/Clp family chaperones are often constitutively expressed in plants, but their expression is developmentally regulated and is induced by different environmental assaults, such as heat, cold, dehydration, high salt or dark-induced etiolation **(Adam and Clarke. 2002)**. **Sharon J. Keeler and co-workers (2000)** demonstrated the association of the expression of cytosolic, as well as the chloroplastic, in a recent report **(Agarwal *et al*., 2003)**, rice HSP100 protein production was correlated with the disappearance of protein granules in yeast cells.

2.5.2.2.1. sub- Class: HSP101

These proteins belong to a family of proteases firstly described in bacteria **(Parankiewicz *et al*., 1999)**, but also in yeast, protozoans, and plants. In particular they are ATPases involved in assembly/disassembly of protein complexes such as the ATP-dependent dissolution of cytosolic or nuclear protein aggregates formed during heat stress **(Lee *et al*., 2004 and Katiyar-Agarwal *et al*., 2001)**. **Tonsor *et al*. 2008** showed that HSP101 is more highly expressed in populations at higher latitude. **Halter (2012)** suggests that HSP101 expression is important in emergency high-temperature tolerance. Studies have shown that HSP101 is necessary for thermotolerance in a variety of species. Researcher have conducted knock out studies of HSP101 in *A. thaliana*. These plants were able to grow normally but had no tolerance of heat stress **(Hong *et al*., 2001)**.

It was further shown that the dissolution of electron-dense granules by Hsp101 takes place during the post-stress phase, implying a role for Hsp100 in the recovery of cell stress. This role was confirmed by a study on HSP101 in Arabidopsis. This protein is part of a molecular complex involving also small HSPs (sHSPs), and has the role of re-solubilizing protein aggregates formed as an effect of the heat stress **(Lee *et al*., 2005 and Agarwal *et al*., 2003)**. **Halter (2012)** also reported that there were trade-offs to high expression of HSP101. These trade-offs include reduced root growth and lower fruit

production. Over expression of Hsp101 in rice plants, that are sensitive to heat stress, resulted in a significant improvement of growth performance during their recovery **(Katiyar *et al*., 2003)**. Thus HSP101 expression can be costly to plant growth and reproduction.

In many plant species (i.e. *Arabidopsis thaliana*, wheat, rice, maize, soybean), many cDNAs and genomic clones, coding for different forms of HSP101, with molecular weights ranging from 100.9 to 109.4 kDa, have been isolated and characterized, suggesting that HSP101 is a member of a small gene family strongly induced by heat **(Agarwal *et al*., 2001 and 2002)**. In maize, **Zhang *et al*., 2005** reported that HSP101identified in maize is a nucleus-localized protein, which belongs to the campylobacter invasion antigen B (CiaB) protein sub-family, whose members promote the renaturation of protein aggregates, and are essential for the induction of thermotolerance, HSP101 forms complexes with HSP70 that could be part of a large multi-chaperone complex, involved in the correct protein folding. Other reports have shown that HSP101 has a central role in establishing thermotolerance **(Hong *et al*., 2001)**. In *Triticum aestivum*, three different HSP101 coding sequences are present in Gene Bank **(Campbell *et al*., 2001)**.

2.5.3 Hsp101 mechanisms for controlling protein aggregation

Hsps/chaperones play complementary and sometimes overlapping roles in protecting proteins from stress. Abiotic stress in plants often causes dysfunction/denaturation of structural and functional proteins. Maintaining proteins in their functional conformations and preventing the aggregation of non-native proteins are particularly important for cell survival under stress conditions. To maintain cellular homeostasis, some members of the Hsp/chaperone families [e.g. small Hsp (sHsp) and Hsp70 stabilize protein conformation, prevent aggregation and thereby maintain the non-native protein in a competent state for subsequent refolding, which is achieved by other Hsps/chaperones (e.g. Hsp60, Hsp70 and Hsp90). When denatured or misfolded proteins form aggregates, they can be resolubilized by Hsp100/Clp followed by refolding, or degraded by protease. Some Hsps/chaperones (e.g. Hsp70, Hsp90) accompany the signal transduction and transcription activation that lead to the synthesis of other members of Hsps/chaperones [e.g. those controlled by heat-shock factor (HSFs)] and other stress-response proteins (e.g. antioxidants).

Expression of heat shock proteins (HSPs) is known to be an important adaptive strategy in this regard **(Feder and Hoffman, 1999)**. It works together as a molecular chaperone machine in order to aid a protein that has been misfolded into its nonnative state. HSP70's are the first to interact with aggregated proteins. They block proteases from binding to the misfolded protein and they also facilitate the transport of the protein to the HSP100/ClpB complex **(Tyedmers *et al*., 2010)**. The HSP100 family has been proven to be necessary in the thermotolerance of several organisms. The job of the HSP100/ClpB family of proteins is to interact with aggregated proteins in order to make the substrate on the nonnative protein exposed for HSP70 to then work with. Another mechanism proposed that protein aggregates can be efficiently resolubilized by Hsp100/Clp family chaperones and are then refolded by the assistance of the Hsp70 system; the final refolding of solubilized proteins into the native form might be completed by members of the Hsp60 family (GroEL–GroES) **(Peres Ben-Zvi and Goloubinoff., 2001)**.

Similar observations have been also reported with plant chaperones. For example, Hsp18.1 from pea (*Pisum sativum*) can stably bind heat-denatured protein and maintain it in a folding-competent state for the further refolding by Hsp70/Hsp100 complexes **(Mogk *et al*., 2003)**. **Sun *et al*., (2002)** reported that Small HSPs are responsible for keeping the misfolded proteins in a form in which they can be refolded. A number of studies have found that expression of sHSPs is correlated to thermotolerance. However, plants can have more than 30 sHSPs and no single sHSP has the same impact on thermotolerance that HSP101 has been demonstrated to have. Together, all of the HSP families work together to assist and support stressed proteins so that they may be refolded and are able to perform their metabolic processes properly.

2.6. Heat shock proteins expression Measures by Quantitative Real- time PCR

Quantitative competitive Real time – polymerase chain reaction (qc RT-PCR) measures the absolute amount (i.e., copy number) of a specific transcript in a sample. Although the amount of product formed is easy to determine, it is difficult to determine the initial copy number of the target molecule because the overall efficiency of the RT-PCR for each sample is unknown. Real-time PCR monitors PCR product formation continuously during the PCR reaction by means of a fluorogenic reporter. This reporter system may take the form of fluorescently labeled PCR primers or involve the use of a fluorescent dye such as Syber Green™, which interchelates double-stranded DNA. Both of these systems require prior optimization of PCR conditions to eliminate primer-dimer formation, which would interfere in subsequent analysis. Because Real-Time PCR combines PCR amplification and product detection in one single step, the technique is very fast and easy to perform, compared to classical RT-PCR techniques. It allows the simultaneous quantification of a number of genes through the use of different flourophores **(Overbergh *et al*., 1999)**.

P. Rampino *et al*., (2007) indicate that Quantitative *HSP* transcription analysis by Real-Time PCR was performed on the six wheat genotypes. *HSP* gene expression was strongly induced in the stressed seedlings, confirming the role of these proteins in the mechanisms of protection activated by plants in response to high temperature. **Mariolina Gulli *et al*., (2007)** reported that, to establish any variation in response of the durum wheat HSP101 genes to heat stress, their expression was compared in different thermal conditions. The level of expression of the different HSP101 genes in T. durum was investigated by Quantitative (Real-Time) RT-PCR. Through this analysis, differences in timing and level of expression for the two isoforms were observed in both cultivars analyzed, thus confirming the different role for TdHSP101C gene with respect to TdHSP101B. In particular TdHSP101C (A and B forms) being massively expressed only after the long term treatment at 34 ˚C, it`s might be the leading actor in the acquisition of thermotolerance.

Moaed *et al*., (2012) reported that there is genetic variability in the synthesis of HSPs in wheat. Consistent with these observations, Quantative RT-PCR revealed that HSP101 is expressed constitively in the tow wheat genotypes examined in the study. In heat tolerant genotype (C306) expression level of HSP101 showed up regulation at all stages and duration of heat treatment. In contrast, heat susceptible genotype (PBW343)

showed considerable reduction in HSP101 transcripts at all three durations of heat stress as compared with their respective controls.

2.7. Single nucleotide polymorphism (SNP)

Single nucleotide polymorphism (SNP) detection technologies are used to scan for new polymorphisms and to determine the allele(s) of a known polymorphism in target sequences. SNPs are single base differences between haplotypes. Once discovered, SNPs can be converted into genetic markers that can be inexpensively assayed in a high-throughput manner **(Gut, 2001)**. Due to their abundance, it is possible to use SNP-based markers to generate very dense genetic maps **(Rafalski, 2002).** Such maps can be used to conduct marker-assisted selection (MAS) programs, construct the specific genotypes required for quantitative genetic studies, and to enhance our understanding of genome organization and function and address fundamental questions relating to evolution and meiotic recombination. SNPs can also be used for genome-wide linkage disequilibrium and association studies that assign genes to specific functions or traits. Furthermore, transcript-associated SNPs can be used to develop allele-specific assays for the examination of cisregulatory variation within a species **(Bray *et al*., 2003; Cowles *et al*., 2002; Guo *et al*., 2003); Stupar and Springer, 2006).**

2.7.1 Significance of SNPs in Plants

SNPs are by far the most common form of DNA polymorphism in a genome. They have been extensively used in genotyping human populations and most human sequence variation is attributable to SNPs. They are used in pharmacogenomics, diagnostic and biomedical research. However, SNPs have not been in regular use yet in plant genotyping. A large amount of SNP data is available in humans but very limited data is available on SNPs in plants. This is mainly due to the enormous cost involved in developing SNPs, but since human geneticists have developed a number of SNP genotyping assays, plant biologists can take advantage and use the already well-developed assays in human studies. SNPs have tremendous potential for germplasm fingerprinting and MAS.

In several crop plants, markers associated with phenotypic traits have been used in selection for desirable traits in plant breeding programmes. SNPs have been already used in *Arabidopsis* to study linkage disequilibrium (LD), which shows decay within 50 kb **(Nordborg *et al*. 2005**). In maize, a high frequency of nucleotide changes (on average, 1 SNP every 30 bp) and a rapid decay of LD within 100–200 bp have been reported **(Tenaillon *et al*. 2001; Remington *et al*. 2001)**. In soybean, **Zhu *et al*. (2008)** provided a comprehensive study on sequence polymorphisms. These authors surveyed about 76 400 bp from 25 diverse genotypes. They found roughly 1 SNP every 273 bp, and concluded that mean nucleotide diversity in cultivated soybean is much lower than that observed in *A. thaliana*, the model selfing species.

The SNP frequency between bread wheat genes from the A, B and C genomes is one SNP per 20 bp **(Wolters *et al*., 2000)**. In addition, they estimated the LD from their data and concluded that, in contrast to reports on maize **(Tenaillon *et al*. 2001; Remington *et al*. 2001)**, there is little decay of LD over distances of about 50 kb; LD declined only at genetic map distances greater than 2.5 cM. In barley, the level of sequence polymorphism

was reported to be approximately 1 mutation per 189 bases **(Kanazin *et al*. 2002**), but was much higher (1 SNP every 50 bp) in another sample of accessions including elite lines, landraces of *Hordeum vulgare*, and wild accessions of *Hordeum spontaneum* (**Roussel *et al*. 2004**).

Homoeologous ⁄ paralogous and varietal singlenucleotide polymorphisms (SNPs) have previously been studied and used in polyploid crops (**Akhunov *et al*., 2009; Bernardo *et al*., 2009; Bundock *et al*., 2009; Edwards *et al*., 2009; Imelfort *et al*., 2009**); however, these studies have also shown that distinguishing intervarietal markers from intergenomic polymorphisms is complicated and prone to error. For example, a previous in silico study by our group showed that of the _71 000 putative SNPs identified in the wheat EST database, only _3500 appear to be intervarietal (5%) with the majority being SNPs in homoeologous and paralogous genes **(Barker and Edwards, 2009**). These results, together with the large size of the wheat genome (_17 300 Mb), mean that despite the global importance of wheat, there are still relatively few validated varietal SNP markers in regular use. While this situation remains, it is likely that, when compared with other crops such as maize and rice, wheat will continue to lag behind in terms of markerassisted selection in breeding programmes (**Mochida and Shinozaki, 2010**).

The development of SNP-based genotyping platforms has lead to an increase in the number of protocols available for analyzing the genetic variation in numerous species (**Perkel, 2008**). However, as with the development of SNPs, large-scale genotyping in polyploid species is still a significant challenge because of the presence of the homoeologous and paralogous genes. Despite these challenges, a number of platforms have recently been developed to perform high-density genotyping (large numbers of SNPs, with small numbers of individual plants), and these have been successfully employed to genotype wheat **(Chao *et al*., 2010)**. However, these technologies can be difficult to optimize and as such they have yet to be generally adopted by the wheat community leaving few options for wheat breeders and geneticists who wish to carry out mediumto low-density genotyping on large or very large numbers of individual plants.

In bread wheat, SNP studies have been limited to single genes or DNA fragments (**Boisson *et al*. 2005**) allowing association studies or genetic mapping. Recently, systematic searches for SNPs in wheat have been initiated and many SNPs are available at http://wheat. pw.usda.gov/SNP/.

2.7.2 SNPs detection by Direct Sequencing

Sequence analysis is the most direct way of identifying SNPs. DNA could be sequenced using various methods available like Sangar dideoxy and other nucleotide sequencing. Direct sequencing is time consuming and costly. The other problem encountered in the use of this method in identifying SNPs is the sequencing error. A sequencing error rate of just one base per 1000 would equal the rate at which SNPs are found to occur. Another significant problem, which arises, is that many plant species are heterozygotes or polyploids. In these cases, direct sequencing would help only highlighting the base difference and not the exact bases that were changed (**Kirk *et al*., 2002; Edward K J & Mogg R. 2001**).

In one of the approaches, direct sequencing of AFLP bands can isolate SNP. This has been applied for the first time to discover 24 SNPs from 10 DNA fragments in 11.11

Kb of genomic DNA of brown trout (*Salmo trutta*). This strategy can be useful for SNP analysis of non-model organisms where sufficient sequence data is not available **(Nicod J & Largiader C R. 2003)**. Locus specific primers are synthesized from available genomic sequences and PCR amplification is done. The PCR products are then sequenced and the sequence differences are used to discover new SNPs.