गेहूँ (ट्रिटिकम एस्टीवम एल.) से आवधिक उष्मीय तनाव मे ऊष्मा प्रेरित माइटोजन सक्रिय प्रोटीन काइनेज जीन की खोज, क्लोनिं गतथा निरूपण

Identification, Cloning and Characterization of Heat-induced MAPK Gene(s) from Wheat (*Triticum aestivum* L.) Under Terminal Heat Stress

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CERTIFICATE

This is to certify that the thesis entitled **“**Identification, Cloning and Characterization of Heat-induced MAPK Gene(s) from Wheat (*Triticum aestivum* L.) under Terminal Heat Stress**”** submitted to the Faculty of the Post-Graduate School, **ICAR-Indian Agricultural Research Institute, New Delhi**, in partial fulfillment of **Master of Science** in **Biochemistry**, embodies the results of *bona fide* research work carried out by Mr. Sachidanand Tiwari, under my guidance and supervision, and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help availed during the course of investigation as well as source of information have been duly acknowledged by him.

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CONTENTS

Sr. No.	TITLE	Page No.
1.	**Introduction**	1-4
2.	**Review of literature**	5-11
3.	**Materials and methods**	11-24
4.	**Results**	25-30
5.	**Discussion**	31-33
6.	**Summary and conclusion**	34-35
7.	**Abstract in English**	36
8.	**Abstract in Hindi**	37
9.	**Bibliography**	i-ix

LIST OF FIGURES

Fig. No.	Title	After Page No.
1	PCR amplification of putative MAPK gene from wheat cultivar HD3059	25
2	Restriction Map of pGEMT-Easy cloning vector	25
3	Blue-White screening of transformed clone harboring MAPK-pGEMT Easy construct	25
4	Restriction analysis of isolated Plasmid from positive transformed colonies (white colonies) harboring MAPK gene pGEMT Easy construct	25
5	Nucleotide sequence of the cloned putative MAPK gene from wheat *cv*. HD3059	26
6	Amino acid sequence in protein of the cloned putative MAPK gene from wheat *cv*. HD3059	26
7	Conserved domain prediction in protein of putative MAPK gene cloned from wheat *cv*. HD3059 using Conserved Domain search tool of NCBI	26
8	Predicted phosphorylation site on protein of putative MAPK gene cloned from wheat *cv*. HD3059 of wheat using NetPhos 2.0	26
9	Phylogenetic tree of putative MAPK gene cloned from wheat *cv*. HD3059	26
10	Predicted protein model of putative cloned MAPK gene from wheat *cv*. HD3059; Predicted using SWISS-MODEL server	26
11	Denaturing agarose gel image of total RNA isolated from leaves of two thermotolerant *cvs*. (HD3059, Halna) and two thermosusceptible *cvs*. (HD2329, PBW550) of wheat	26

	exposed to differential heat treatment	
12.a	Expression profiling of putative MAPK transcripts in wheat *cvs*. Halna and HD3059 and thermosusceptible wheat *cvs*. HD2329 and PBW550 of wheat under differential heat stress at grain-filling stage; C-22ºC; T1- heat stress of 30ºC for 2 h, T2- heat stress for 38ºC for 2 h; β-actin gene was used as endogenous gene control; relative fold express was calculated by Pfaffl method	27
12.b	Semiquantitative-PCR of putative MAPK transcripts in thermotolerant *cvs*. HD3059, Halna and thermosusceptible *cvs*. PBW550 and HD2329 of wheat under differential heat stress at grain-filling stage; C-22ºC; T1- heat stress of 30ºC for 2 h, T2- heat stress for 38ºC for 2 h	27
13.a	Photosynthetic rate of thermotolerant wheat *cvs*. Halna and HD3059 at milky-ripe and mealy-ripe stage under differential heat stress treatment ; C-22ºC; T1- heat stress of 30ºC for 2 h, T2- heat stress for 38ºC for 2 h	27
13.b	Photosynthetic rate of thermosusceptible wheat *cvs*. HD2329 and PBW550 at milky-ripe and mealy-ripe stage under differential heat stress treatment ; C-22ºC; T1- heat stress of 30ºC for 2 h, T2- heat stress for 38ºC for 2 h	27
14.a	Estimation of Total Antioxidant Capacity (TAC) in thermotolerant wheat *cvs*. Halna and HD3059 at milky-ripe and mealy-ripe stage under differential heat stress treatment ; C-22ºC; T1- heat stress of 30ºC for 2 h, T2- heat stress for 38ºC for 2 h	28
14.b	Estimation of Total Antioxidant Capacity (TAC) in thermosusceptible wheat *cvs*. PBW550 and HD2329 at milky-ripe and mealy-ripe stage under differential heat stress treatment ; C-22ºC; T1- heat stress of 30ºC for 2 h, T2- heat stress for 38ºC for 2 h	28
15.a	Guaiacol peroxidase (GPX) activity assay in thermotolerant wheat *cvs*. Halna and HD3059 at milky-ripe and mealy-ripe stage under differential heat stress	28

	treatment; C-22ºC; T1- heat stress of 30ºC for 2 h, T2- heat stress for 38ºC for 2 h heat stress at grain-filling stage	
15.b	Guaiacol peroxidase (GPX) activity assay in thermosusceptible wheat *cvs*. HD2329 and PBW550 of wheat at milky-ripe and mealy-ripe stage under differential heat stress treatment; C-22ºC; T1- heat stress of 30ºC for 2 h, T2- heat stress for 38ºC for 2 h	28
16.a	Superoxide dismutase (SOD) activity assay in thermotolerant wheat *cvs*. Halna and HD3059 at milky-ripe and mealy-ripe stage under differential heat stress treatment ; C-22ºC; T1- heat stress of 30ºC for 2 h, T2- heat stress for 38ºC for 2 h	28
16.b	Superoxide dismutase (SOD) activity assay in thermosusceptible wheat *cvs*. PBW550 and HD2329 at milky-ripe and mealy-ripe stage under differential heat stress treatment ; C-22ºC; T1- heat stress of 30ºC for 2 h, T2- heat stress for 38ºC for 2 h	29
17.a	Catalase activity assay in thermotolerant wheat *cvs*. Halna and HD3059 at milky-ripe and mealy-ripe stage under differential heat stress treatment ; C-22ºC; T1- heat stress of 30ºC for 2 h, T2- heat stress for 38ºC for 2 h	29
17.b	Catalase activity assay in thermosusceptible wheat *cvs*. HD3059 and HD2329 at milky-ripe and mealy-ripe stage under differential heat stress treatment ; C-22ºC; T1- heat stress of 30ºC for 2 h, T2- heat stress for 38ºC for 2 h	29
18.a	Estimation of Proline content in thermotolerant wheat *cvs*. Halna and HD3059 at milky-ripe and mealy-ripe stage under differential heat stress treatment ; C-22ºC; T1- heat stress of 30ºC for 2 h, T2- heat stress for 38ºC for 2 h	29
18.b	Estimation of Proline content in thermosusceptible wheat *cvs*. PBW550 and HD2329 of wheat at milky-ripe and mealy-ripe stage under differential heat stress treatment; C-22ºC; T1- heat stress of 30ºC for 2 h, T2- heat stress for 38ºC for 2 h	29

19.a	Estimation of Reactive oxygen species (ROS) in thermotolerant wheat *cvs*. Halna and HD3059 at milky-ripe and mealy-ripe stage under differential heat stress treatment ; C-22ºC; T1- heat stress of 30ºC for 2 h, T2- heat stress for 38ºC for 2 h	30
19.b	Estimation of Reactive oxygen species (ROS) in thermosusceptible wheat *cvs*. PBW550 and HD3059 at milky-ripe and mealy-ripe stage under differential heat stress treatment; C-22ºC; T1- heat stress of 30ºC for 2 h, T2- heat stress for 38ºC for 2 h	30

Chapter 1 INTRODUCTION

Wheat (*Triticum aestivum* L.) is the primary cereal crop of the Poaceae (Gramineae) family. It is cultivated over a wide range of agro-climatic region and exceeds in area and production in comparison to other grain crop (including rice, maize, etc.). It is one of the most important cereal grain crops of the world. It is the most vital and staple source of nourishment for 36% of the world population. At the global level, 55% of the carbohydrate and 20% of the calories is derived through consumption of wheat crop (Breiman and Graur, 1995). Wheat grain contains all essential nutrients; kernel contains about 12% water, 60-80% of carbohydrates mainly as starch, 8-15% proteins with all essential amino acids except lysine, tryptophan and methionine, 1.5-2% fats, 1.5-2% minerals, vitamins and 2.2% crude fibres. On the basis of chromosome number (genome), wheat is categorised into three groups - diploids 2n= 14 (n=7), tetraploids 2n= 28 (n=14) and hexaploids 2n=42 (n=21). Most of the currently cultivated wheat varieties belong to allohexaploid wheat (*Triticum aestivum* L.). It evolve as a tetraploid wheat (*Triticum turgidum* L.: AABB) through hybridization under natural condition with the D genome donor *i.e. Aegilops tauschii Coss*. (Kihara, 1944). India ranks second in wheat production followed by China.

Climatic change over the past few decades have been fairly rapid in many agricultural regions around the world. Air temperature is increasing in most of the major cereal growing regions around the world. Climate change and agriculture are dependent upon each other. Current estimate indicate that average temperatures will rise by 3–5°C in the next 50–100 years, severely affecting global agricultural systems (IPCC, 2014). Plants interact with various climatic factors during their growth and development. Plants are exposed to various abiotic stresses because of global climatic changes, which adversely affect their growth and development. These abiotic stresses may include drought stress, heat stress, chilling injury, salinity stress, etc. Since, ambient temperature of globe is increasing at a very rapid rate and is predicted to continue rising under climate change, heat stress is major abiotic stress which possess severe threat to crop production (Farooq *et al.,* 2011). Fluctuation in the temperature has severe effect on the growth and development of plant. Seed germination is inhibited or slowed down if subjected to high temperature.

Heat stress may adversely affect photosynthesis, respiration, water relations and membrane stability (Wahid *et al.,* 2007). High temperature is an important limitation to the cultivation of important cereal crops, such as wheat in large areas of the world (Kumar *et al.*, 2013). The optimum temperature for anthesis and grain-filling stage ranges from 12 to 22ºC. Exposure of plant to temperature above the ambient significantly reduces the grain yield (McDonald *et al.*, 1983; Macas *et al.*, 2000; Mullarkey *et al.*, 2000 and Tewolde *et al.*, 2006). Heat stress during anthesis period cause poor development of flower buds along with their sterility (Wardlaw and Wrigley, 1994). High temperature during the reproductive stage can lead to pollen sterility, tissue dehydration, lower CO_2 assimilation and increased photorespiration. High temperature also reduces the phenology of wheat crop, as a result of which grain yield is reduced in significant amount (Wardlaw and Moncur, 1995; Zahedi and Jenner, 2003). High temperature during grain-filling period shortens the duration by 0.4 day for each 1℃ increase in the mean temperature from optimum temperature (Tahir and Nakata, 2005). The reduction in the yield of wheat under stress is due to decline in the number of grain per ear as well as 1000 grain weight (Test weight). Every 1℃ rise in temperature above ambient condition during grain-filling results in yield reduction by 3-4% (Reynolds *et al.*, 1994 and Wardlaw *et al.*, 1989). The high temperature at the start of grain-filling and ripening stage has a negative impact on yield as measured by kernel weight, 39.8% reduction in case of susceptible varieties and 26.7% reduction in tolerant varieties has been reported by Modarresi *et al.*, (2010).

Food security under present scenario of climate change is one of major challenges for the agricultural research. So, there is a need to develop the heat tolerant wheat genotype which can withstand the high temperature without any significant loss in the yield. Plants exhibit a characteristic set of cellular and metabolic responses required for them to survive under the high temperature conditions (Guy, 2008). These responses leads to changes in the cellular organization, including organelles, cytoskeleton, and membrane functions (Weis and Berry, 1988) which is accompanied by decrease in the synthesis of normal proteins and the accelerated accumulation of heat shock proteins (HSPs) (Bray *et al.*, 2000). Membranes are very sensitive to environmental signals; probably enclose sensory devices capable of detecting stimuli and transducing them into cellular response.

Later on calcium ions activate signalling mechanism of heat shock proteins and mitogen activated protein kinase pathway which make the cell to withstand the effect of external stimuli. Some major tolerance mechanisms, includes ion transporters, osmolyte accumulation, scavenging of free radicals, late embryogenesis abundant proteins. Heat stress imparts its effects at various levels, including plasma membrane and biochemical pathways functional in the cytoplasm or in cytosolic organelles (Sung *et al.*, 2003). Primary effects of heat stress cause fluidity of plasma membrane which up regulate different signalling pathways of cell including mitogen activated protein kinase pathway, calcium dependent protein kinase pathway and Ca^{2+}dependent salt overly sensitive (SOS) signalling (Kaur *et al.*, 2005). Signalling of these cascades at nuclear level leads to the production of compatible osmolyte for osmotic adjustment of cell and antioxidant for nullifying the effect of ROS. The antioxidant defence mechanism leads to heat-stress adaptation, and its strength is interrelated with level of thermotolerance in plants (Maestri *et al.*, 2002).

Eukaryotic mitogen activated protein kinase (MAPK) cascades have evolved to convert environmental and developmental signals into adaptive and programmed responses (Kultz *et al.*, 1998). Plant MAPK cascades regulate numerous processes, including stress and hormonal responses, innate immunity and developmental programs. MAPK cascades communicate and intensify signals through three types of reversibly phosphorylated kinases by phosphorylating the downstream protein components, and mediate a wide range of responses together along with changes in gene expression (Cristina *et al.*, 2010). MAPKs are serine/threonine kinases which further phosphorylate a wide range of substrates in downstream pathway of signalling cascade, which include various other kinases and transcription factors (Chang, *et al.,* 2001). The MAP kinases play universal role in signalling pathways which is activated by abiotic and biotic stress. Major abiotic stress which activate MAPK signalling cascade are cold (Jonak *et al.,* 1996), salt (Jayaram *et al.,* 2008), touch (Mizoguchi *et al.,* 1996), wounding (Wang *et al.,* 2007), heat (Sangwan *et al.,* 2002), UV (Agrawal *et al.,* 2002), osmotic shock (Zong *et al.,* 2009), heavy metals (Rao *et al.* 2010), etc. The first heat shock activated MAPK (HAMK) is identified in *Medicago sativa* by Sangwan *et al.,* (2002). Later on heat stress induced MAPK is reported in many plants like *Solanum tuberosum* (Blanco *et al.*, 2006), *Nicotiana tabacum* (Suri *et al.,* 2008), *Orzya sativa* (Rao *et al.,* 2009). About 20 MAPK

homologues has been cloned in several plant species, including Arabidopsis (Mizoguchi *et al.,* 1993, 1994), *Nicotiana tabacum* (Wilson *et al.,* 1993; Zhang *et al.,* 1997), *Medicago sativa* (Duerr *et al.,* 1993; Jonak *et al.,* 1993, 1995), *Pisum sativum* (Stafstrom *et al.,* 1993), and *Petunia* × *atkinsiana* (Decroocq *et al.,* 1995).

Extensive work done has been carried out to elucidate the role of MAPK in different abiotic stress like drought stress, salinity stress, cold stress, wound stress, heavy metal accumulation very limited information is available on its role in heat stress. MAPK form a linear cascade of protein kinases in their signal transduction pathway. Components of cascade are - (a) MAP kinase kinase kinase (MAPKKK), (b) MAP kinase kinase, and (c) MAP kinase (MAPK). These components mediate sequential phosphorylation in downstream manner during signal transduction pathway. Heat stress induced MAPK has been identified and characterized from various crops like rice, maize, tobacco, potato and flowering plants; but very limited information is available in wheat. The first draft of wheat genome has been recently sequenced and available on public domain. Wheat has not much been characterized because of complexity of the genome, as compared to other closely related species. The mechanism underlying heat stress-tolerance has yet not been elucidated in wheat. The candidate members associated with stress tolerance has not been explored much. Keeping the above points in view, the present study is carried out with the following objectives -

1. Identification, cloning and *in-silico* characterization of heat-induced novel MAPK gene from wheat.
2. Spatio-temporal expression analysis of candidate MAPK(s) gene in wheat under the heat stress.

The relevant literature on the different aspects studied during the course of this investigation is reviewed here briefly under the following headings:

2.1 Impact of Climate Change on Agriculture

As a result of increased anthropogenic activity on planet, Earth's climate is changing in a very abrupt manner. Due to increased emission of greenhouse gases (GHG's) and its accumulation in atmosphere, temperature of Earth is increasing in a very steady manner. CO_2 is most significant contributor to global warming, commonly emitted during fossil fuel burning or from thermal power plants or heating system of buildings. Temperature rise due to greenhouse intensely effect the Earth's climate system. Average surface temperature of globe is increased by 0.74°C, since the 19th century and predicted to increase by 1.4-5.8°C at the end of 2100 AD (IPCC, 2007). Change in temperature pattern cause increase in both maximum and minimum temperature along with significant change in rainfall pattern (IPCC 2008). Global warming leads to increase in frequency of extreme climate events like heat waves, heavy rainfall melting of polar ice caps thus affecting the global hydrological cycle. Thus heat stress is immediate consequence of global warming which is increasing both in intensity and duration possessing threat to plants. Agriculture is drastically effected by changes in meteorological parameters. Due to pronounced effect of global warming mean temperature is projected to increase by 0.4–2.0°C during kharif and to 1.1–4.5°C in rabi season by 2070. Similarly average rainfall is probable to increase by up to 10% during kharif and rabi season by 2070. Increase in atmospheric carbon dioxide has a fertilization effect on crops with C_3 photosynthetic pathway. On the other hand increase in temperature can cause reduction in crop phenology, increase evapotranspiration, decrease fertilizer use efficiency, alters the general equilibrium position of insect population leading to serious threat in crop production. For every degree increase in mean temperature, grain yield in wheat crop shows reduction by 428 kg/ha (Kalra *et al.*, 1996). Major growing crops like wheat, rice and maize suffers great reduction in yield due to increase in ambient temperature. Increase of 2°C temperature from optimum will reduced the grain yield of wheat by 18.7%, rice by 7.4% and maize by 14.6% (Hundal *et al.,* 1996). So, the

changing scenario of global climate hold serious threat to agriculture sustainability and production.

2.2 Heat stress and wheat

Heat stress is prevalence of high temperature beyond an ambient level for period of time enough to cause permanent damage to crop growth and development. Heat stress is multipartite function of exposure duration, magnitude and rate of increase of temperature. Temporary increase of 10-15°C temperature above the ambient value is considered as heat stress (Wahid *et al.,* 2007). Constant or short lived high temperature result morphological, biochemical and physiological changes affecting growth and yield of plant, thus possess serious risk to crop production (Hall, 2001). Effects of heat stress is prevalent throughout the life cycle of plant. For case at the time of seed germination high temperature slow down or completely inhibit the germination. During later stages heat stress unfavourably affect photosynthesis, imbalance water relations and cell membrane stability along with modulation in level of hormones and metabolites. High temperature during reproductive phase results in floral abortion (Guilioni *et al.,* 1997)

Wheat, (*Triticum aestivum* L.) is the most broadly grown cereal crop in the world after rice and maize (Asseng *et al.,* 2011). Wheat crop is very sensitive to heat stress (Slafer and Satorre, 1999). Increasing temperature in wheat growing season in wheat dominated area has been reported by Alexander *et al.,* (2006) and Hennessy *et al.,* (2008). High temperature shows varying degree of effects at different growth stages of wheat but terminal heat stress i.e. at reproductive stage is more harmful than heat stress at vegetative stage due to direct effect on grain (Wollenweber *et al.,* 2003). It was reported by Burrell (2003), that some wheat varieties can lose 10-15% of yield with every 5°C increase in temperature. In a study, found that high temperatures during grain growth shortened and compressed stages of grain filling, reduced duration of dry matter accumulation and reduced kernel weight by 50%.

2.3 Response of wheat to heat stress

The response of wheat against the elevated temperature includes morphological, molecular and physiological mechanism which can be exploited for improvisation of crop against heat stress.

2.3.1 Morphological response of wheat under heat stress

High temperature causes blistering of leaves and twigs, discoloration and damage of leaf, senescence and abscission along with inhibition of root and shoots growth (Ismail and Hall, 1999). Ferris *et al.,* (1998) reported severe reduction in intermodal length during heat stress condition which result in premature death of wheat crop. High temperature results reduction in both grain number per spike and grain weight causing significant loss in yield of wheat. Temperature above 20°C between spike initiation and anthesis stage cause significantly reduction in number of grain per spike (Saini and Aspinall, 1982). Peet *et al.,* (1998) reported that high temperature impair the pollen and anther development which cause decreased in grain yield of wheat. Reduced number of tillers with promoted shoot elongation was observed in wheat plant under heat stress. In wheat green leaf area and productive tillers/plant were drastically reduced under heat stress (Djanaguiraman *et al.,* 2010) Elevated temperature changes the phenology of wheat, reducing the duration between anthesis and physiological maturity which result in loss of grain weight (Warrington *et al.,* 1977).

2.3.2 Physiological response of wheat under heat stress

Plant water status of plant is severly varied under heat stress condition. High temperature during daytime lead to high vapour pressure deficit results in high transpiration rate. Decrease in water potential of plants affecting many physiological processes. Heat stress is commonly related with reduced water availability to plants (Simoes-Araujo *et al.,* 2003). Heat stress is commonly related with reduction in water availability. To cope with this problem certain crops including wheat accumulate certain low molecular mass organic compounds (osmolytes) like proline, sugars, sugars alcohol, tertiary and quaternary amines (Sairam and Tyagi, 2004.). Accumulation of such osmolytes contribute to enhance heat tolerance for plants. Integrity and functions of biological membranes are sensitive to high temperature, as it alters the tertiary and quaternary structures of membrane proteins. Such changes enhance the membrane permeability as evident from increased loss of electrolytes. The increased solute leakage, indicate the decreased cell membrane thermostability (Wahid and Shabbir, 2005)

Photosynthesis is the most subtle physiological process to be sensed at elevated temperature (Wahid *et al.,* 2007) and any reduction in photosynthesis rate affects growth and grain yield of wheat (Al-Khatib *et al.,* 1990). It has been reported that heat stress reduces photosynthesis by causing disruptions in the structure and function of chloroplasts, and thus reduction in chlorophyll content (Xu *et al.,* 2006). The inhibition of photosynthesis due to high temperature is mostly attributed to increases in the rate of photorespiration. Exposure to heat stress above 40°C also damages the photosynthetic apparatus due to synthesis of reactive oxygen species which further degrades it and ultimately reduces the photosynthates produced by the plants (Sharkey, 2005). However, under modest heat stress no inhibition of photosystem II (PSII) has been observed, even though there was a substantial reduction in carbon assimilation (Sharkey, 2005).

2.3.3 Biochemical response of wheat under heat stress

Enzymes are key to metabolic pathway which are efficient in narrow range of temperature. Abiotic stress, mainly heat stress uncouple the metabolic pathways and enzymes which lead to accumulation of reactive oxygen species (ROS). ROS include hydroxy radical (OH^-), superoxide radical (O_2^-) and hydrogen peroxide radical (H_2O_2) which cause oxidative stress in cell (Liu and Huang, 2000). These reactive species oxidise the membrane lipids, leading to loss of semi-permeable nature of cell membrane. Superoxide radical is generated in organelle like chloroplast and mitochondria. Superoxide dismutase scavenge the superoxide radical and convert in hydrogen peroxide which is further detoxified through ascorbate peroxidase activity or catalase activity. More toxic hydroxy radical is generated in organelle through Haber-Weiss reaction which can potentially damage protein, DNA, lipids and other important macromolecules leading to limitations in yield and growth of crop. Ascorbate peroxidase (AOP) and Superoxide dismutase (SOD) are overexpressed during heat stress condition for mitigating the effect of reactive oxygen species. Overexpression of antioxidant activity is facilitated by signalling molecules like calcium dependent protein kinase and mitogen activated protein kinase (Gong *et al.,* 1997). Under heat induced oxidative stress membrane peroxidation is significantly increased, reducing membrane stability by 28% to 54% and resulting electrolyte leakage in wheat plants (Savchenko *et al.,* 2002).

2.3.4 Molecular response of wheat under heat stress

Heat stress lead to increased synthesis and accumulation of heat shock protein (HSP's). HSP's prevent denaturation of cellular proteins against the high temperature and also act as molecular chaperones which insure native configuration and functionality of protein. Other heat stress induced protein includes ubiquitin (Sun *et al.,* 1997), cytosolic Cu-Zn SOD (Herouart *et al.,* 1994) and Mn-POD (Brown *et al.,* 1993). Initial effect of heat stress includes the fluidity of membrane, leads to Ca^{2+} influx in cytosol. This influx will result in up regulation of various signalling cascade including calcium dependent protein kinase and mitogen activated protein kinase. Signalling of these cascade leads to acclimation of cell towards heat stress.

2.4 Mitogen Activated Protein kinase: A key signalling molecule

Mitogen activated protein kinase (MAPK) cascades are major components in downstream path of receptors that transduce extracellular stimuli into intracellular responses. The MAPKs are acknowledged as signalling machinery for regulation of developmental and physiological responses such as cell growth, hormone signalling cell differentiation, pathogen infection, drought, low temperature, wounding, high salinity etc. (Jonak *et al.,* 1999). Structurally the MAPKs form the cascade of three protein kinases in a series. The three components of the cascade includes (i) MAP kinase kinase kinase (MAPKK kinase or MAPKKK or MAP3K), (ii) MAP kinase kinase (MAPK kinase or MAPKK or MAP2K) and (iii) MAP kinase (MAPK or MPK) which are functionally related and mediate phosphorylation reactions in sequential manner. MAPKKK is an upstream activator of MAPKK which further activate MAPK in downstream pathway. Generally, this activation occurs by the phosphorylation on serine/threonine residues on MAPKK in a conserved signature sequence (S/T-X3-5-S/T). The phosphorylated MAPKK in turn activates MAPK, the downstream next component of the cascade, by phosphorylating threonine and tyrosine residues in TEY or TDY motif in the activation loop. Generally, activation occurs by the phosphorylation on two serine/threonine residues on MAPKK in a conserved motif. In plants, these protein phosphorylation cascades link extracellular stimuli to a broad range of cellular responses. Downstream phosphorylation substrate include transcription regulators, transcription factors, protein kinase and splicing

factors. Signal transduction pathway starts with signal perception, followed by generation of second messengers like reactive oxygen species (ROS) and inositol phosphates (IP). These second messengers modulate intracellular Ca^{2+} levels which initiate phosphorylation cascades controlling specific sets of stress regulated genes.

2.4.1 Role of MAPK's in various stress

Specific sensors sense specific stress stimuli and transmit signals to activate specific transcription factors accordingly. When plant cells perceive any stress stimulus, they immediately activate signalling machinery comprising protein kinases that direct gene expression consequently to change their physiological status as a defence mechanism. According to Rodriguez *et al*., (2005) signal transduction networks for cold, drought and salt stress can be divided into three major signalling types: (i) osmotic/oxidative stress signalling (ii) Ca^{2+} dependent signalling and (iii) Ca^{2+} dependent salt overlay sensitive (SOS) signalling. Analyses of MAPK's in Arabidopsis and tobacco have suggested that MAPK pathway components can function in several combinations and have defined functions in different biological condition. Depending on the stimulus a given plant MAPKK can interact and activate several different MAPKs (Cardinale, 2002).

Plants respond to biotic stress by activating defence responses which include rapid production of reactive oxygen species (ROS), strengthening of cell walls, induction of the hypersensitive response (HR) and the necrotic effect on sites of infection**.** Plant defence responses also include synthesis of pathogen-related proteins (PrP) and phytoalexins. Studies of the infection of tobacco leaves by TMV (tobacco mosaic virus) has confirmed that both stress induced protein kinase (SIPK) and wound induced protein kinase (WIPK) are activated during infection. In tobacco, SIPK and WIPK become activated by various ROS and overexpression of SIPK in plants cause hypersensitive reaction towards infection. (Zhang *et al.,* 2000). In Arabidopsis, MPK3, MPK4 and MPK6 are all activated by bacterial and fungal infection (Zhang *et al.,* 2000). Osmotic stress signalling is MAPK module for abiotic stress signalling in plants. The osmotic signalling involves MAPK pathway, which is activated by protein tyrosine kinases and lead to production of compatible solutes. In Arabidopsis MPK3, MPK4 and MPK6 genes are induced in response to osmotic stress (Droillard, 2002). Tomato MPK1, MPK2 and MPK3 genes are orthologues of Arabidopsis MPK3 and MPK6 genes which are expressed during wound stress

(Holly, 2003). Arabidopsis mutant with increased sensitivity to the chemical mutagen methyl methanesulfonate and UV-C confirm the role of MPK1 gene against genotoxic stress (Ulm, 2002).

2.4.2 Role of MAPK in heat stress

MAPK cascades are important parts of signal transduction pathway in plants during heat stress. As a signalling response to heat stress condition, cytosolic Ca^{2+} sharply rises (Larkindale and Knight, 2002), which is linked to the acquisition of tolerance by transducing high temperature-induced signals to MAPK. MAPK cascades are important parts of signal transduction pathways in plants and thought to function ubiquitously in many responses to external signals (Gupta and Kaur, 2005). A heat-shock activated MAPK (HAMK) has been identified, which is triggered by changes in membrane fluidity resulting in cytoskeletal remodelling (Sangwan *et al*., 2002). Ca^{2+} influx coupling with action of Ca^{2+} dependent protein kinases (CDPK) have been closely related with the expression of HSPs. Membrane fluidity is directly, reversibly affected by temperature. The actin cytoskeleton is known to be connected to the plasma membrane and heat activated Mitogen Kinase (HAMK) is activated through reshuffling of actin filaments. HAMK activation in downstream pathway require calcium influx. MAPK cascades transmit and amplify signals via three types of reversibly phosphorylated kinases leading to the phosphorylation of substrate proteins, which mediate a wide series of responses, including changes in gene expression. Plant MAPK cascades regulate numerous processes, including stress and hormonal responses, innate immunity, and developmental program.

2.5 Cloning and characterization of MAPK in plants

The MAPK genes were first identified in 1993 as D5 kinase in *Pisum sativum* (Stafstrom *et al.,* 1993) and as MsERK1 in *Medicago sativa* (Duerr *et al.,* 1993). Afterwards many MAPKs have been cloned in many plant species like Arabidopsis, maize, rice etc. Cloning of AtMPK3, AtMPK4 and AtMPK6 genes in Arabidopsis under oxidative stress is reported (Droillard, 2002). A putative rice MAPKKK gene in rice (*dsm1*) is characterized (Xiong *et al.,* 2003). Peng *et al*., (2006) investigated the expression patterns of MaMAPK in Malus under drought tolerance. Sangwan *et al.,* (2002) identified the first plant heat shock activated MAPK (HAMK) from *Medicago sativa* plant. In *Solanum tuberosum* heat responsive StMPK1 gene

(MAPK homolog) is characterized (Blanco *et al.,* 2006). A novel rice (*Oryza sativa* L.) multiple stress responsive MAP kinase gene, OsMSRMK2 gene is reported (Agrawal *et al.,* 2002). ZmMPK5 gene, homologue of MAPK functioning during oxidative stress in *Zea mays* is characterized (Hai *et al.,* 2009). Exogenous abscissic acid (ABA) and hydrogen peroxide (H_2O_2) induced calcium-dependant ZmMPK7 gene in maize is reported (Zong *et al.,* 2009). PsMPK2 gene, a homologue of MAPK was isolated from *Pisum sativum* (Masia *et al.,* 2008). Genome-wide identification and expression analysis of mitogen-activated protein kinase and mitogen-activated protein kinase kinase genes in *Capsicum annuum* is reported (Liu *et al*., 2015). Expression analysis of MAPK and MAPKK gene family in *Malus domestica* is reported by Zhang *et al*., (2013). Analysis of expression profiles of MAPKKK gene in maize was reported (Kong *et al*., 2013).

3.1 Plant materials and heat stress treatment

Four popular wheat cultivars *i.e.* Halna, HD3059 (thermotolerant) and HD2329, PBW550 (thermosusceptible) were used in the present study. Seeds of these four wheat *cvs*. were collected from the Division of Genetics, Indian Agricultural Research Institute (IARI), New Delhi. Seeds were treated with Bavistin (0.5%) before sowing in pots. Seeds were sown in 36 pots (nine for each variety) having equal quantity of perlite to FYM mixture inside the regulated chamber (22±3°C, relative humidity of 75%, and 8 h light with intensity of 250 μmol m^{-1} s^{-1} Photosynthetic Active Radiation) in the National Phytotron Facility, IARI, New Delhi. Irrigation was done at regular intervals, and plants at the milky-ripe and mealy-ripe sub-stage of grain-filling (selected based on the Feekes scale; three pots from each variety) were exposed to heat stress of 30°C and 38°C for 2 h, whereas other three pots in each group served as control (22±3°C). The heat stress was given in a sinusoidal mode using microprocessor-regulated controller with an increase of 1°C/10 min, till the temperature reaches the 30° or 38°C, and it was maintained for 2 h; further, temperature decreases to 22°C in the same fashion. Samples (stem, and flag leaf) were collected in triplicates from both the cultivars and stored at -80°C for further downstream analysis.

3.2 *De novo* assembly for the identification of Mitogen-activated Protein Kinase transcripts

An RNA-Seq experiment was executed in our lab for the identification of novel heat-responsive stress-associated genes in wheat *cvs*. HD2985 and HD2329 under control and HS-treated condition at grain-filling stage using *de novo* transcriptome sequencing on Illumina HiSeq 2000 platform. The raw data generated using Next-Generation Sequencing (NGS) of control and HS-treated samples of HD2985 and HD2329 cultivars of wheat (NCBI BioProject Database: PRJNA171754) was assembled using Velvet and Oases v 2.0 and was mined for the identification of MAPK transcripts based on the presence of conserved domain in the transcripts as well as homology based search. Based on the *in-silico* analysis, 40 transcripts showed homology with MAPK gene reported from plant source. The digital gene

expression (DGE) of the identified transcripts showing homology with MAPK was analyzed, and further transcript having maximum DGE were targeted for the cloning.

3.3 Cloning of identified MAPK transcript

3.3.1 Total RNA isolation-

Total RNA was isolated by using Trizol reagent (Invitrogen, UK) and the steps involved in protocol are as follows:

- Grind 100 mg of tissue to fine powder in liquid nitrogen using pre-chilled mortar and pestle.
- Add 1 mL of Trizol reagent and keep at room temperature for 5 min till it get thaw.
- Transfer it to 1.5 mL RNase-free eppendorf tube and add 200 μL chloroform into tube and vortex it for short duration.
- Keep the tube at room temperature for 5 min and later centrifuge it at 13000 rpm for 15 min at 4°C.
- Transfer the aqueous phase to fresh RNase-free eppendorf tube and add 0.6 volume of pre-chilled isopropanol.
- Mix well the tubes by hand vortexing and keep at room temperature for 10 min. Centrifuge at 13000 rpm for 10 min. at 4°C and discard supernatant.
- Add 500 μL of 70% chilled ethanol to pellet and centrifuge it at 13000 rpm for 5 min. at 4°C.
- Discard the supernatant and air dry the pellet for 10-15 min.
- Add 40 μL RNase-free water and keep on ice till pellet gets dissolved.
- Store the RNA at -80°C for future use.

3.3.2 Qualitative and quantitative assessment of total RNA by NanoDrop

To estimate the quantity and quality (in terms of protein and DNA contamination) of isolated RNA, spectrophotometry was performed and the data was analyzed using software N.D. (V.3.3.0). One microliter RNA sample was loaded into the well of Nanodrop spectrophotometer (Thermo Scientific, U.S.A.) and the concentration and purity of the total extracted RNA was determined as the 260/280 nm and 260/230 nm ratio automatically calculated by the software. Only RNA samples with A260/A280 and A260/A230 ratios between 1.8 and 2.2 and between 2 and 2.2, respectively, were subsequently used for the cDNA synthesis.

3.3.3 Complementary DNA synthesis

cDNA synthesis was carried out by using Revert Aid H Minus First stand cDNA synthesis kit (Fermentas, UK) as per the instructions given by the manufacturer –

Component	Volume
Total RNA (100 ng to 5 µg)	1 µL
Oligo dT Primer (100 µM)	1 µL
RNase free water	10 µL
Total volume	12 µL

The above component was mixed in tube, and kept at 65°C for 5 min. After that take out the tube, spin it and immediately transfer to ice. Further, following components were added for initiating the reaction -

Component	Volume
5X Buffer	4 µL
Reverse Transcriptase (200 U/µL)	1 µL
dNTP Mix (10 mM)	2 µL
RibolockRNase Inhibitor (20 U/µL	1 µL
Total volume	20 µL

Mix all the component and incubate the mixture at 42°C for 1 h and terminate the reaction at 72°C for 5 min.

3.3.4 Primer designing

The transcript of MAPK identified for the NGS data mining based on the Digital gene expression were targeted for the cloning. The transcript sequence was subject to clustal W2 alignment and conserved region was used for the oligo designing. Forward and Reverse primers were designed using Genefisher2 primer designing software (http://bibiserv.techfak.uni-bielefeld.de/genefisher2/), and quality was checked using Oligo Analyzer (Integrated DNA Technologies, USA).

Code	**Primer sequence (5'-3')**	**Tm (°C)**
MAPK-F	TAAAAAGGGCTCGGATAAGG	58.3
MAPK-R	CAGTGTGGTTCATCGATCTG	57.5

3.3.5 Reverse Transcriptase-PCR amplification of candidate MAPK gene

The reaction mixture was prepared for the RT-PCR by adding the following components-

Components	Concentration	Volume
cDNA	(200 ng/ µL)	1 µL
Forward primer	10 µM	1 µL
Reverse primer	10 µM	1 µL
dNTP	10 mM	2 µL
Taq polymerase	5U/µL	1 µL
Buffer	10X	5 µL
RNase free water		39 µL
Total volume		50 µL

Briefly spin the tube and set the following program for PCR

3.3.6 Steps of RT-PCR reaction

Step	**Temperature (°C)**	**Duration**
Initial Denaturation	95	3 min
Denaturation	94	30 sec
Annealing	59.3	30 sec
Extension	72	1 min
	Repeat step 2 to 4 for 35cycles	
Final Extension	72	10 min
Hold	4	∞

3.3.7 Visualization of amplified product on agarose gel

Prepare 1% agarose gel, by weighing 1 g of agarose and melt in 100 mL of 1X TBE, pH-7. Sample (50 µL) was mixed with 6X loading dye and was loaded onto gel. The gel was run at 80 V for 30 min, and visualized under UV transilluminator.

3.3.8 Purification of amplicon

Purification was carried out by PCR clean-up kit (Promega, UK) as per the protocol provided in the kit.

- Amplified amplicon band was excised from the gel.
- Weigh the gel slice and add required amount of membrane binding solution (10 µL membrane binding solution per 10 mg of gel).
- Incubate the gel in water bath at 65°C until the gel gets completely dissolved.
- Transfer melted gel into membrane binding column provided and centrifuge at 12000 rpm for 1 min.
- Discard the flow through and wash the column with 700 µL washing solution and centrifuge at 12000 rpm for 1 min.
- Add 500 µL washing solution for another wash and centrifuge at 12000 rpm for 5 min.
- Discard the flow through and centrifuge at 12000 rpm for 1 min. to dry the column.
- Add 20 µL pre warm nuclease free water and leave at room temperature for 5 min. and centrifuge at 12000 rpm for 2 min.
- Store the eluted product at -20°C.

3.4 Cloning and transformation

The eluted product was ligated into pGEM-T Easy vector (Promega, UK) using the T_4 DNA ligase enzyme along with the other component as described below

Component	**Concentration**	**Volume**
Buffer	2X	5 µL
pGEM-T Easy vector	50 ng/µL	1 µL
T_4 DNA Ligase enzyme	3 Weiss U/µL	1 µL
Insert DNA	150 ng	1 µL
Nuclease free water	-	2 µL
Total volume		10 µL

The reaction was incubated at 4°C temperature for overnight.

3.4.1 Transformation of ligated DNA into *E. coli* DH5α strain

Transformation was carried out as per the protocol described by Sambrook *et al.*, (1989) as follows:

- Thaw a tube of DH5α competent cells on ice for 10 min.
- Add 3 µL of ligated product to competent cells; mix gently by flicking the tube 4-5 times (do not vortex). Place the mixture on ice for 30 min. without mixing.
- Heat shock at 42°C for 30 seconds.
- Transfer tubes on ice for 5 min.
- Add 400 µL Luria Broth (LB) media to tubes.
- Place the tube at 37°C for 60 min. Shake vigorously the tubes.
- Pre-warm LB agar plates to 37°C.
- Spread 100 µL of the cells onto the plates with ampicillin antibiotic.
- Incubate at 37°C for overnight.

3.4.2 Preparation of Master plate

The transformed plates with white colonies were picked up and streaked on fresh agar plate with ampicillin antibiotic. A number was assigned to each colony on the LA/Amp plate. Each colony was cultured in LB broth and incubated at 37ºC for 8-10h.

3.4.3 Plasmid isolation

Plasmid was isolated by High yield plasmid mini kit (Real Genomics).

Method of isolation:

- Transfer 1.5 mL of bacterial culture to an eppendorf tube and centrifuge for 1 min. at optimum speed (10,000 rpm), discard the supernatant.
- Add 200 µL of PD1 Buffer (RNase A added) to the tube and resuspend the cell pellet by vortexing or pipetting.
- Add 200 µL of PD2 Buffer and mix gently by inverting the tube 10 times. Do not vortex, to avoid shearing genomic DNA.
- Keep it at room temperature for 2min. until lysate clears.

- Add 300 µL of PD3 Buffer and mix immediately by inverting the tube 10 times. Do not vortex.
- Centrifuge the tube for 3 min at 14,000 rpm.
- Place a PD column in a collection tube and apply the clear lysate (supernatant) to the PD column. Centrifuge it at 13000 rpm for 30 seconds.
- Discard the flow-through and place the PD column back in the collection tube.
- Add 400 µL of W1 buffer in the PD column, centrifuge at 13000 rpm for 30 seconds.
- Discard the flow-through and place the PD column back in the collection tube.
- Centrifuge again for 2 min. at 14000 rpm to dry the column matrix.
- Transfer dried PD column onto a clean micro centrifuge tube.
- Add 50 µL of elution buffer or nuclease free water into the centre of the column matrix. Stand for 2 min. until elution buffer is absorbed by the matrix.
- Centrifuge for 2 min. at full speed to elute purified DNA.

Restriction of plasmid DNA:

Restriction of plasmid DNA was carried out by fast digest *Eco*RI enzyme (Thermo Fisher Scientific, USA) as follows:

Component	Concentration	Volume
Buffer	(10X)	2 µL
Plasmid DNA	(200ng)	4 µL
*Eco*RI enzyme	5U/µL	1 µL
Nuclease free water		13 µL
Total volume		20 µL

Mix all the components in eppendorf tube and incubate it at 37°C for 20 min. Restriction was confirmed on 1% agarose gel

Sequencing of cloned gene by Sanger's Di-deoxy method

The cloned gene was sequenced by Sanger's Di-deoxy method using T7 and SP6 as forward and reverse primer.

***In-silico* characterization of cloned gene**

The sequenced genes were subjected to nucleotide based homology search using BLASTn tool of NCBI (http://blast.ncbi.nlm.nih.gov). Similarly BLASTp was also carried out to know the homology at protein level. The nucleotide sequence was used for the identification of open reading frame using ORF finder (http:// www.ncbi.nlm.nih.gov/gorf/gorf.htmL). The Expasy tool (http://www.expasy.org) was used to find out the amino acid sequence, composition of the protein was determined by Expasy ProtParam (http://web.expasy. Org /cgi-bin /protparam / prot pa -ram). The sequence of amino acid was used for the identification of the conserved domain using the tool of NCBI conserved domain search (http:// www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Further, the identification of phosphorylation sites and kinase specific phosphorylation sites were predicted using the NetPhosK. The nucleotide sequence was submitted to NCBI GenBank using Bankit tool of NCBI.

3.5 Expression analysis of cloned gene using quantitative RT-PCR

Expression level of MAPK gene transcripts was evaluated in different genotype of wheat viz. HD3059, Halna (thermotolerant) and HD2329, PBW550 (thermosusceptible) under differential heat stressed treatment at different reproductive stages.

3.5.1 RNA isolation and cDNA synthesis

Total RNA were isolated using Trizol method as mentioned in earlier section. The quality and quantity of isolated total RNA was checked using NanoDrop 2000.

3.5.2 Designing of primers for quantitative Real-Time PCR

Forward and reverse primers were designed using Genefisher2 primer designing software (http://bibiserv. techfak.uni-bielefeld.de/genefisher2). Further, the quality was checked using Oligo Analyzer (Integrated DNA Technologies, USA).

Code	Primer sequence (5'-3')	Tm (°C)
qMAPK-F	GAAATCGGTGCTAAGGGTGA	60
qMAPK-R	CGTCACCGATGTGTAACCTG	60

3.5.3 Quantitative Real-Time PCR

For the expression analysis, qRT-PCR was performed using the Kappa SYBR Fast qPCR master mix kit on the CFX96 platform (Bio Rad, UK). The cDNA template (100 ng/ µL) was uniformly taken for the expression analysis. Reaction mixture was prepared with the following components-

Component	Concentration	Volume for single reaction
KAPA SYBR Fast qPCR master mix	2X	10 µL
Forward primer	200 nM	0.4 µL
Reverse primer	200 nM	0.4 µL
cDNA	100 ng	1 µL
DEPC-treated water		8.2 µL
Total volume		20 µL

Reactions were run in Bio Rad qRT-PCR CFX96 platform (Bio Rad, UK) using following standard program:

Step	Temperature(°C)	Duration
Initial denaturation	95	3 min
Denaturation	95	10 sec
Annealing	60	30 sec
	Plate read and repeat step 2 to 3 for 40 cycles	
Melt curve	55-95 with increment of 1ºC for every 5 sec followed by plate read	1min
Hold	4	∞

Wheat β-actin gene was used as endogenous control gene for normalizing the Ct value. The relative expression was calculated by the Pfaffl method (Pfaffl, 2002).

3.5.4 Relative quantification

Quantification of gene was achieved using the Ct (Cycle Threshold) comparative method and is expressed as ‘‘n-fold up or down regulation of transcription’’ in relation to a calibrator which is represented by the smallest signal detectable for that specific gene. For relative quantification by the comparative Ct method, values are expressed relative to a reference sample, called the calibrator (0 hour sample). The expression of selected genes was calibrated by that of the reference gene, β-actin at each time point and converted to the relative expression ratio (fold of expression).

$$\text{Fold of Expression} = 2^{-\Delta\Delta CT}$$

Where,

$\Delta\Delta C_t$ = Average ΔCt of target - Average ΔCt of calibrator

ΔC_t = Average C_T of target - Average C_T of endogenous control.

Analysis of gene expression

C_t values data analyzed by CFX software v3.0 designed to perform relative quantification using comparative C_t ($\Delta\Delta C_t$) method by Bio-Rad for relative gene expression of target genes. Algorithm of this model is one of the features of the CFX 3.0 software provided by Bio-Rad, thus this bioinformatics tool was used for relative quantification.

Detailed mathematical explanation of ΔΔCt data analysis method:

Comparative C_t (threshold cycle) method is a gene quantitation approach. This involves comparing the C_t values of the samples of interest with a control or calibrator such as a non-treated sample or RNA from normal tissue. The C_t values of both the calibrator and the samples of interest are normalized to an appropriate endogenous housekeeping gene for Actin for transcripts. The comparative C_t method is also known as the $2^{-\Delta\Delta C_t}$ method, where

$$\Delta\Delta C_t = \Delta C_t \text{ sample} - \Delta C_t \text{ reference}$$

3.6 Estimation of biochemical parameters

3.6.1 Estimation of total antioxidant capacity

The thermal characteristics of all the four wheat cultivars exposed to heat stress are correlated with their antioxidant capacity. The FRAP (ferric reducing ability of plasma) method described by Benzie and Strain (1999) was followed for estimation of total antioxidant capacity. This method is based on the reduction of a ferric-tripyridyltriazine complex to its ferrous colored form in the presence of antioxidants. The values were expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1mmol L^{-1} $FeSO_4$.

3.6.2 Estimation of free amino acids

The total free amino acid content was determined by the ninhydrin test, according to the method of Sircelj *et al*., (2005). A known weight of dry powdered plant material of each sample was extracted in 80% ethanol and centrifuged at 8,000x g for 15 min. The supernatant containing the alcohol-soluble fraction was collected. A reaction mixture, consisting of 0.1 mL extract, 0.9 mL of double-distilled water and 1mL of ninhydrin reagent was shaken vigorously and then heated in a boiling water bath for about 20 min. and added to 5mL diluents (equal volumes of water and n-propanol). After cooling the tubes to room temperature, the colour intensity was measured at 570 nm. The blank set was prepared by adding 0.1 mL of 80% ethanol in place of sample extract. The standard curve was prepared by using leucine as a standard amino acid. The results were expressed in terms of mg of amino acid per g of dry tissue.

3.6.3 Catalase activity assay

Activities of catalase enzyme were measured as described by Chance and Maehly, (1955). Fresh leaf material (1 g) was crushed in 5 mL of ice-cold 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM sodium-EDTA and 1% (w/v) polyvinyl-pyrrolidone (PVP). The homogenates were centrifuged at 10,000 g (4°C) for 10 min. The tissue extracts were used for the quantification of soluble protein content by using Bradford method and analysis of catalase activity. Catalase activity was measured in a reaction mixture (3 mL) containing 100 mM Na_2HPO_4 buffer pH 6.8 (2 mL), 30 mM H_2O_2 (0.5 mL) and 0.5 mL enzyme. For assaying CAT activity,

the decomposition of H_2O_2 was followed by decline in the absorbance at 240 nm as catalase enzyme catalyzes the reaction

3.6.4 Guaiacol peroxidase (GPX) activity assay

The samples (control and heat stressed sample) collected from wheat cultivars HD2329, Halna, PBW550 and HD3059 were used for the guaiacol-peroxidase (GPX) activity assay following the method of Evers *et al.*, (1994) with slight modification. Fresh leaf material (1 g) was crushed in 5 mL of ice-cold 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM sodium-EDTA and 1% (w/v) polyvinyl-pyrrolidone (PVP). The homogenates were centrifuged at 10,000 g (4°C) for 10 min. The tissue extracts were used for the quantification of soluble protein content and analysis of peroxidase activity. Coomassie blue dye-binding assay was used for protein estimation and bovine serum albumin (BSA) for standard curve preparation. The oxidation of guaiacol into tetraguaiacol was estimated by measuring the absorbance at 470 nm against the reagent blank, using extinction coefficient of 26.6 mM^{-1} cm^{-1}.

3.6.5 Superoxide dismutase activity (SOD) assay

The samples (control and HS-treated) collected from wheat *cvs*. HD2329, HD3059, PBW550 and Halna were used for the SOD activity assay based on the pyrogallol autoxidation method. Briefly, reaction medium was prepared using the 50 mM Tris-Cl buffer with 1 mM EDTA (pH 8.2). Crude extract was added to 0.2 mM pyrogallol (dissolved in pH 6.5, 50 mM PPB) to initiate the reaction, and the absorbance decrease of pyrogallol was monitored at 420 nm. The percentage inhibition of pyrogallol autoxidation was calculated by the following formula:

% inhibition of pyrogallol autoxidation= $[1-(\Delta A/\Delta A_{max})] \times 100$, where

ΔA= Absorbance change due to pyrogallol autoxidation in the sample reaction system

ΔA_{max} = Absorbance change due to pyrogallol autoxidation in the control

3.6.6 Estimation of proline content

Free proline content estimation in the leaves was determined by following the protocol of Bates et al. Leaf samples (0.1 g) were homogenized in 2.5 mL of sulphosalycylic acid (3%) using mortar and pestle and centrifuged at 12000 rpm.

About 1 mL of extract was taken in test tube and to it 2 mL of glacial acetic acid and 2 mL of acidic ninhydrin reagent were added. The reaction mixture was boiled in water bath at 100 °C for 1hr. After cooling the reaction mixture, 4 mL of toluene was added and vortexing was done. After thorough mixing, the chromophore containing toluene was separated and absorbance read at 520 nm in spectrophotometer against toluene blank. Concentration of proline was estimated by referring to a standard curve of proline.

3.6.7 Estimation of Reactive Oxygen Species

Hydrogen peroxide content (H_2O_2) was estimated by estimating the absorbance of titanium hydroperoxide complex. Leaf samples (0.5 g) were homogenized in 10 mL of cold acetone. The homogenate was filtered through Whatmann No.1 filter paper. To whole of the extract 4 mL of titanium reagent was added followed by 5 mL of concentrated ammonium solution to precipitate hydroperoxide-titanium complex. After centrifugation for 5 min. at 10000 rpm the supernatant was discarded and precipitate was dissolved in 1 M sulphuric acid. It was re-centrifuged to remove undissolved material and absorbance was recorded at 415 nm against blank. Concentration of H_2O_2 was determined using standard curve plotted with known concentration of H_2O_2.

4.1. Identification of transcripts predicted to be Mitogen Activated Protein Kinase

Whole transcriptome sequencing of wheat was carried out in our lab for the identification of novel heat-responsive genes at grain-filling stage using *de novo* assembly. The raw data generated using Next-Generation Sequencing (NGS) of control and HS-treated samples of HD2985 and HD2329 *cvs.* of wheat (NCBI BioProject Database: PRJNA171754) was assembled using Velvet and Oases v 2.0, and was mined for the identification of novel MAPK transcripts based on the domain search and homology with MAPK gene. Differentially expressed genes were functionally annotated using the available databases as NCBI non-redundant, TriFLDB and classified into different categories according to their putative functions generated by Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis. We observed 40 novel transcripts showing homology with MAPK gene.

4.2. Molecular cloning and *in-silico* characterization of candidate MAPK gene

The identified MAPK transcripts mined from the NGS data were characterized for their Digital gene expression (DGE). Based on the DGE, transcript_MAPK was targeted for the cloning. Transcript-specific forward and reverse primers were used along with cDNA template synthesized from the isolated RNA from HD3059 *cv*. in HS condition at 38°C for 2 h (T2). RT-PCR showed amplification of ~1.3 Kb, as visualised on 0.8% agarose gel (Fig. 1). The amplicon was excised from the gel and purified using PCR clean-up kit before being used for the ligation and transformation. The purified amplicon was ligated in pGEMT-Easy vector (Fig. 2) and transformed using *E. coli* DH5α strain. The positive transformants (white colonies) were used for the preparation of master-plate by streaking on to LA/Amp plate (Fig. 3). The plasmids were isolated from the positive bacterial transformants by Rapid Alkaline Lysis method. The plasmids were subjected to restriction analysis (RE) in order to check the release of the target MAPK gene. We observed release of ~1.3 kb fragment from the plasmid on 0.8% agarose gel (Fig. 4). The isolated plasmid showing release of target gene was sent for the sequencing by Sanger's di-deoxy method using universal primers of T7 and SP6. Sequencing followed by

curing showed the presence of 1333 nucleotides in the amplified sequence. BLASTn (NCBI) search showed maximum homology with transcript cloned from *Hordelum vulgare* (acc. no. AK353742). BLASTp analysis showed maximum (100%) homology with protein of MAPK gene reported from *Zea mays* (acc. no. NP_001167676). The gene sequence was submitted in National Centre for Biotechnology Information (NCBI) GenBank with acc. no. KT835664 (Fig. 5).

Open reading frame was predicted using ORF finder (NCBI). A total of 6 ORF were predicted, longest ORF was of 1110 bp which encodes for 314 amino acids (Fig. 6). Conserved domain search showed the presence of Serine/Threonine kinase which is conserved functional domain of MAPK family (Fig. 7). Phosphorylation sites in protein of cloned MAPK gene was predicted by NetPhosK software (Fig. 8). Proteins of this family perform diverse cellular functions. Phylogenetic relationship of candidate MAPK gene is analysed through ClustalW software. Cloned MAPK gene show maximum homology with *Hordeum vulgare* (acc. No: AK353742) (Fig. 9). Protein was modelled based on the SWISS-MODEL template library. The target sequence was searched with BLAST against the primary amino acid sequence. A total of 500 templates were found and template with highest quality was used for 3-D modelling of protein (Fig. 10).

4.3. Expression profiling of cloned MAPK gene at grain-filling stage under heat stress

Two thermotolerant (Halna and HD3059) and two thermosusceptible (HD2329 and PBW550) wheat *cvs.* were treated in differential condition of heat stress at mealy-ripe stage. RNA was isolated from all the four wheat *cvs.* under control and HS condition of 30°C for 2 h (T1) and 38°C for 2 h (T2) (Fig. 11) and expression analysis was done using quantitative Real-Time PCR (qRT-PCR). Relative expression of putative MAPK gene transcripts was observed maximum at 38ºC in all the *cvs.* percent increase was observed significantly higher in thermotolerant *cvs.* (Halna and HD3059), as compared to thermosusceptible *cvs.* (HD2329 and PBW 550). Halna showed relative expression of 2.1 and 4.1 in response to HS of 30ºC for 2 h (T1) and 38ºC for 2 h (T2) respectively as compared to control conditions. Similarly, HD3059 showed 1.98 fold increase in the expression under HS of 30ºC for 2 h (T1) ,which further up-regulated to 3.45 in response to HS of 38ºC for 2 h (T2). In HD2329, the relative fold expression was observed 1.45 increase in

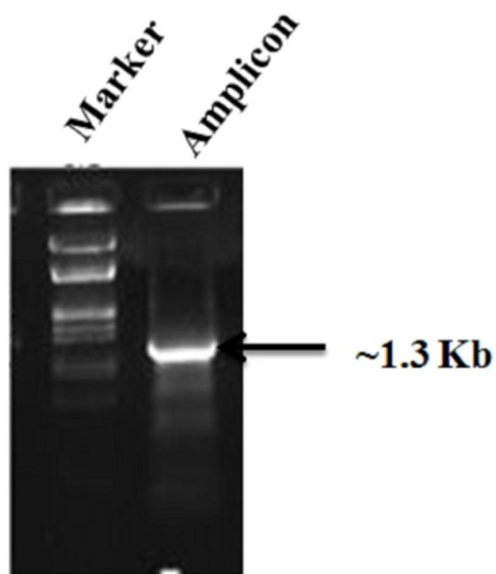


Figure 1: PCR amplification of putative MAPK gene from wheat *cv.* HD3059

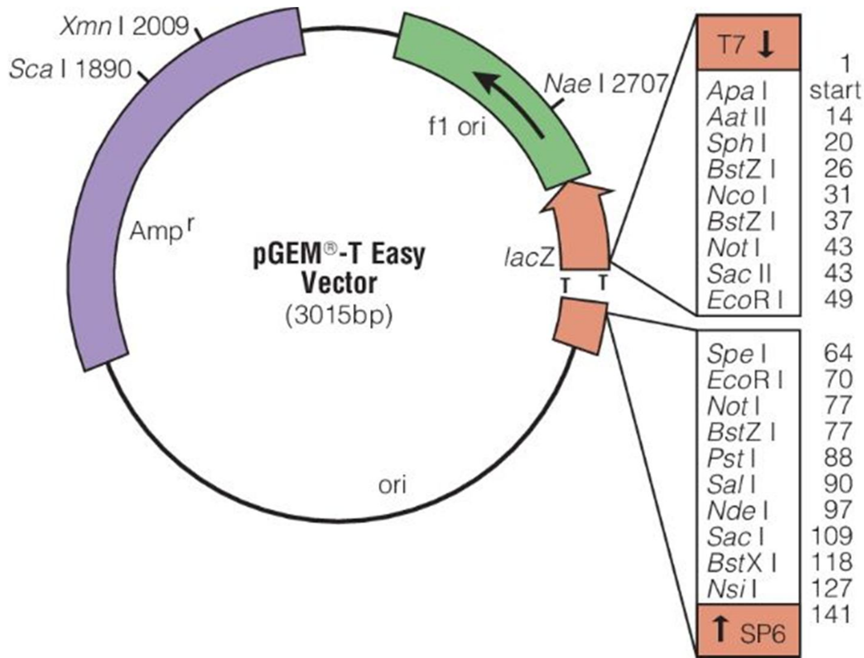


Figure 2: Restriction map of pGEM-T cloning vector

Positive Transformants

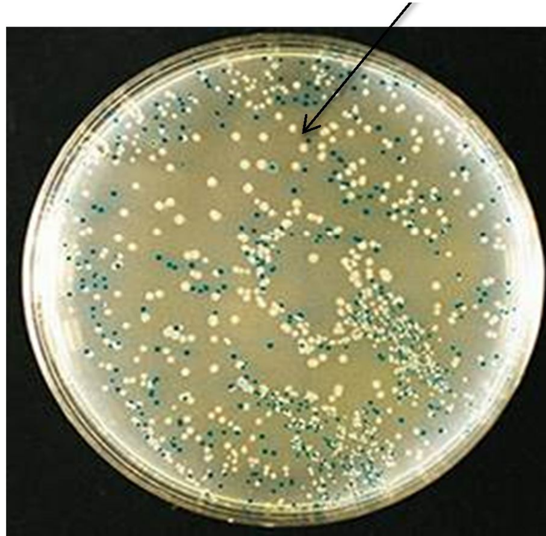


Figure 3: Blue-White screening of transformed clone

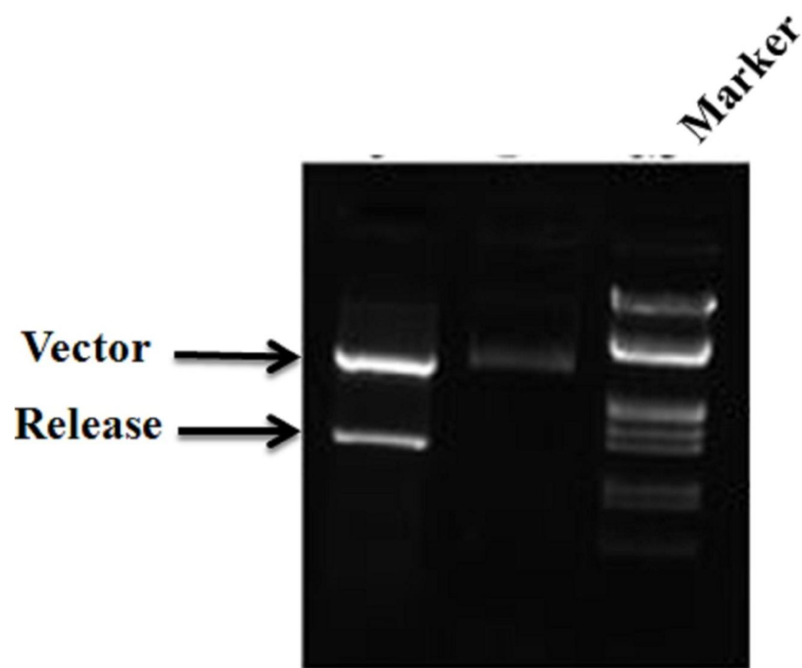


Figure 4: Restriction analysis of isolated Plasmid from positive transformed colonies (white colonies) with MAPK pGEM-T Easy construct.

5'-ATTCGAAAATGGCAATGCTGGTGGATCCTCCGAATGGCATGGGAAACCAAGGGAAGCACTACTACTCAA
TGTGGCAAACCTTGTTTGAGATTGACACCAAGTATGTGCCTATCAAGCCCATTGGCCGAGGAGCTTATGG
AATAGTTTGCTCATCCATAAACCGTGAGACAAACGAGAAAGTAGCGATAAAGAAGATACATAATGTATTC
GACAACCGTGTGGATGCACTAAGGACCTTGCGGGAGCTGAAACTCCTCCGGCATCTCCGCCATGAGAATG
TTATTTCTTTGAAGGATATAATGATGCCTGTACAAAGGAGGAGCTTTAAGGATGTGTACTTGGTTTATGA
GCTCATGGATACAGACCTGCATCAGATAATCAAATCGCCTCAGGGGCTTTCCAACGACCACTGCCAATAT
TTTCTTTTTCAGTTGCTTCGAGGACTGAAATACCTCCATTCAGCAGAGATACTCCACAGAGACCTAAAAC
CTGGGAACCTACTGGTGAATGCAAACTGTGATCTGAAGATATGTGATTTTGGTCTTGCACGTACAAACAG
TAGTAAAGGCCAGTTTATGACTGAATACGTCGTCACCCGCTGGTATAGGGCTCCTGAATTGCTGCTTTGC
TGTGACAACTACGGCACTTCCATCGATGTTTGGTCTGTTGGCTGCATCTTTGCTGAGCTACTTGGCCGCA
AGCCTATTTTTCCCGGGACAGAGTGCCTGAATCAGCTAAAACTGATAGTCAATGTTCTTGGCACCATGAG
CGAGTCTGACCTGGAGTTCATCGACAACCCGAAGGCTCGCAGATATATCAAGACCCTTCCCTACACTCCT
GGTGTTCCACTCGCAAGTATGTACCCACATGCGCACCCTCTTGCCATCGATCTGTTACAGAAGATGCTCA
TCTTCGATCCTACCAAAAGGATCAGTGTTACCCAGGCCCTTGAGCACCCTTACATGTCTCCTCTGTATGA
CCCAAGTGCAAACCCTCCCGCACAAGTGCCCATCGATCTCGACATAGATGAGAACATCAGCTCAGAGATG
ATCAGGGAAATGATGTGGCAGGAGATGCTTCACTACCATCCTGAAGCCGCAGCTGCAGTAAACATGTGAT
GATGTTCTTGCAGTGCCCCGGGAAGAACCCGGCAGGCTCACTACTTTTTTCCTCGAAAAGACTACTGCGA
TTATCGCACCTATTAAGTAACCACGACGTGCAGTGTGGAGAGCTATCTCCGTGTAAATATGCAGTGCGAT
AAGAACCGCATGGATAGTTCTTGTTATGGACCACTATTCGGTGTATGTATACTGTTGTGTTGTTGTATGA
GCTCATGAAAGAACTGTTGAAGCGA-3'

Figure 5: Nucleotide sequence of the cloned putative MAPK gene from wheat *cv*. HD3059

MAMLVDPPNGMGNQGKHYYSMWQTLFEIDTKYVPIKPIGRGAYGIVC
SSINRETNEKVAIKKIHNVFDNRVDALRTLRELKLLRHLRHENVISLKDI
MMPVQRRSFKDVYLVYELMDTDLHQIIKSPQGLSNDHCQYFLFQLLR
GLKYLHSAEILHRDLKPGNLLVNANCDLKICDFGLARTNSSKGQFMTE
YVVTRWYRAPELLLCCDNYGTSIDVWSVGCIFAELLGRKPIFPGTECL
NQLKLIVNVLGTMSESDLEFIDNPKARRYIKTLPYTPGVPLASMYPHAH
PLAIDLLQKMLIFDPTKRISVTQALEHPYMSPLYDPSANPPAQVPIDLDI
DENISSEMIREMMWQEMLHYHPEAAAAVNM

Figure 6: Amino acid sequence in protein of the cloned putative MAPK gene from wheat *cv*. HD3059

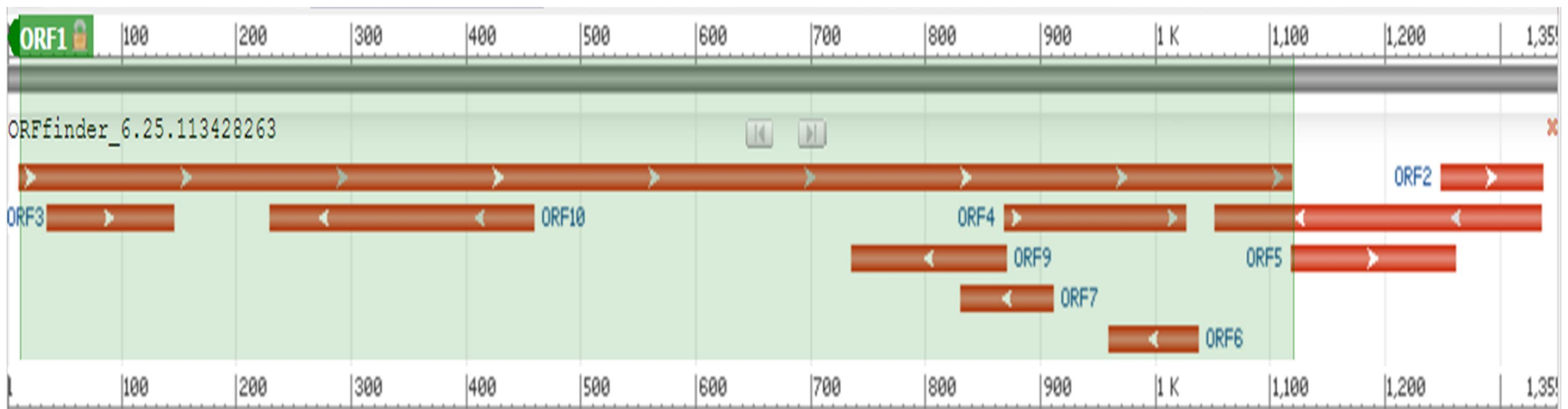


Figure 7: Predicted Open Reading Frame (ORF) in putative MAPK gene cloned from wheat *cv.* HD3059 using ORF Finder tool of NCBI (http://www.ncbi.nlm.nih.gov/orf finder/)

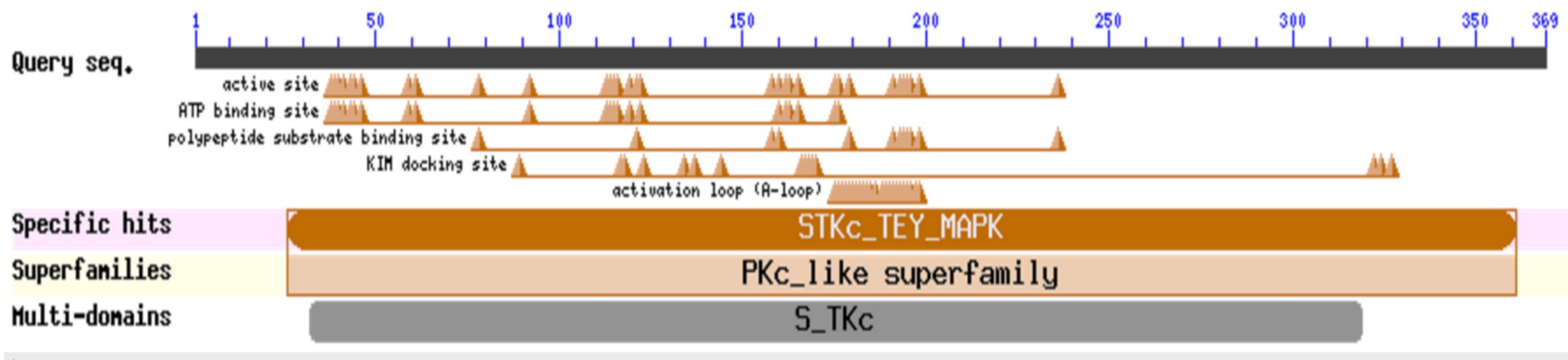


Figure 8: Conserved domain prediction in protein of putative MAPK gene cloned from wheat *cv.* HD3059 using Conserved Domain search tool of NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi)

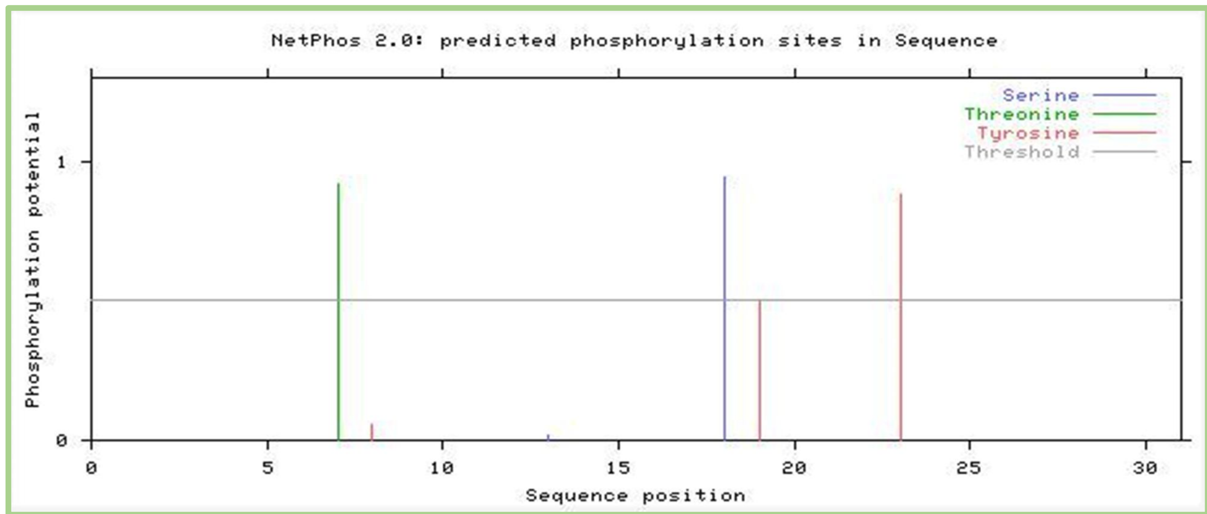


Figure 9: Predicted phosphorylation site on protein of putative MAPK gene cloned from wheat *cv.* HD3059 using NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/)

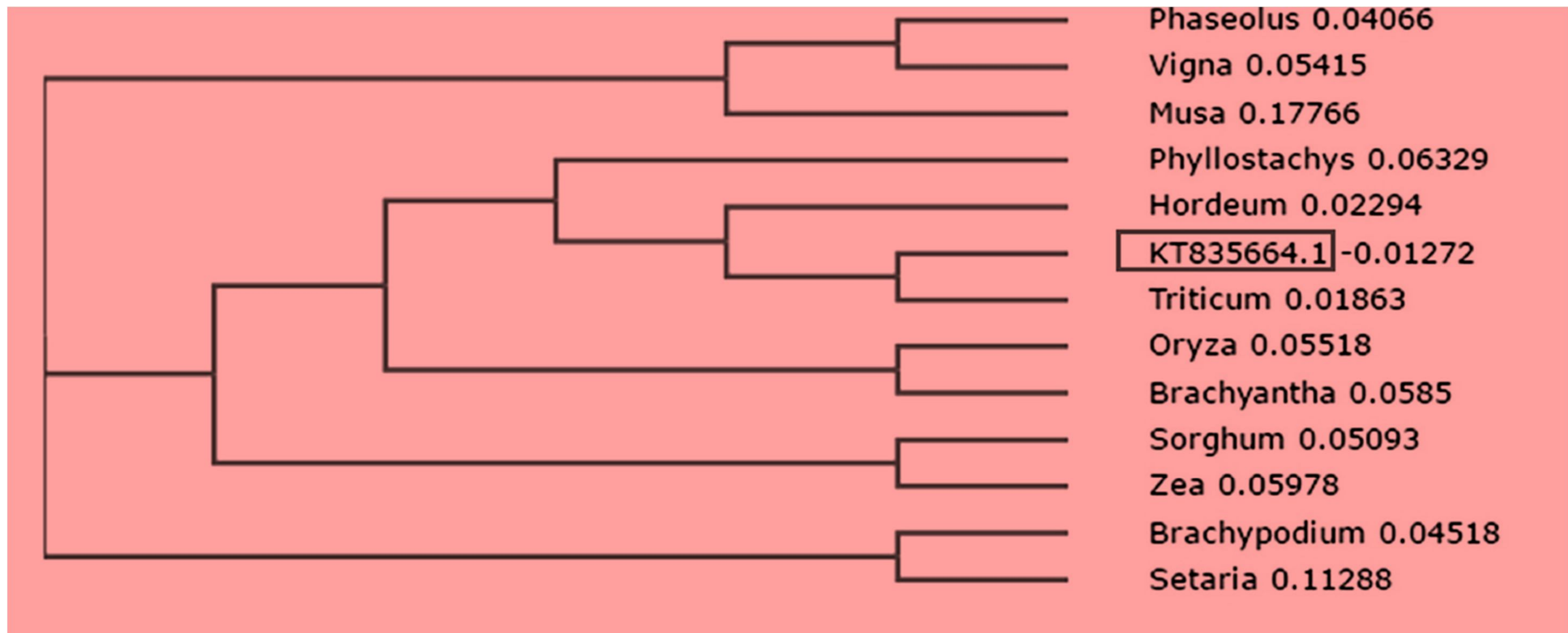


Figure 10: Phylogenetic tree of putative MAPK gene cloned from wheat *cv.* HD3059

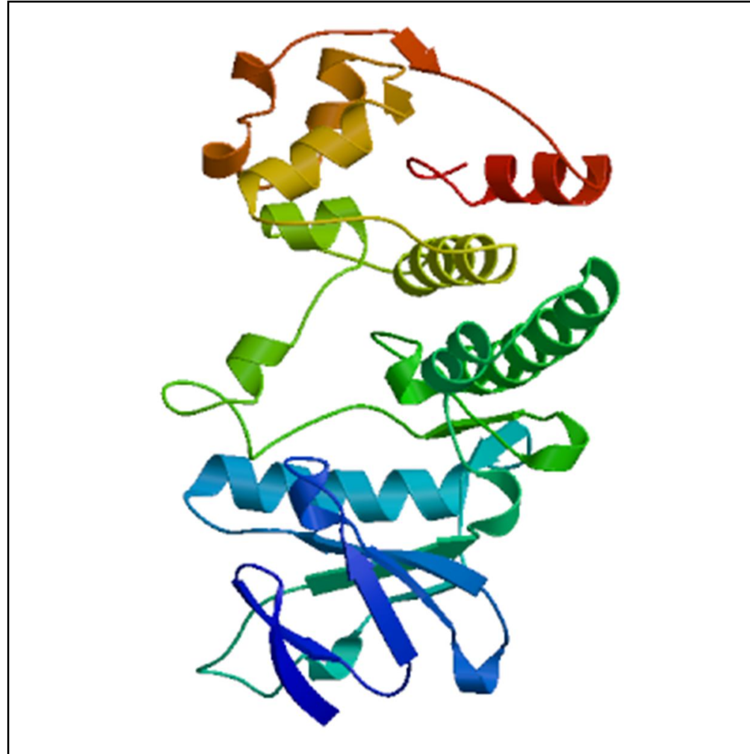


Figure 11: Predicted protein model of putative cloned MAPK gene from wheat cultivar HD3059; predicted using SWISS-MODEL server (http://swissmodel.expasy.org/)

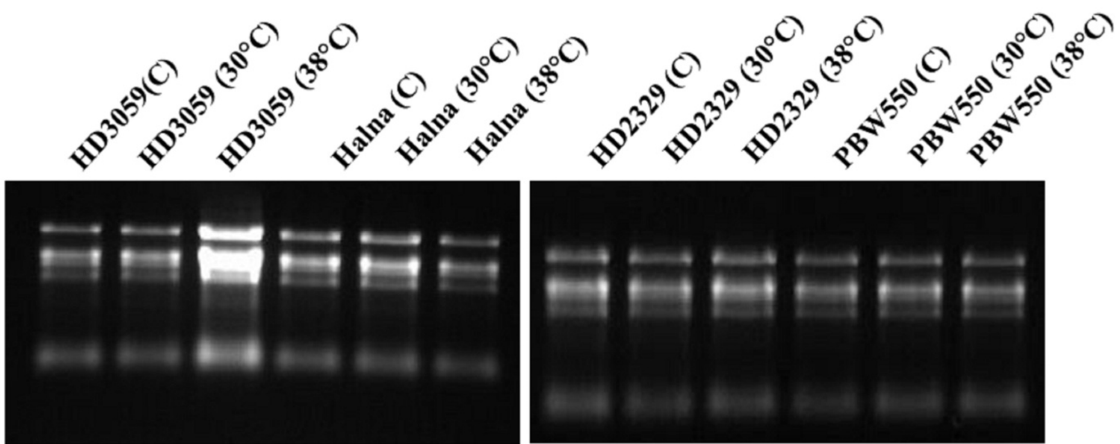


Figure 12: Denaturing agarose gel image of total RNA isolated from leaves of two thermotolerant *cvs*. (HD3059, Halna) and two thermosusceptible *cvs.* (HD2329, PBW550) of wheat exposed to differential heat treatment

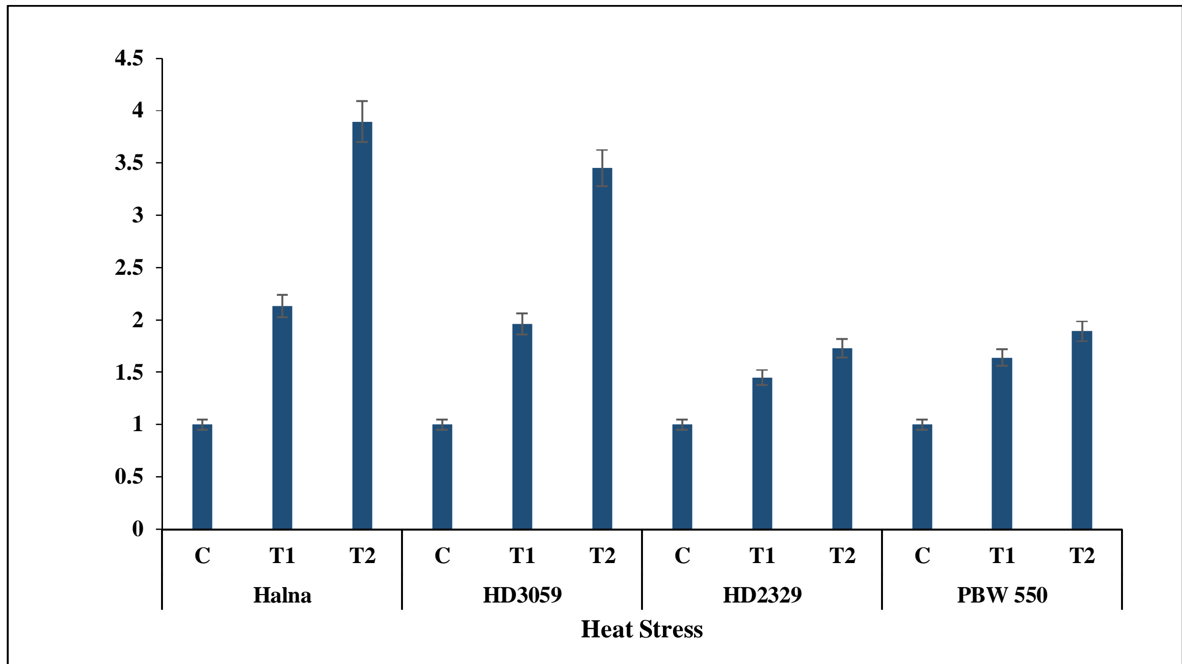


Figure 12(a): Expression profiling of putative MAPK gene transcripts in wheat *cvs.* Halna and HD3059 and thermosusceptible wheat *cvs.* HD2329 and PBW550 of wheat under differential heat stress at grain-filling stage; C-22℃; T1- heat stress of 30℃ for 2 h, T2- heat stress for 38℃ for 2 h; β-actin gene was used as endogenous gene control; relative fold express was calculated by Pfaffl method (Pfaffl, 2002).

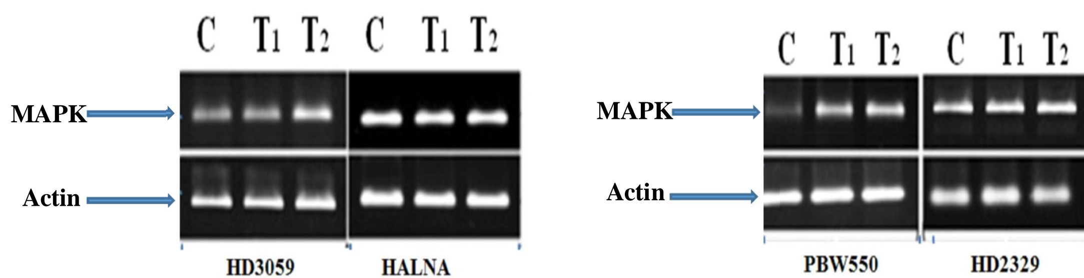


Figure 12(b): Semi quantitative-PCR of putative MAPK gene transcripts in thermotolerant wheat *cvs.* HD3059, Halna and thermosusceptible wheat *cvs.* PBW550 and HD2329 of wheat under differential heat stress at grain-filling stage; C-22℃; T1- heat stress of 30℃ for 2 h, T2- heat stress for 38℃ for 2 h.

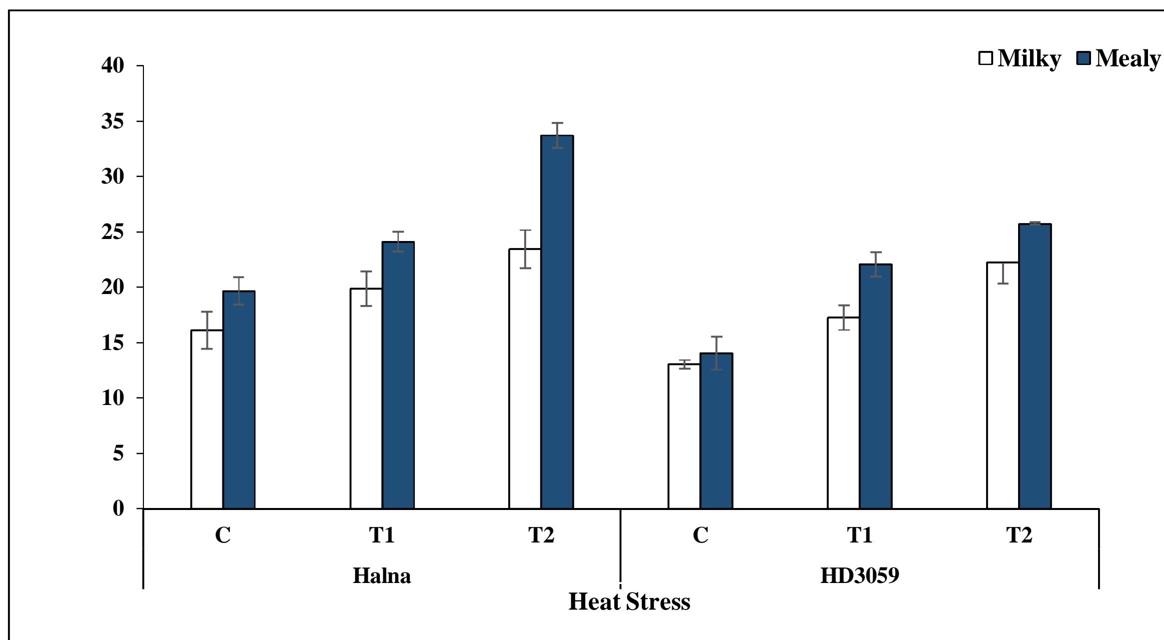


Figure 14(a): Estimation of Total Antioxidant Capacity (TAC) in thermotolerant wheat *cvs.* Halna and HD3059 at milky-ripe and mealy-ripe stage under differential heat stress treatment ; C-22℃; T1- heat stress of 30℃ for 2 h, T2- heat stress for 38℃ for 2 h.

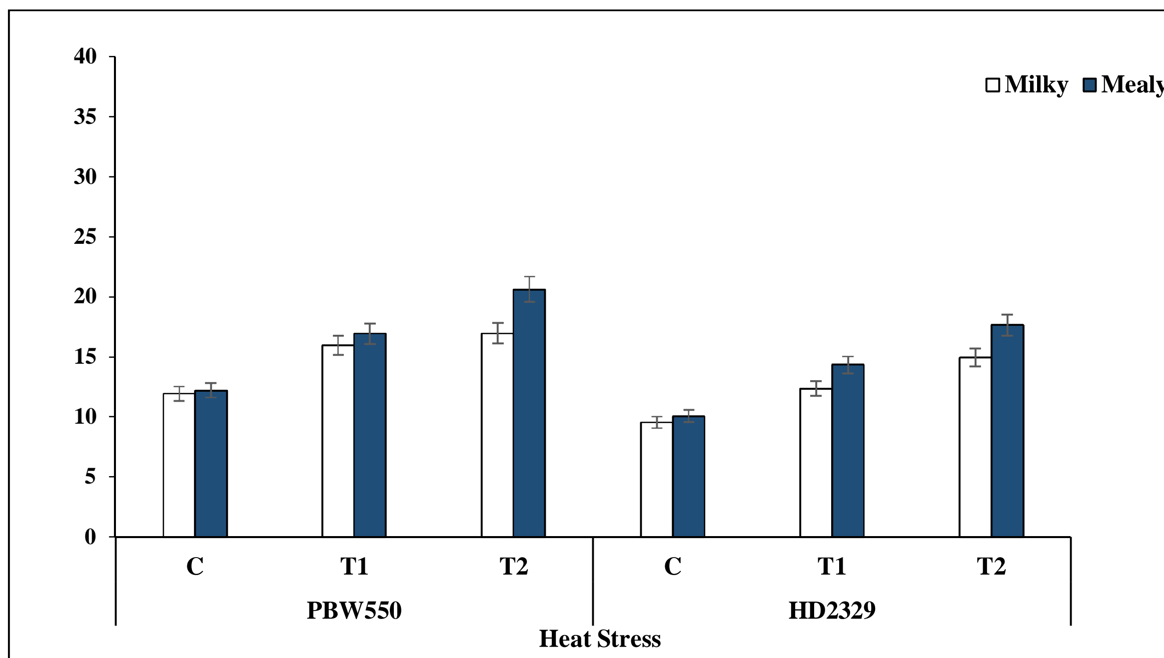


Figure 14(b): Estimation of Total Antioxidant Capacity (TAC) in thermosusceptible wheat *cvs.* HD2329 and PBW550 of wheat at milky-ripe and mealy-ripe stage under differential heat stress treatment; C-22℃; T1- heat stress of 30℃ for 2 h, T2- heat stress for 38℃ for 2 h.

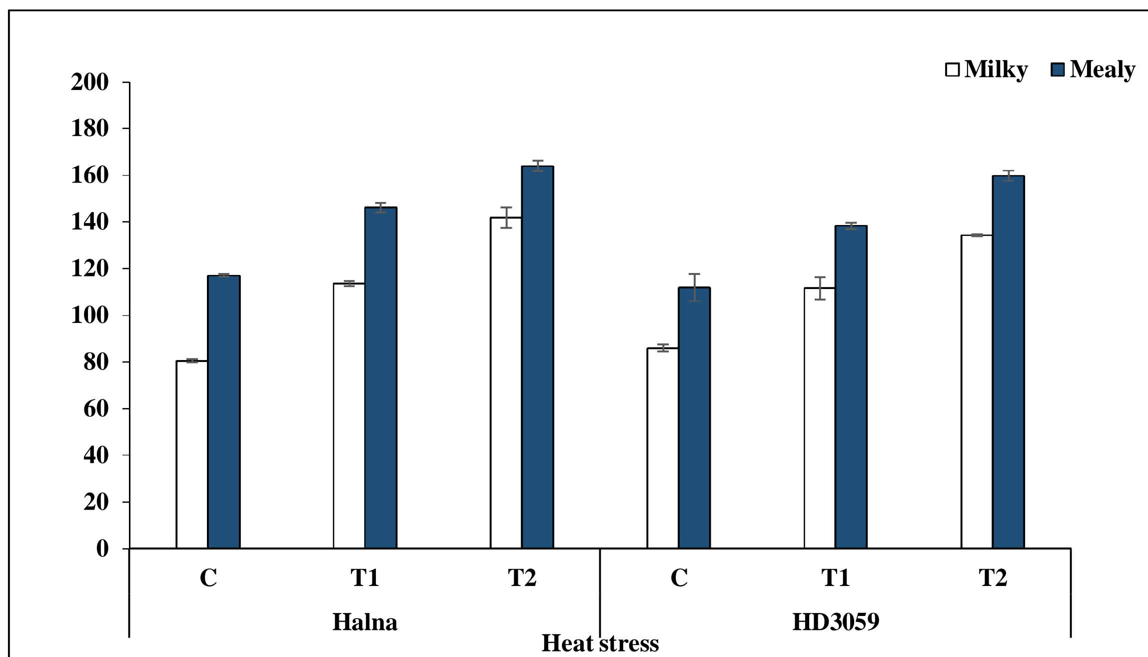


Figure 15(a): Guaiacol peroxidise (GPX) activity assay in thermotolerant wheat *cvs.* Halna and HD3059 at milky-ripe and mealy-ripe stage under differential heat stress treatment; C-22℃; T1- heat stress of 30℃ for 2 h, T2- heat stress for 38℃ for 2 h.

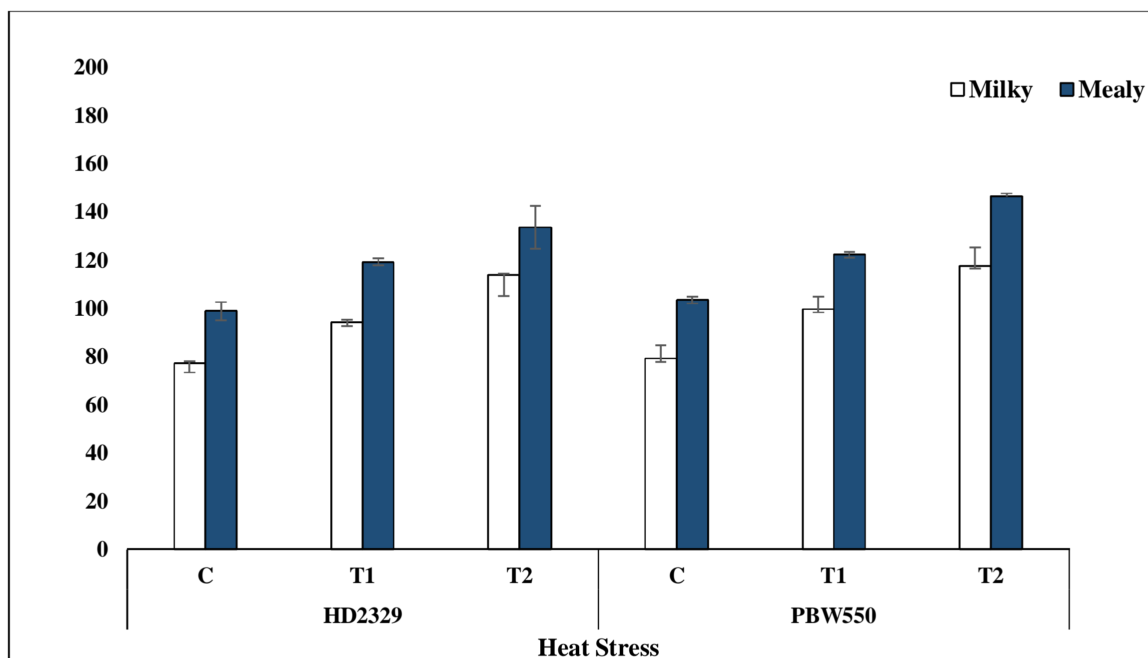


Figure 15(b): Guaiacol peroxidase (GPX) activity assay in thermosusceptible wheat *cvs.* HD2329 and PBW550 of wheat at milky-ripe and mealy-ripe stage under differential heat stress treatment; C-22℃; T1- heat stress of 30℃ for 2 h, T2- heat stress for 38℃ for 2 h.

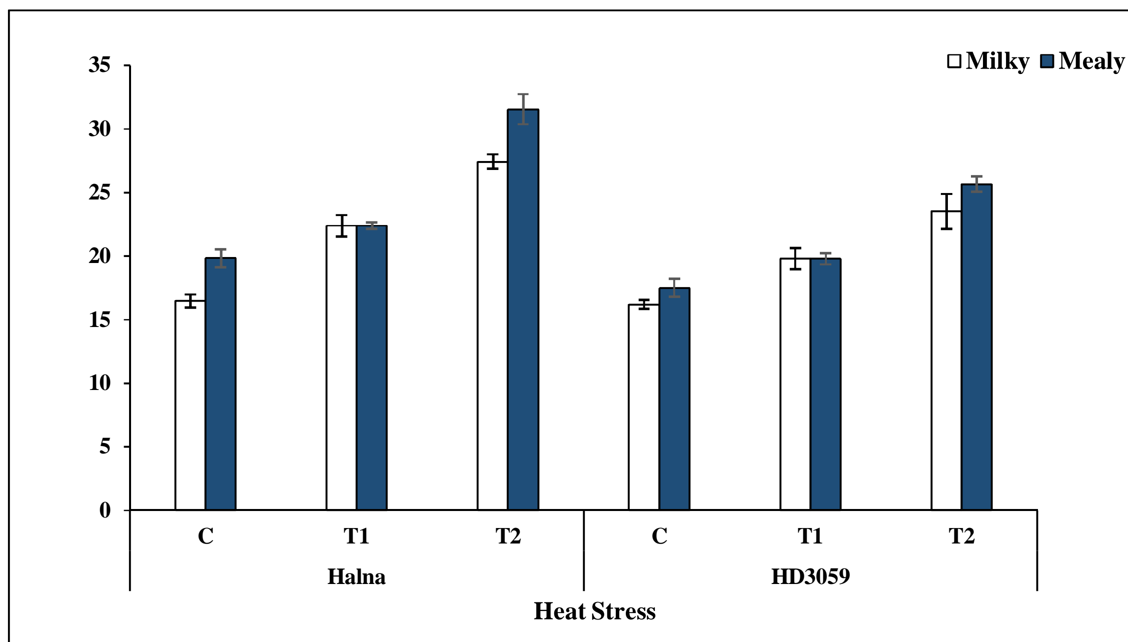


Figure 16(a): Superoxide dismutase (SOD) activity assay in thermotolerant wheat *cvs.* Halna and HD3059 at milky-ripe and mealy-ripe stage under differential heat stress treatment ; C-22ºC; T1- heat stress of 30ºC for 2 h, T2- heat stress for 38ºC for 2 h.

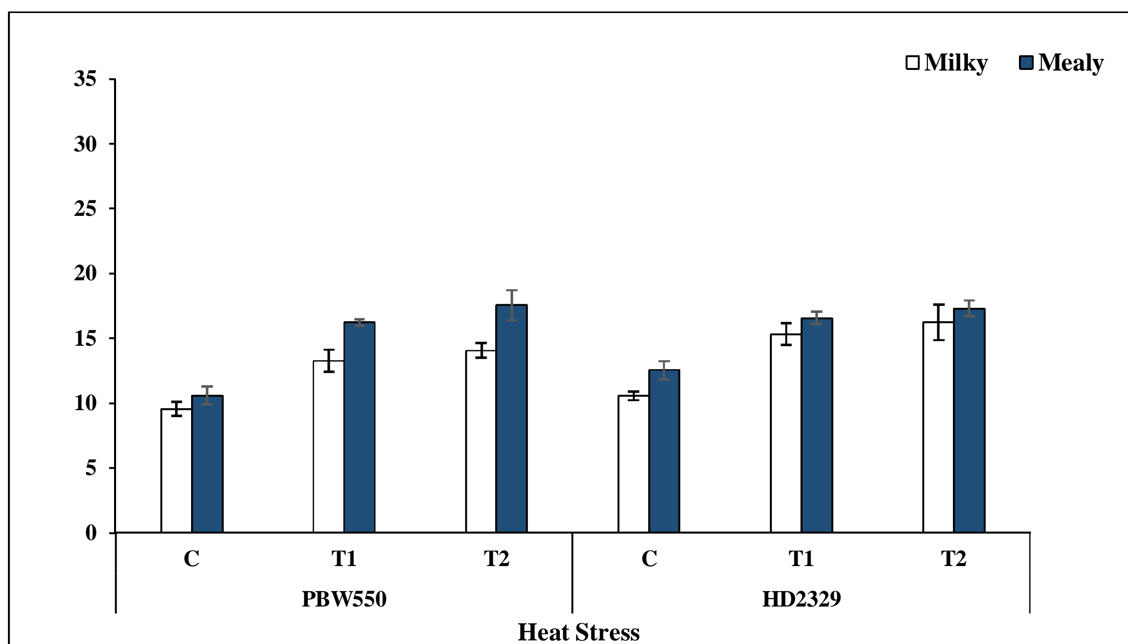


Figure 16(b): Superoxide dismutase (SOD) activity assay in thermosusceptible wheat *cvs.* PBW550 and HD2329 at milky-ripe and mealy-ripe stage under differential heat stress treatment ; C-22ºC; T1- heat stress of 30ºC for 2 h, T2- heat stress for 38ºC for 2 h.

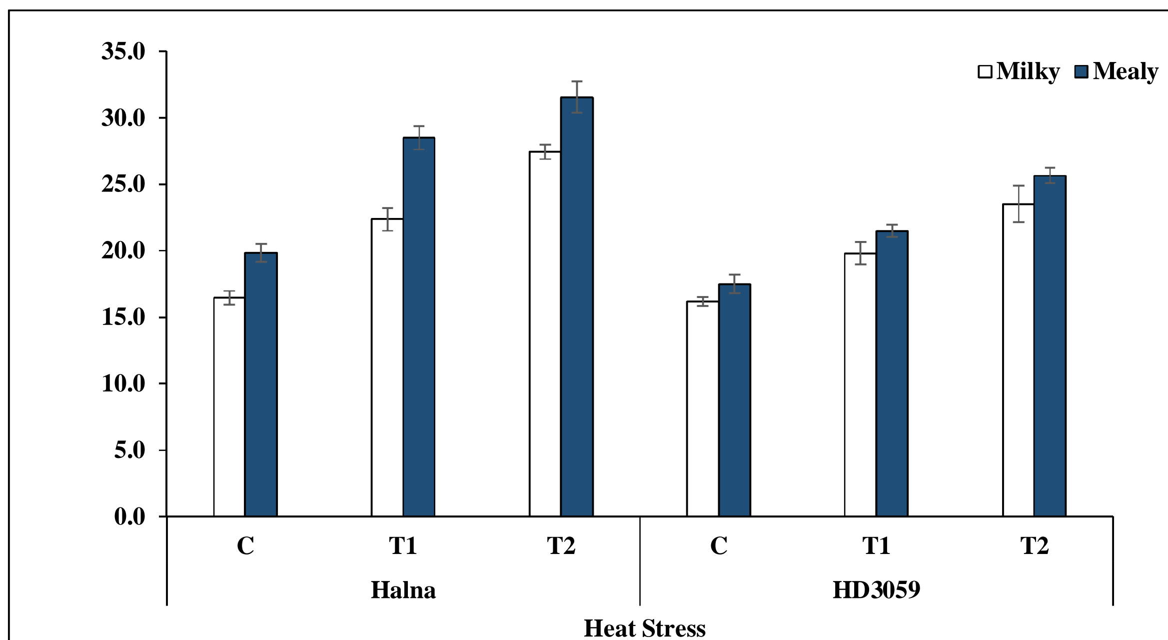


Figure 17(a): Catalase activity assay in thermotolerant wheat *cvs.* Halna and HD3059 at milky-ripe and mealy-ripe stage under differential heat stress treatment ; C-22ºC; T1- heat stress of 30ºC for 2 h, T2- heat stress for 38ºC for 2 h.

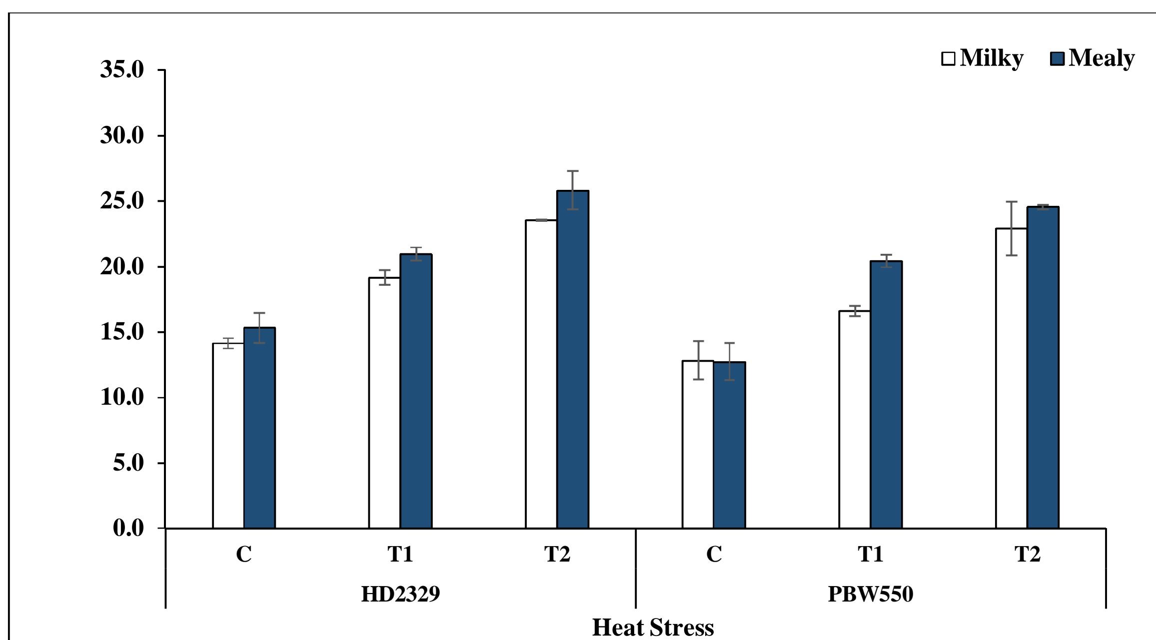


Figure 17(b): Catalase activity assay in thermosusceptible wheat *cvs.* HD2329 and PBW550 of wheat at milky-ripe and mealy-ripe stage under differential heat stress treatment; C-22ºC; T1- heat stress of 30ºC for 2 h, T2- heat stress for 38ºC for 2 h.

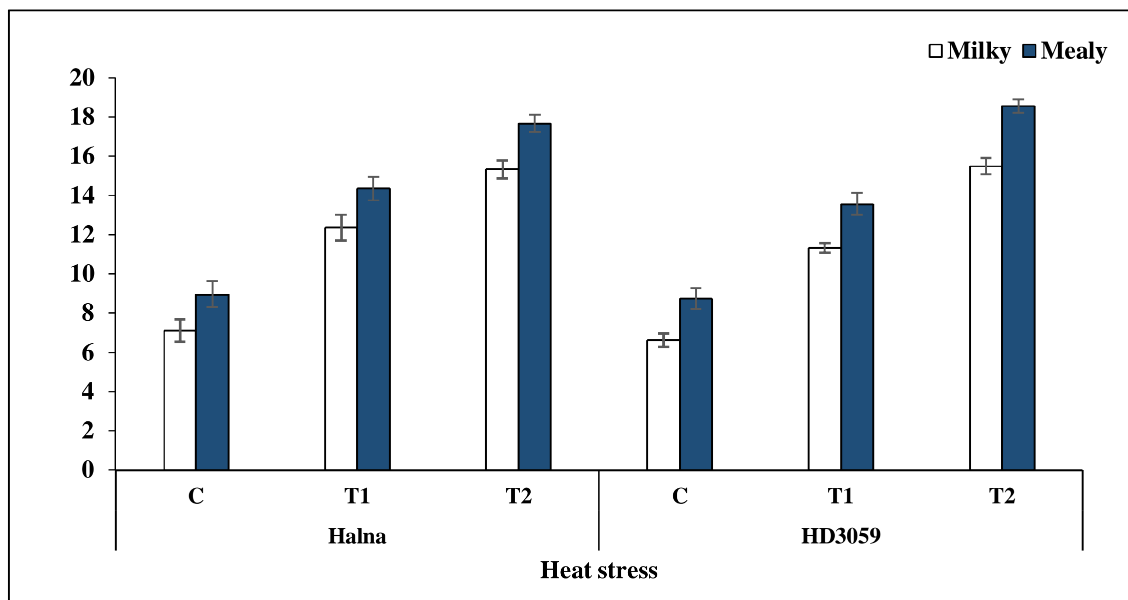


Figure 18(a): Estimation of Proline content in thermotolerant wheat *cvs.* Halna and HD3059 at milky-ripe and mealy-ripe stage under differential heat stress treatment ; C-22ºC; T1- heat stress of 30ºC for 2 h, T2- heat stress for 38ºC for 2 h.

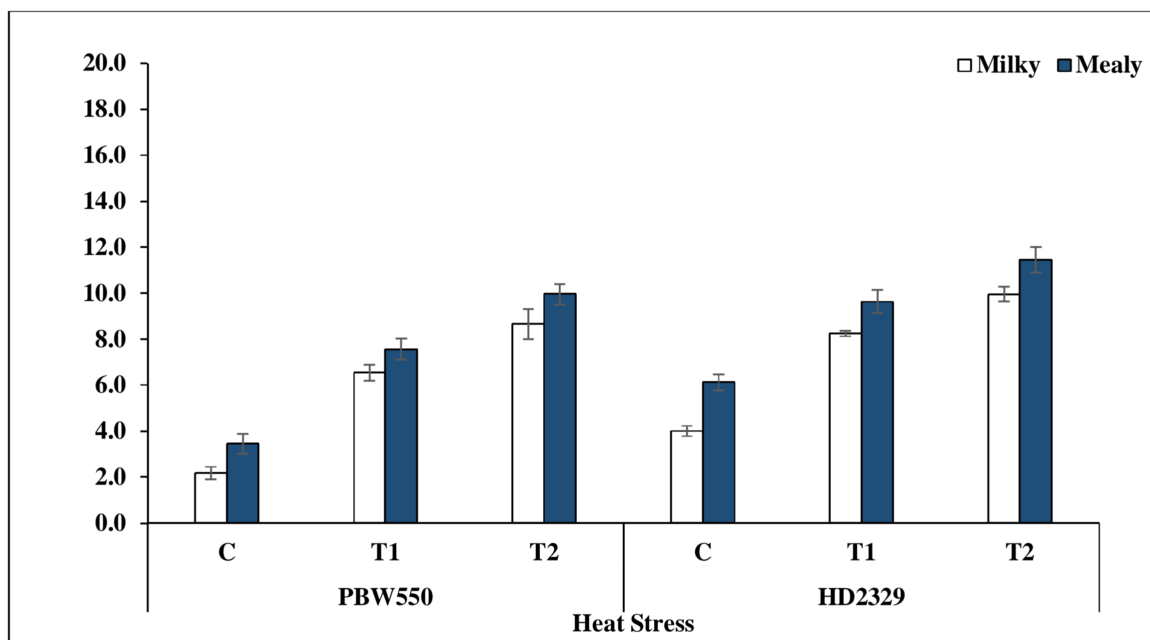
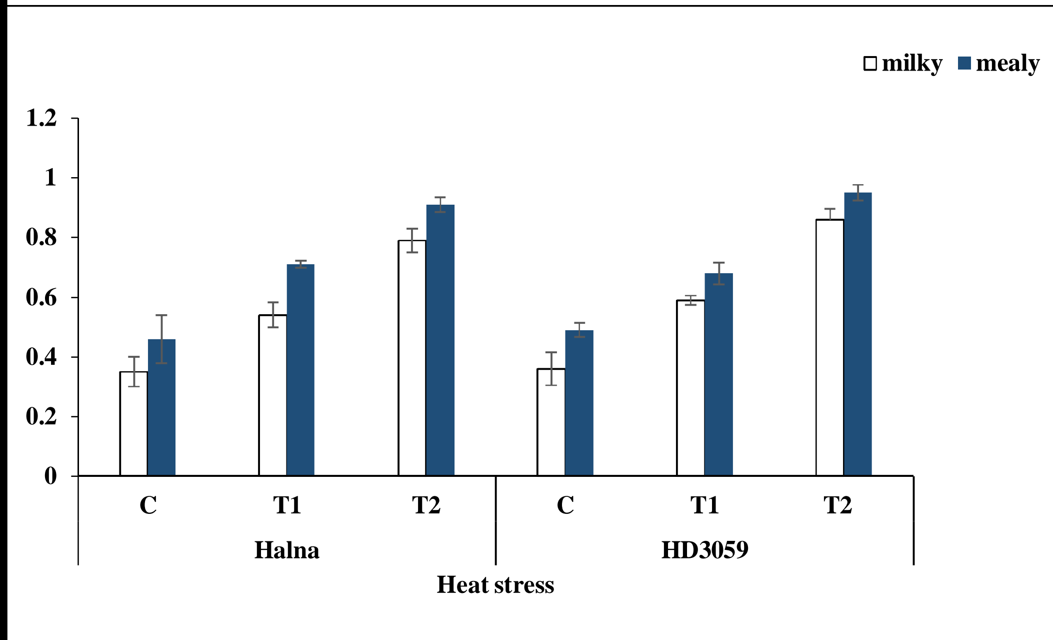
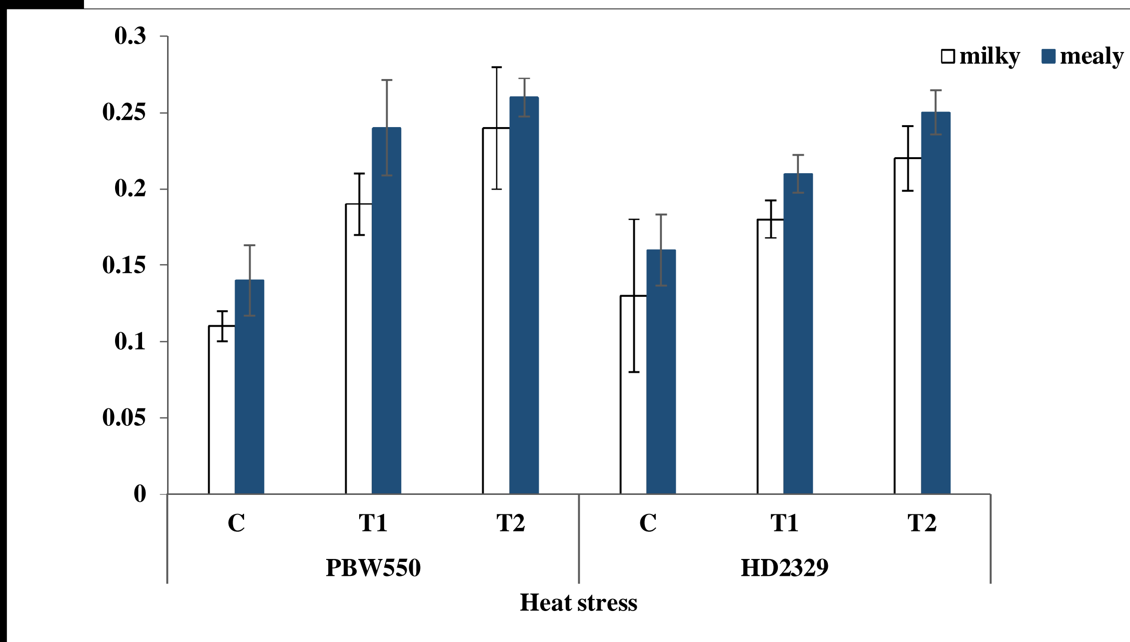


Figure 18(b): Estimation of Proline content in thermosusceptible wheat *cvs.* PBW550 and HD2329 of wheat at milky-ripe and mealy-ripe stage under differential heat stress treatment; C-22ºC; T1- heat stress of 30ºC for 2 h, T2- heat stress for 38ºC for 2 h.



e **19(a):** Estimation of Reactive oxygen species (ROS) in thermotolerant wheat *cvs.* Halna D3059 at milky-ripe and mealy-ripe stage under differential heat stress treatment ; C-22ºC; at stress of 30ºC for 2 h, T2- heat stress for 38ºC for 2 h.



e **19(b):** Estimation of Reactive oxygen species (ROS) in thermosusceptible wheat *cvs.* 50 and HD2329 at milky-ripe and mealy-ripe stage under differential heat stress treatment; C; T1- heat stress of 30ºC for 2 h, T2- heat stress for 38ºC for 2 h.

Plants are constantly exposed to both abiotic and biotic stress that has severely effect both quality and quantity of the grains and ultimately cause reduction in yield. Plants have evolved different mechanisms to cope-up with the vagaries of the nature. The defense responses of plants to these stresses are complex and involved numerous cellular, physiological and molecular adaptations. Abiotic stress especially heat and drought has huge impact on the growth and other biological feature of cereals and is responsible for severe losses in the field. Plant defense or adaptation lies in the signaling pathways operating in response to the stress which allow the plant to respond in a rapid and efficient manner. Plants are responding in a specific manner when they have to face more than one stress simultaneously and the response cannot be predicted based on the plants response to the individual single stresses. Mitogen-activated protein kinase (MAKP) are important regulators in the pathway of MAPK signaling, which play very crucial roles in plant growth and stress-responses. MAPK activity is important to maintain the normal biological activity inside plant cells under stress condition. Inactivation of specific MAPK gene causes dysfunction of signaling pathways related to the tolerance which makes plant succumb to the stress. Various MAPK genes are characterized from various crops like *Oryza sativa* (Xiong *et al*., 2003) *Medicago sativa* (Sangwan *et al*., 2002) and *Solanum tuberosum* (Blanco *et al*., 2006). Cloning of AtMPK3, AtMPK4 and AtMPK6 gene from *Arabidopsis thaliana* under oxidative stress was reported by (Droillard, 2002). A putative rice MAPKKK gene (*dsm1*) in *Oryza sativa* was reported (Xiong et al., 2003). MAPK are highly conserved in all eukaryotes and are responsible for the signal transduction of diverse cellular processes under various abiotic and biotic stress-responses. It was also reported that MAPK pathways are also activated by pathogen attack and signaling is mediated by salicylic acid. Here our results with MAPK expression related study under the heat stress condition in wheat reported that the MAPK plays crucial role during the regulation of plant responses against environmental stresses such as heat, drought stress and salinity. We selected the four different *cvs.* of wheat in which two are thermotolerant (Halna and HD3059) and two are thermosusceptible (HD2329 and PBW550) for our research work.

We identified and cloned the MAPK gene of ~1.3 kb length from wheat *cv.* HD3059. Various other heat responsive MAPK gene in wheat has been reported by Xu *et al.*, (2007), Sangwan *et al.*, (2002), Lee *et al.*, (2012), and Schnable *et al.*, (2012). Expression profiling of MAPK gene show fold increase in HS condition. Thermotolerant Halna *cv.* showed 4.1 fold increase in MAPK gene transcript at HS condition of 38°C for 2 h (T2). Thermosusceptible wheat *cv.* PBW550 show 2.34 fold increase in MAPK gene transcript at HS condition of 38°C for 2 h (T2). Our result is in confirmation with Sangwan *et al.*, (2002) who reported 2.2- 4.5 fold increase in MAPK gene transcript during heat stress condition. Jonak *et al.*, 1996 also reported 1.5 fold increase in MAPK gene transcript during oxidative stress. Kong *et al.*, (2013) has also reported 3.64 fold increase in MAPK gene transcript during oxidative stress in Maize. Total antioxidant potential has been used as indirect parameter for analyzing the thermotolerance capacity of the plant. In present investigation, we observed very high TAC in thermotolerant *cvs.* Halna showed maximum TAC in response to HS of 38°C for 2 h (T2) during milky-ripe and mealy-ripe stage which is in conformation with the finding of Kumar *et al.*, (2013). Catalase enzyme showed maximum activity in *cv.* Halna at mealy-ripe stage under HS condition. Thermosusceptible *cvs.* (PBW550 and HD2329) showed increase in the catalase activity in HS condition of 30°C for 2 h (T1). Wahid *et al.*, (2002) and Kumar *et al.*, (2012) reported increase in catalase enzyme activity during HS condition in wheat. Superoxide dismutase (SOD) follows same trend in activity as catalase enzyme. Maximum activity of SOD is observed in thermotolerant *cv.* Halna in HS condition. Our result is supported by Kumar *et al.*, (2012). Wahid *et al.*, (2002) reported accumulation of peroxides increases during HS condition which results in increased activity of peroxidase enzyme. We observed highest activity of peroxidase enzyme in Halna *cv.* in HS condition of 38°C for 2 h (T2) during mealy-ripe stage. Heat stress condition in wheat result in increased accumulation of proline. In HS condition increased accumulation of Reactive oxygen species was reported in wheat by Kumar *et al.*, (2012). We also observed the increase in ROS content in wheat under HS condition. Maximum accumulation of ROS is observed in thermotolerant *cv.* Halna. High ROS content during heat stress can be correlated with high expression of MAPK gene transcript in HS condition. Higher accumulation of ROS leads to high expression of MAPK gene. Our observation is

supported by Moon *et al*., (2002) who reported increase in level of NDP kinase 2 gene (MAPK homologue) in *Arabidopsis thaliana* under heat stress condition.

As a result of increased anthropogenic activity on planet Earth's climate is changing in a very abrupt manner. Due to increased emission of greenhouse gases (GHG's) and its accumulation in atmosphere, temperature of Earth is increasing in a very steady manner. High temperature leads to severe loss in grain yield quality in wheat. MAPK, key signalling molecule during heat stress condition play very significant role in adaptation of plants against heat stress conditions. The present investigation on the "Identification, cloning and characterization of heat-induced MAPK gene(s) from wheat (*Triticum aestivum* L.) under the terminal heat stress" was undertaken at the Division of Biochemistry at IARI, New Delhi during 2014-2016. Plants at the milky-ripe and mealy-ripe sub-stage of grain-filling (selected based on the Feekes scale; three pots from each variety) were exposed to heat stress of 30°C and 38°C for 2 h, whereas other three pots in each group served as control (22±3°C). Heat responsive MAPK gene of 1.3 kb size was cloned from HD3059 cultivar under HS condition of 38°C for 2 h (T2). The gene sequence was submitted in National Centre for Biotechnology Information (NCBI) GenBank with accession no. KT835664. The cloned sequence was blasted using BLASTn (NCBI) which showed maximum homology with transcript *Hordelum vulgare* (acc. no. AK353742). An open reading frame of 314 amino acids was identified. Conserve domain analysis showed homology of cloned MAPK with Serine/Threonine type kinase. Protein of cloned MAPK transcript is predicted to be localized in cytoplasm. Further, expression analysis of cloned transcripts using qRT-PCR showed significant variations in expression heat stress condition. Thermotolerant *cvs.*HD3059 and Halna showed relative more fold in expression of MAPK transcript in HS condition, than thermosusceptible *cvs.* HD2329 and PBW550. Expression level of MAPK transcript showed 2-4 fold increase with increase in temperature from 30 to 38°C.

Expression of MAPK during heat stress condition is regulated by levels of oxygen radicals in cell. To correlate the expression of MAPK gene with oxygen radicals we performed analysis of parameters such as Total antioxidant activity (TAC), Estimation of Reactive oxygen species (ROS) along with activity assay of antioxidant enzymes including Catalase, Peroxidase, and Superoxide dismutase (SOD). In HS condition increase in TAC is observed in all *cvs.* Highest increase in

TAC was observed in Halna *cv*. Activity of other antioxidant enzymes showed increase in activity at mealy stage in HS condition of 38°C for 2 h (T2) in Halna *cv.* Thermosusceptible *cv.* HD2329 showed lowest accumulation of ROS along with significantly less increase in activity of antioxidant enzymes as compared with thermotolerant *cv.* Halna under HS condition. Proline accumulation was observed highest in thermotolerant *cv.* Halna and lowest in thermosusceptible *cv.* PBW550.

To conclude we have cloned a putative MAPK gene of size ~ 1.3 kb from wheat *cv*. HD3059. *In-silico* characterization of putative MAPK gene showed conserved signature sequence S/T-X3-5-S/T in Serine/Kinase motif. Expression analysis of cloned MAPK gene transcript is showed higher fold increase in thermotolerant *cvs.* as compared to thermosusceptible *cvs.* under HS condition. We have also observed higher fold increase in MAPK gene expression in mealy stage as compared to milky stage during HS of 38°C for 2 h (T2). Biochemical characterization of SOD, Catalase and peroxidase showed increase in activity under HS condition of 38°C for 2h (T2) at mealy-ripe stage. Higher accumulation of ROS and Proline was observed during mealy stage under 38°C HS for 2 h (T2) in case of all four wheat *cvs.* Significant increase in ROS and Proline was observed more in thermotolerant wheat *cvs.* (Halna and HD3059) as compared with thermosusceptible wheat *cvs.* (PBW550 and HD2329).

MAPK play very important role in signalling cascade during heat stress condition. Thus there is a need to explore different isoforms of MAPK in order to elucidate the signalling mechanism in wheat under heat stress condition. It will help to understand the tolerance mechanism of wheat under HS condition and also in development of heat tolerant wheat crop.

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Identification, Cloning and Characterization of Heat-induced MAPK Gene(s) from Wheat (*Triticum aestivum* L.) Under Terminal Heat Stress

AbstrAct

Wheat is highly sensitive to heat stress and a wide-diversity has been observed among different genotypes for HS-tolerance. MAPK is key component in signal transduction during biotic and abiotic stress. Heat stress activate the signalling cascade of MAPK in wheat, resulting in activation of various transcription factors and protein kinase for better adaptation of wheat towards heat stress. In the present study, we have identified 37 novel transcripts showing homology with MAPK through *de novo* transcriptomic approach. Based on the digital fold expression, transcript_MAPK was targeted for the cloning. MAPK gene of 1.3 kb size was cloned from wheat cultivar HD3059 under HS condition. BLASTn search showed maximum homology with MAPK gene transcript of *Hordeum vulgare* (acc. no. AK353742). BLASTp analysis showed maximum homology with MAPK protein in *Zea mays* (acc. no. NP_001167676.1). The MAPK gene sequence was submitted in NCBI GenBank (acc. no. KT835664.1). ORF analysis showed the presence of 314 amino acid. Conserved domain search showed the presence of Serine/Threonine type kinase (STK) domain in the sequence. Expression analysis of MAPK gene showed significantly higher transcript level during mealy-ripe stage than at milky-ripe stage in HS-treated thermotolerant *cvs.* (HD3059 and Halna). Halna *cv.* showed maximum increase in TAC under HS of 38°C for 2 h (T2) during mealy-ripe stage. Percentage increase in TAC was observed minimum in HD2329 under HS of 38°C for 2 h (T2) in compared to other *cvs*. We observed increase in activity of antioxidant enzymes like SOD, CAT and GPX in response to HS of in all the wheat *cvs.* During grain filling stage Halna showed maximum activity of all the three antioxidant enzymes, as compared to other *cvs.* ROS showed maximum accumulation in Halna under HS of 38°C for 2 h (T2) during mealy-ripe stage. There is, however, need to explore novel MAPKs from contrasting wheat cultivars in order to characterize their role in signalling and thermotolerance of wheat. Over-expression of candidate MAPK in desirable lines of wheat can pave the way for the development of 'climate-smart' wheat.

गेहूँ (ट्रिटिकम एस्टीवम एल.) से आवधिक उष्मीय तनाव मे ऊष्मा प्रेरित माइटोजन सक्रिय प्रोटीन काइनेज जीन की खोज, क्लोनिंग तथा निरूपण|

सारांश

गेहूँ ऊष्मा तनाव के प्रति बेहद संवेदनशील है और उष्मयी तनाव के लिये विभिन्न जीनोटाइप के बीच एक व्यापक विविधता देखी गई है। माइटोजन सक्रिय प्रोटीन काइनेज जैविक और अजैविक तनाव के दौरान संकेत पारगमन का महत्वपूर्ण घटक है। उष्मयी तनाव गेहूँ मे संकेतन को सकृय करता है जिसके परिणामस्वरूप उष्मयी तनाव के दिशा मे गेहूँ की बेहतर अनुकूलन के लिए विभिन्न प्रतिलेखन कारक और प्रोटीन काइनेज सक्रिय होते है। वर्तमान अध्यन मे हमने नवीन ट्रांस्क्रिप्टोमिव माध्यम द्वारा मयपकेय से अनुरूपता दिखाने वाले ३७ नवीन अनुलेख की खोज की। डिजिटल गुणी अभिवयक्ति के आधार पर अनुलेख मापकेय को क्लोनिंग के लिए चयनित किया गया। १.३ केबी आकार के मयपकेय जीन को उष्मयी तनाव मे गेहूँ की एचडी-३०५९ किस्म मे क्लोन किया गया। ब्लास्ट न खोज ने होरदेउम वल्गर अनुलेखो के साथ अधिकतम अनुरूपता दिखाई (ए सी सी नंबर आ के ३५३७१८)| ब्लास्ट प विश्लेषण ने ज़ेया मेज़ मयपेक प्रोटीन के साथ अधिकतम अनुरूपता दिखाई (ए सी सी नंबर नप ००१७६७ ६१)| मेपकय जीन अनुक्रम को एन सी बी आई जीन बैंक (परिग्रहण संख्या आर टी ८३५६६४) मे प्रस्तुत किया गया। संरक्षित डोमेन खोज के अनुक्रम मे सेरीन /थ्रिओनिन प्रकार के काइनेज (एस टी के) डोमेन की उपस्थिति देखी गई। मैयपक अभिवयक्ति विश्लेषण मे उषमसहिष्णु प्रकार (एच डी ३०५९ और हलना) मे दूधिया परीपकव अवस्था की तुलना में मीली राइप अवस्था के दौरान अनुलेखन स्तर बढ़ा हुआ मिला। हलना में २ घंटा (टी २) के लिये ३८ ° सेल्सियस के उष्मयी तनाव तहत टी सी ए में अधिकतम वृद्धि देखी गई। दूसरी किस्मों की तुलना मे २ घंटा (टी २) के लिये ३८ ° सेल्सियस उष्मयी तनाव पर एच डी २३२९ मे टी सी ए की न्यूनतम प्रतिशत वृद्धि देखी गई। हमने उष्मयी तनाव की प्रतिक्रिया मे मिली सभी किस्मों मे प्रशक्षिकरक एंजाइम की सक्रियता जैसे एस ओ डी, सी अ टी और जी पी एक्स मे अधिकतम सक्रियता देखी| दूसरी किस्मों की तुलना मे २ घंटा (टी २) के लिये ३८ ° सेल्सियस उष्मयी तनाव पर एच डी २३२९ मे टी सी ए की न्यूनतम प्रतिशत वृद्धि देखी गई। दूसरी किस्मों की तुलना में हलना मे इन तीनो प्रशक्षिकरक एंजाइम की सक्रियता अधिकतम देखी गई। मीली राइप

चरण मे २ घंटे (टी २) के लिए ३८ ॰ सेल्सियस के ऊष्मए तनाव मे हलना मे आर.ओ.एस अधिकतम संचय दिखाई दिया। मयपकेय की अधिक अभिव्यक्ति गेहूं की वांछनीय लाइनों 'जलवायु-स्मार्ट' गेहूं के विकास के लिए मार्ग प्रशस्त कर सकते हैं।

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