IN-SILICO IDENTIFICATION AND CHARACTERIZATION OF WZYb PROTEIN ENCODING GENE FAMILY IN WHEAT

Thesis Submitted for the Award of the Degree of

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in

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By

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2024

DECLARATION

I, hereby declared that the presented work in the thesis entitled "***IN-SILICO* IDENTIFICATION AND CHARACTERIZATION OF WZYb PROTEIN ENCODING GENE FAMILY IN WHEAT**" in fulfilment of degree of **Doctor of Philosophy (Ph. D.)** is outcome of research work carried out by me under the supervision Dr Gurmeen Rakhra, working as Assistant Professor, in the School of Bioengineering and Biosciences of Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of another investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

(Signature of Scholar)

Name of the scholar: Ridhi Joshi

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CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled "***IN-SILICO* IDENTIFICATION AND CHARACTERIZATION OF WZYb PROTEIN ENCODING GENE FAMILY IN WHEAT"** submitted in fulfillment of the requirement for the reward of degree of **Doctor of Philosophy (Ph.D.)** in the Biotechnology of School of Bioengineering and Biosciences, is a research work carried out by Ridhi Joshi, 11719504, is bonafide record of his/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

(Signature of Supervisor)

Name of supervisor: Dr Gurmeen Rakhra

Designation: Assistant Professor

Department/school: School of Biosciences and Bioengineering

University: Lovely Professional University

Abstract

Drought stress has become a global issue for the crops and is affecting their growth as well as productivity. Wheat is one of the most consumable rabi crop all over the world and has been known to be affected by the various abiotic stress conditions, including drought. In the present study, stress responsive genes, dehydrins have been identified which helps in improving the crop productivity. Dehydrins are well known stress tolerant genes in plants but their structure, function and multitasking roles in plants are still unknown. WZYb is a boiling soluble protein which belongs to LEA2 dehydrin family. It is well known for expression in cellular dehydration and plays an important role in response to various abiotic stresses including drought. Therefore, in this study, an attempt has been made to carry out the genome wide analysis of a protein belonging to WZYb dehydrin family in wheat in order to conceptualize the diverse functions based upon the structural properties and evolutionary patterns. The identification and characterization of this dehydrin protein was done using a recently available wheat genome sequence from IWGSC and this revealed a total of 48 dehydrin genes encoding for 48 different dehydrin proteins. The identified proteins ranged from 9.65 kDa to 101.60 kDa. Nomenclature of proteins was done as TaDHN, Ta indicates *Triticum aestivum* while DHN indicated dehydrin which was then followed by the respective molecular weight and chromosome number of the identified proteins. Majority of these genes (46) were distributed on the 15 chromosomes while the remaining two genes were found to be distributed on an unidentified chromosome. The number of introns in the open reading frames for majority of the genes varied from 0-2 while the four genes lacked the introns. The predicted pI value of these proteins ranged between 5.19 to 10.7. It was also observed that 35 TaDHNs were single domain proteins consisting of a single dehydrin like domain whereas the remaining 13 dehydrins were found to possess additional domains, and hence were known as multidomain proteins. The TaDHNs were predicted to be localized mostly in cytoplasm (35) and nucleus (5) while some of them were secretory in nature. Analysis of protein- protein interaction networks of multi-domain dehydrins revealed that their functional partners are involved in different cellular functions. The homolog proteins were identified with the help of multiple alignment using Clustal omega and the phylogenetic analysis revealed the existence of

3 major clades of TaDHNs. Expression of the dehydrin genes was analyzed using qRT-PCR and it was found that 8 out of 17 genes showed an enhanced expression under 3-day drought conditions. Also, during western blotting, significant upregulated expression was observed in the stressed wheat seedlings at different time intervals and also in the post stress 24 h harvested samples. Promoter analysis revealed the presence of various biotic, abiotic, stress responsive, hormonal responsive and developmental responsive cis elements. The digital expression analysis done using the microarray data also revealed the up-regulated expression of dehydrins under drought and heat stress at different time intervals. Based upon the findings, it can be inferred that this study provides new insights into the structure properties of dehydrins and its family in wheat which can be of paramount importance to understand their role in developmental processes under drought conditions, and this can be a valuable source in the future research to increase the crop productivity.

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Chapter-1

Introduction

Unusual fluctuations in weather nowadays often lead to various abiotic stress conditions like high and low temperature, drought, osmotic and soil salinity etcetera(A. Gupta *et al*., 2020). The factors like inadequate rainfall, depletion in soil moisture and high evaporation from leaves are the predisposing elements responsible to cause turgor loss of the cell and disruption of the cell membrane (Rorat, 2006). Countries like India, Australia, North America, Africa, Brazil and China are primarily facing the challenges imposed by drought which is categorized under the class related to water stress (Miyan, 2015). The drought conditions are known to primarily affect the major arable crops and as a result, the agricultural sector is facing the challenge of meeting the demand for food and has led to human starvation and agricultural failure (Pereira, 2016; Wens, 2022).

It is well known that when environmental equilibrium reaches drying, then any moderate change in air equilibrium results in a lethal effect on various plants and animals since water is known to regulate the functioning of intracellular macromolecules and to maintain the membrane structure of cells (Alpert, 2005). Thus, water removal from the different cells of desiccation-sensitive organisms disintegrate cell membranes and organelles, and also cause irreversible accumulation of essential macromolecules (Tweddle *et al*., 2003) (Figure 1).

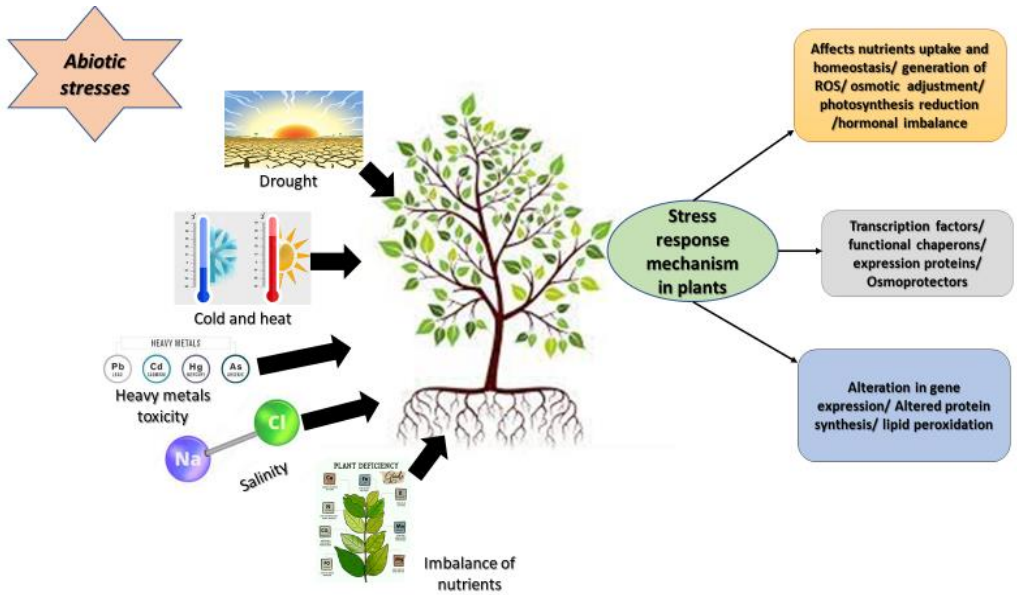


Figure 1:Physiological Responses of Different Abiotic Stress on Plants

The plant cells' membrane receptors initially detect the drought stress, and the signal is then sent further down, where it is converted into secondary messengers like calcium, reactive oxygen species (ROS), damaging lipid peroxides, and inositol phosphates. These secondary messengers work with Ca^{2+} sensors, or calcium-binding proteins, to control the intracellular calcium level. The primary stress-responsive genes or the transcription factors governing these genes are subsequently the target of these proteins' interactions with the correct partners, which start a phosphorylation cascade (M. Ali *et al*., 2020). To increase stress tolerance, multiple downstream stress-responsive genes are activated by the overexpression of transcription factor genes. Additionally, the molecules that respond to stress can intensify the original signals and start a second wave of signalling that can use the similar or alternative signalling pathways (Isah & Isah, 2019; Mahajan & Tuteja, 2005). Ultimately, by directing plant adaptability and survival, these stress gene products enable plants to thrive under unfavourable situations.

LEA proteins

The literature has unveiled that plants produce diverse, significant and hydrophilic proteins in small amounts during their exposure to various environmental conditions like drought, cold, heat shock, salinity etc(Battaglia & Covarrubias, 2013). Among these, Late Embryogenesis Abundant (LEA) proteins have been well comprehended. These LEA proteins were first identified in cotton in high amounts in plants during late embryogenesis(Mertens *et al*., 2018). Additionally, these proteins are produced during abiotic stress conditions induced by drought, cold, and exposure to high salinity (Ingram & Bartels, 1996). It is notable that most LEA proteins are intrinsically disordered proteins (IDPs). (M. Hara, 2010a). The "lock and key" paradigm of structured proteins proposed by Emil Fischer has been widely accepted over the past few decades. However, of lately, researchers have also started to gain a key interest in understanding the role and mechanism of action of unstructured proteins like LEA in different organisms. Extensive research in this direction has revealed that approximately 24-30% of proteins found in eukaryotes are disordered, with around half of these proteins containing a significant region of disorder (Uversky *et al*., 2005)

(Dunker & Obradovic, 2001). Moreover, intrinsically disordered proteins have been found to have typically four conformations, i.e., coil-like, molten globule, native (ordered) and pre-molten globule (Figure 2) (Uversky, 2020). The extensive studies on these IDPs have revealed that these proteins have a wide range of biological functions(Fong *et al*., 2009)and are known to contain sites for regulatory proteolytic attacks and post-translational modifications(Dunker & Obradovic, 2001).

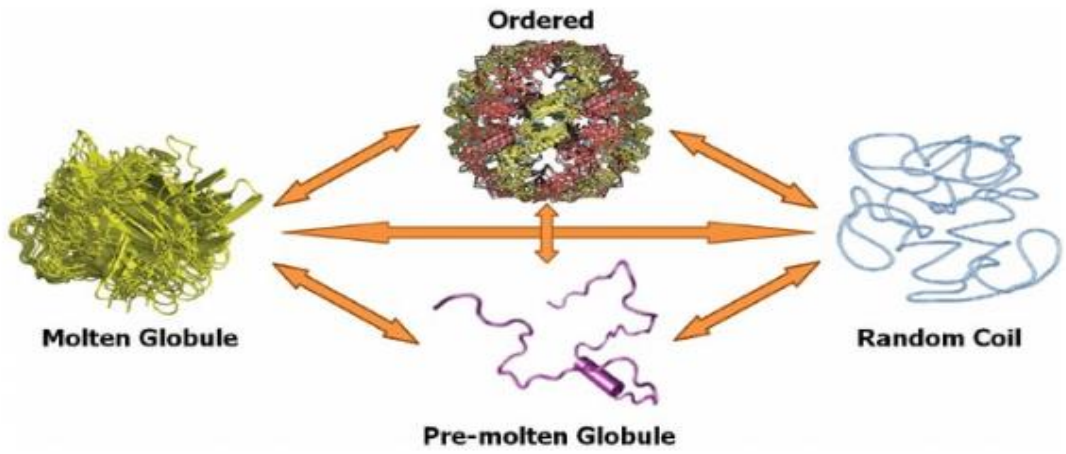


Figure 2:Four conformations of intrinsically disordered proteins (Uversky, 2020)

Dehydrins

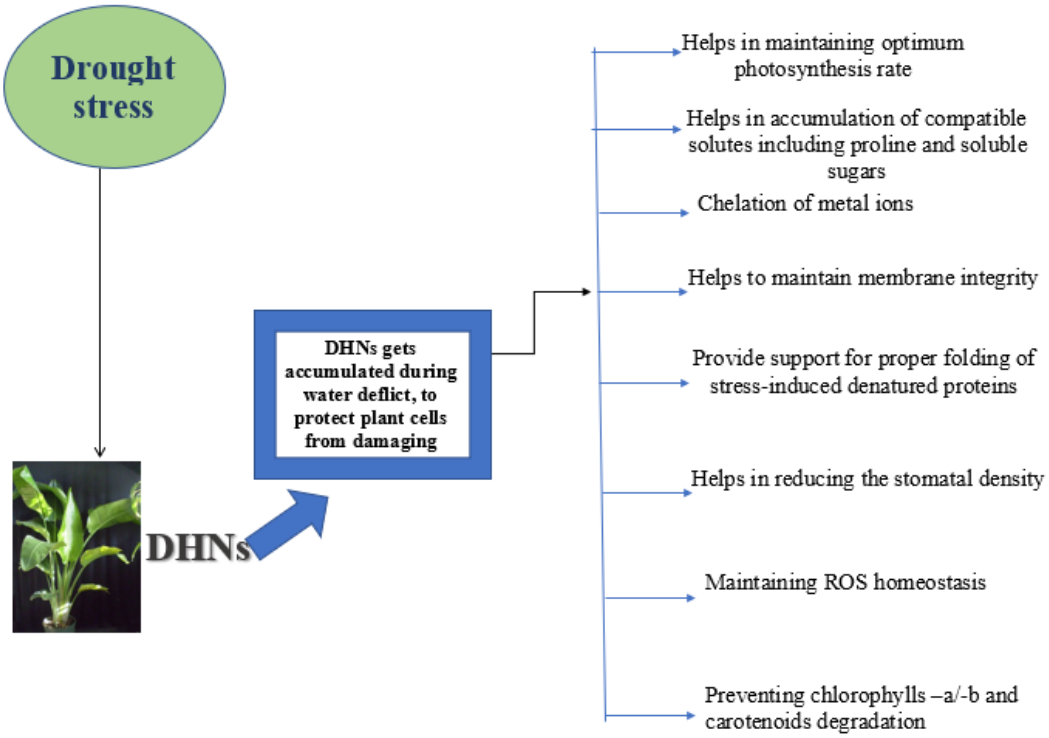


Figure 3: Different roles of dehydrins in plants

Dehydrins are one family of LEA proteins that play a vital role in stress responses(Kosová *et al.*, 2016) (Figure 3). These proteins have been found in photosynthetic organisms like cyanobacteria, ferns and vascular plants. In vascular plants, the presence of dehydrins is recorded in conditions like embryogenesis, and vegetative tissues under dehydration due to osmolytes or evaporation and low temperature (Campbell & Close, 1997). The presence of proteins similar to dehydrins has also been accorded in rotifers, algae and cyanobacteria (Berjak, 2006). Furthermore, dehydrin protein concentration has been correlated with the state of relative desiccation tolerance (Campbell & Close, 1997), (R. Li *et al.*, 1998), (Tunnacliffe *et al.*, 2010). Typically, dehydrin proteins have been found to contain a high number of hydrophilic amino acids and are thermostable, which explains their solubility in water even at 100 °C (Clarke & Graether, 2015). The occurrence of these proteins has been observed in chloroplasts (Mueller *et al.*, 2003), cytoplasm (Rinne *et al.*, 1999), vacuoles (Heyen *et al.*, 2002), mitochondria (Borovskii *et al.*, 2002) and nuclear regions like heterochromatin, euchromatin, nucleoplasm and nucleoli (Godoy *et al.*,

1994). These dehydrin proteins have molecular weights within the range of 9 kDa to 200 kDa (Azarkovich, 2020).

The sequence of dehydrin protein families is known to contain three conserved motifs, i.e. K-, S- and Y-segments (Graether & Boddington, 2014). All the dehydrins have Lys-rich K-segment (EKKGIMDKIKEKLPG)(Z. Yu *et al*., 2018), present adjacent to the C terminal and aid in forming A2 amphipathic ά- helix. On the other hand, conserved Y-segment (DEYGNP) homologous to nucleotide binding chaperones of plants and animals is present at the N-terminal (Vazquez-Hernandez *et al*., 2021). Furthermore, in dehydrins, there might be a S- segment(sequence of 3-9 consecutive serine residues) which occurs either on the C- or N- terminal (Fattash *et al*., 2021)(Figure 4).

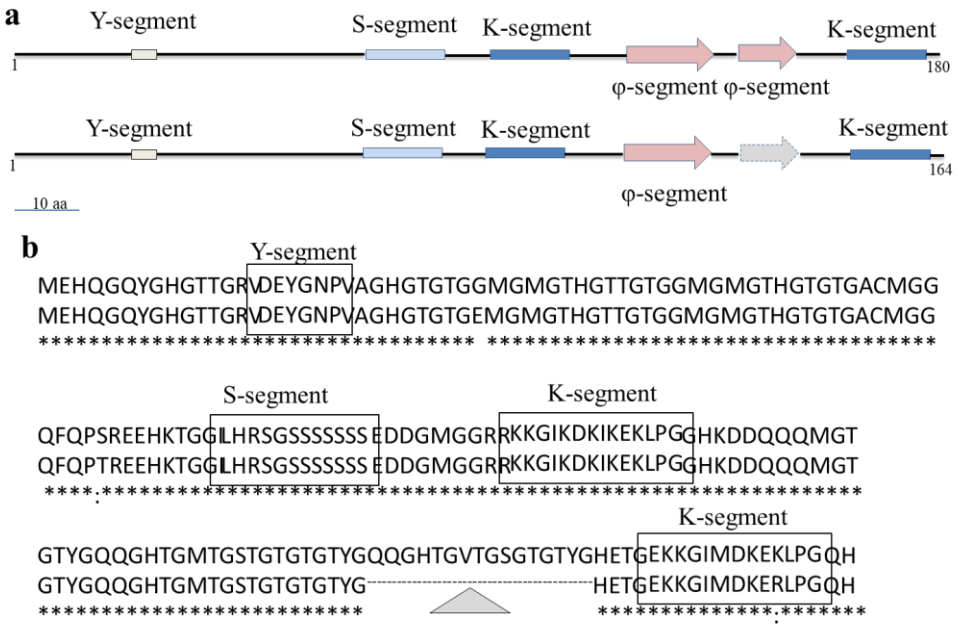


Figure 4: (a, b) Schematic representation of dehydrin protein containing K-, Y- and S-segment (Fattash *et al*., 2021)

The previously published literature has unravelled that plant-specific dehydrins usually express themselves during the induction of water stress conditions (Tunnacliffe

et al., 2010). These dehydrins like the LEA proteins are generally intrinsically disordered proteins lacking a well-defined 3D structure (Eriksson & Harryson, 2011).

Lately, extensive studies on dehydrins have uncovered that they are actively involved in stabilizing enzymes, membranes and nucleotides under abiotic stress conditions (Sharma, 2022). The dehydrins have been proposed to bind with the membrane and stabilize it by acting as scavenging metals, molecular shields, and reactive oxygen species (Graether & Boddington, 2014). For instance, DHN1 from *Zea mays* is reported to bind to PA lipids via forming an α-helical structure (Koag *et al*., 2003). Moreover, *Thellungiella salsuginea* dehydrin 1 (TsDHN-1) has been reported to interact with liposomes thereby leading to a disordered-ordered structure transition in its secondary structure (Rahman *et al*., 2011). Several transgenic studies have provided evidence that the overexpression of dehydrin proteins can lead to improved growth parameters in plants under various stress conditions, indicating their significant role in stress tolerance (H. Liu *et al*., 2015; Vuković *et al*., 2022). In precision, the actual function of dehydrins in the in-vivo condition is still unknown, but in stress conditions, plants produce these dehydrins in a tremendous amount, which indicates that these dehydrins have a vital role in the survival of plants (Rorat, 2006).

Research has indicated that different members of the boiling soluble protein (BSP) family, known as LEA proteins, may play a role in regulating various cellular processes in wheat. However, the precise functions of these stress-responsive proteins have yet to be fully understood. Therefore, it is crucial to characterize the different LEA genes present in wheat's BSP family to determine their physiological roles and potential for crop improvement through conventional breeding and transgenic methods. The wheat plant is a hexaploid species that originated from natural crosses between three ancestral species, resulting in a genetically diverse and heterogeneous genome. The complexity and vastness of the wheat genome make studying it a challenging task. For the related species like *Triticum dicoccoides* (Avni *et al*., 2017), *Triticum urartu* (Thomas *et al*., 2011) and *Aegilops Tauschii* (Mayer *et al*., 2014) genome sequences have been released, (Zan *et al*., 2020).

WZYb is boiling soluble protein belonging to group 2 of LEA family (dehydrin) (Rakhra *et al*., 2017). It is one of known dehydrin which participates significantly in

stabilizing the enzymes, membranes and nucleotides in cells under abiotic stresses. Various studies related to transgenic plants proved the overexpression of *wzyb* dehydrin gene that enhances the tolerance to environmental stresses including drought. The expression of a WZYb (wheat dehydrin) can be stimulated by the drought, salinity and ABA stresses (Yang *et al*., 2015; Yu *et al*., 2018a; Zhu *et al*., 2014). Various *in-silico* tools have been used in present study for identification and characterization of proteins belonging to WZYb family, which resulted in functional analysis of particular gene in response to drought stress.

Rationale of the study:

Wheat crop is sensitive to different stress conditions, including heat and drought stress, especially at the flowering and grain development stages, which negatively impacts the yield as well as quality (Kulkarni *et al*., 2017). Therefore, to develop climate resilient wheat, it is imperative to identify genes that impart tolerance to different stresses in this plant. Despite of having significant genetic and protein evidence supporting the importance of dehydrins against water deflecting conditions, a detailed and comprehensive representation vis-à-vis the function of these proteins is still in its infancy. Also, because the plant responses to abiotic stresses are complex and multi- genic, the function of many of the stress-induced dehydrin genes still a matter of interest. Therefore, determining the function of dehydrins and mechanism involved in regulation of stress is the foremost goals for understanding stress acclimation. In the light of these observations, a study has been proposed to identify and characterize the WZYb dehydrin (a type of LEA protein) in wheat using various bioinformatics tools in order to have an insight into their functional aspect.

In the current work, we have thoroughly identified and systematically named the wheat dehydrins (TaDHNs) for the first time. Furthermore, we have analysed the physiochemical properties, chromosome distribution, domain and motifs, evolutionary relationships, gene duplication, gene structure, subcellular localization, disordered nature, and secondary structure using *in-silico* tools. To validate the *in-silico* findings, expression analysis was also done using real-time PCR and western blot and mapped to the digital expression analysis.

Overall, this study aimed to elucidate the drought stress-responsive nature of TaDHNs in wheat for genetically improving the abiotic stress tolerance based upon their structural and functional properties. With regard to this, dehydrin genes were identified for a better understanding of gene function and to provide a theoretical foundation for breeding stress-resistant wheat varieties.

Chapter-2

Review of literature

With the changing environment conditions, plants are unceasingly facing the different environmental stresses which are subsequently affecting the crop yield and production. In recent years, irregular fluctuation in the weather has largely affected crops yield which led to heavy loss worldwide in agricultural sector. Consecutively, the rising population and reduction in arable land are imposing the challenge to meet the demand of food production.

Lately, it has been determined that 50% loss in yield of major crop plants is due to abiotic stresses such as drought, extreme temperature and salinity (Rejeb *et al.*, 2013). Moreover, it has been estimated that by 2050, production of food globally needs to be doubled to meet the demand of food for growing population (Tilman *et al.*, 2002). Owing to which, there has been significant increase in exploration and research on understanding the mechanism of plant responses to different abiotic stresses and generation of tolerance in plants against different abiotic stresses. The abiotic stresses like drought, temperature and salinity primarily leads to dehydration of cells via different mechanisms. In general, the alteration in water potential primarily affects the proteins and cell membranes of plant which progressively leads to death due to these conditions. Out of these, drought is stated to be multidimensional and highly widespread abiotic stress as it affects the plants at different level (Rahman *et al*., 2011; Zlatev & Lidon, 2012) and is proclaimed to be primary limiting factor in crop production especially in tropical region. Technically, the characteristics of drought stress are reduced water potential of leaf and turgor loss causing the closing of stomata, reduction in exchange of gases, decrease in water content, cell enlargement and growth.

Currently, development of tolerance against drought in wheat has become the primary goal in breeding program around the globe, as some areas of world suffers from water scarcity in growing season (Mansour *et al.*, 2020). Although, the breeders have gained success in increase the yield of crop in highly sophisticated environment, but as its well known that genetic amendments done in crops faces difficultly in agricultural lands due to environmental factors like low water availability and high temperature (Ali *et al.*, 2021). According to consensus, approximately 32 % of wheat produced in different parts of world especially, developing countries undergoes varied level of stress

imposed due to drought condition (Kirigwi *et al.*, 2004). The erratic nature of drought and complexity of genetics to regulate the response of plant in water deficits conditions enables us to develop high yielding cultivars adapted to grow under different stress conditions (Blum, 1996).

The extensive studies on abiotic stress like cold and freezing temperature, drought, and salinity leads to water deficit in plants at cellular level, which further leads to aggregation of highly hydrophilic proteins i.e., LEA proteins (Battaglia & Covarrubias, 2013). Furthermore, presence of these LEA proteins has been reported in seeds of various plants which shares the sequence homology with cotton LEA protein. Additionally, certain reports reported the increase in expression of different LEA genes during desiccation of descaling seedlings and artificial exogenies via ABA treatment (Dalal *et al*., 2009; Hanafy *et al*., 2013).

LEA protein Family and its classification

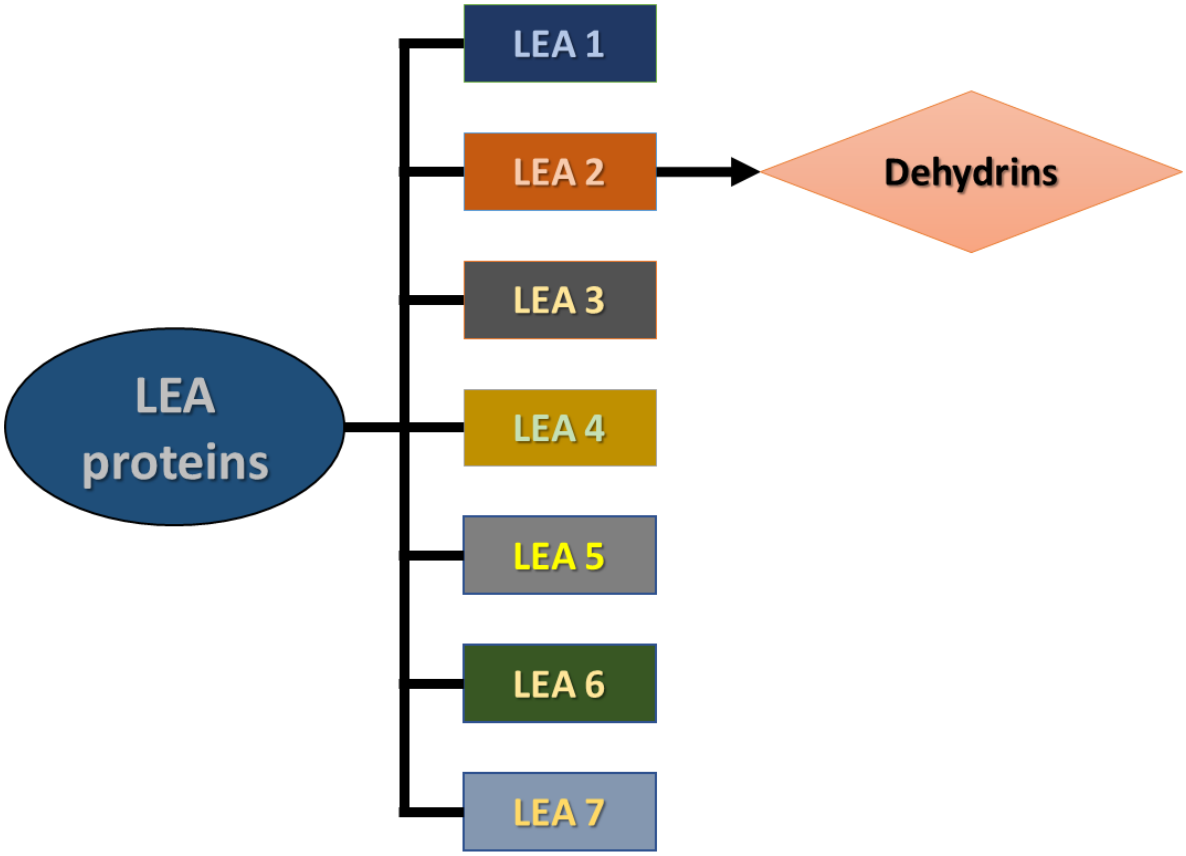


Figure 5: Classifications of LEA proteins

Leon Dure conducted a study in 1981 on the embryonic stage of cotton (*Gossypium hirsutum* L.) and wheat (*Triticum aestivum* L.), and observed the rapid accumulation of different protein families during the late stages of seed development in both plant species. This led to the discovery of LEA proteins. Later, presence of LEA proteins was reported in vegetative tissue of the plant under stress, in few bacteria which possess the desiccation tolerance ability and invertebrates (Dure *et al.,* 1981). These proteins are generally found in the matrix of plant seed mitochondria, providing protection against cold, desiccation and salinity (Grelet *et al.,* 2005; Nguyen *et al.,* 2018). But, the physiological and biochemical characteristics of these proteins are still unknown. Whereas, gene expression of these proteins suggests about their vital role in stress imposed by dehydration. In general, these proteins are intrinsically unorganised and localized in the mitochondrial matrix, which under dehydration conditions folds itself into a class A α-helical structure that later enters inner membrane to offer protection from dehydration (Popot, 2018). Thus, LEA are categorized in the gene family that are known to play vital role in building tolerance against drought. In general, LEA proteins are also stated as "Late Embryogenesis Abundant" proteins that are usually found in plant embryos and have been found to improve the tolerance of embryos against drought (Hanin *et al.,* 2011; Liang *et al.,* 2016). Different studies on LEA proteins have confirmed their presence in plants such as barley, tomato, and wheat during periods of drought. This discovery has led to further investigations and a deeper understanding of the regulatory mechanisms governing these LEA genes. As a result of these investigations, the role of LEA genes in regulating ABA, drought, and salinity conditions has been revealed. Currently, ongoing research is focused on gaining a better understanding of the controlling elements involved in ABA-induced gene expression. In water-stress conditions, the most finely regulated cis element is the ABA-responsive element (ABRE), which contains a palindromic motif sequence of CACGTC. Additionally, another responsive element, the Dehydration-responsive element (DRE) A9, is present in drought conditions due to the presence of a conserved sequence of TACGACAT (Ali *et al*., 2020).

Majority of late embryogenesis abundance (LEA) proteins have low molecular weight within the range of 10-30 kDa and their expression is recorded during the late phase of seed development. The presence of these LEA proteins was first reported in 1981 in cotton seeds by Dure and Chlan. Henceforth, extensive research was done in this direction by researchers, which found the presence of these LEA proteins in *Arabidopsis, Brassica napus,* Barley, maize, rice, soybean, sunflower and wheat (Dalal *et al*., 2009; Hundertmark & Hincha, 2008; Li *et al*., 2020; Liang *et al*., 2016; Lu *et al*., 2021; Minh *et al*., 2019; Sasaki *et al*., 2014; Z. Wang *et al*., 2018). The presence of such stress responsive proteins has been extensively observed in nucleus and cytoplasm. Moreover, in higher plant seeds, the presence of these proteins is recorded in the encapsulated portion of root seedlings as well as also in other organs (Arroyo-Becerra *et al.,* 2022).

Advancements in the field of computational biology have allowed for the creation of a computational database called LEAPdb, which uses bioinformatic tools for *in-silico* analysis of late embryogenesis abundance proteins. Presently, the database contains over 700 unique and non-redundant sequences of LEA proteins across various organisms. Analysis of these proteins has revealed comprehensive information on their physicochemical properties (Shi *et al.,* 2020).

LEA proteins is further classified into seven different groups and families (Figure 4). The Group 1 to 7 except 5 are typically hydrophilic group of proteins (Liu *et al*., 2019). While, LEA protein group 5 is classified as “hydrophobic” or “atypical” protein (Battaglia & Covarrubias, 2013; Pantelić *et al.,* 2022). Homologues of group 1 and 3 have also been found to be present in few invertebrates and bacteria(H. Liu *et al.*, 2019). This classification system depends on amino acid sequence and RNA homology, which determines whether it belongs to the nuclear region or cytoplasm. According to conventional classification, proteins belonging to LEA group are categorized on the basis of conserved amino acid/motifs sequences, probability profiles of proteins or oligonucleotide sequences (Y. Wang *et al*., 2020)

LEA proteins Group 1 (D-19)

First group of LEA proteins are recognized based on the presence of an internal 20-mer sequence, and due to the presence of highly charged residue, these proteins exhibit high hydrophilicity. Seeds, especially in dry conditions, primarily accumulate LEA proteins of Group 1 during embryonic development. Pollen grains have also been reported to accumulate LEA proteins of group 1 during dehydration conditions. These LEA proteins of group 1 have characteristic genes which respond to water-deficient conditions and ABA. The in-vitro experiment was conducted using recombinant wheat (*Triticum aestivum*) which revealed that Em proteins have the ability to regulate the production of LDH (lactate dehydrogenase) and citrate synthase (CS) and prevent their inactivation and aggregation at very low temperature. This TaEm protein has also been expressed in *Saccharomyces cerevisiae,* which has prevented its reduced growth in high osmolarity yeast-growing mediums.

The first identified LEA proteins in cotton seeds were D-19 and D-132. These proteins are characterized by a high glycine content of around 18%, which contributes to their random coil-like disordered structure in an aqueous solution. Circular dichroism (CD) analysis has demonstrated that these proteins have an extended helical PII conformation that is left-handed. Nuclear magnetic resonance (NMR) spectroscopy analysis has also confirmed that these proteins are unstable and flexible. A hydrophilic conserved sequence of 20 amino acids is the foundation that links group 1 and other taxa of LEA proteins. This sequence can also occur as multiple copies of tandem repeats. (Ali *et al.,* 2020).

Interestingly, the distinctive representation of LEA protein of group is observed in different taxonomic domains such as archaea, bacteria and eukaryotes. This conclusion has been made based on the identification of the homologous hydrophilic 20-mer conserved sequence of LEA protein of group 1 in different species like *Artemia franciscana* (crustacean), archaea (methanogenic) and *Bacillus subtilis* (Battaglia *et al.,* 2008). LEA proteins of group 1 present in bacterial species have tendency to inactive the enzyme during in-vitro freeze-thaw treatment. They are accorded to perform similar functions to that of LEA proteins in plants (Raga-Carbajal *et al.,* 2022).

LEA proteins Group 2 (D-11)

The group 2 LEA proteins are generally referred to as Dehydrins (DHNs), and these proteins were also primarily discovered in embryos of cotton seeds. In fact, this group of LEA proteins has been well-comprehend in contrast to other LEA proteins. Moreover, the dehydrins display high hydrophilicity due to highly charged amino acid proportions. The hydrophilicity of these proteins further improves due to the low number of nonpolar hydrophobic amino acids and reduced percentage of cysteine (Cys) and tryptophan (Trp) residues. LEA proteins classified under group 2 are identified by the presence of a K-segment (EKKGIMDKIKEKLPG) which is a Lysine-rich motif containing 15 residues and can appear in 1-11 repeats on a polypeptide. Additionally, these proteins also contain a Y-segment consisting of 1-35 tandem repeats and a serine-rich S-segment. Some conserved hydrophilic and polar sequences are also found between the K- and S-segments. The sub category of group 2 LEA proteins includes K-subgroup, SK-subgroup, KS-subgroup, YK-subgroup, and YSK-sub-group. NMR and CD spectra analysis of LEA proteins of group 2 revealed that they have an unstructured and hydrophilic confirmation in aqueous form. These proteins are induced by water-deficit conditions and seed desiccation. They are also distributed throughout plant vegetative tissues during ideal growth conditions. The K-segment of these proteins' forms alpha-helical amphipathic structures, which is their main protective mechanism. In dehydrated conditions, these proteins adopt a more organized form due to the high solute concentration and ionic content, explaining their role in plant responses to water-deficit conditions. They are called cold-responsive (COR) proteins because they are expressed in response to stress induced by cold. These proteins may be ABA-dependent or independent depending on the surrounding conditions. They mainly accumulate in the cytoplasm and nucleus. The phosphorylated SK2 proteins of the S-segment may act as a possible nuclear localization signal (NLS). Some proteins have a His-rich region that allows binding with metals like Ni2+ and Cu2+, while the acidic form shows binding with Ca2+. These proteins act as calcium-dependent chaperon-like molecules or calcium buffers. Group 2 LEA proteins have been found to have a metal binding potential which may contribute to their ability to detoxify the oxidative stress. (Ali *et al*., 2020; Battaglia *et al*., 2008; Battaglia & Covarrubias, 2013).

LEA proteins Group 3 (D-7/D-29)

In this group of LEA proteins, the presence of an 11-amino acid repeating motif has been determined and this is the reason due to which there is molecular mass difference among this protein. Other than this, these proteins also contain few other conserved sequences which rarely repeat in the sequence and the sequence is different form this 11-mer motif. Moreover, the LEA proteins of group 3 are highly diverse in contrast to another LEA protein group. LEA proteins are a group of proteins that play a crucial role in protecting plants and some animals from various environmental stresses, particularly drought stress. Among the different groups of LEA proteins, group 3 is known to be particularly important in providing protection against dehydration stress.

Group 3 LEA proteins are subdivided into two subgroups, 3A and 3B, based on the variation in the conserved amino acid sequence of their 11-mer motif. Subgroup 3A is well-conserved, whereas subgroup 3B is more heterogeneous due to high inconsistency in their 11-mer motif sequence. Structural analysis of group 3 LEA proteins reveals that they are amphipathic, right-handed α-helical coiled-coil dimers. These proteins do not change their conformation into secondary structure in aqueous solution, but transform themselves to form secondary coiled-coil structure on exposure to ethylene, glycol, glycerol, methanol, or fast drying conditions.

The drying rate and dehydration conditions regulate the structural confirmation of group 3 LEA proteins.

Besides plants, some animals such as *Caenorhabditis elegans*, *Deinococcus radiodurans*, and *Haemophilus influenzae* have also been reported to contain group 3 LEA proteins. AavLEA1 is the well-characterized LEA protein of group 3 in plants. In general, this protein is less compact and unstructured in aqueous form because of high hydration, though its reversal ability to an α-helical form has been noted on dehydration. These proteins are widely distributed among most plants, seedless vascular and non-vascular plants, as well as algae, suggesting that they could have a variety of intracellular targets and localizations.

Extensive research on group 3 LEA proteins indicates their substantial role and contribution in overcoming the undesirable effects imposed by drought stress in most plants, including. wheat (Ali *et al.*, 2020; Battaglia *et al.*, 2008).

LEA proteins Group 4

LEA proteins of group 4 are widely distributed in both non-vascular and vascular plants. These proteins have a conserved N-terminal region of about 70-80 amino acids, while the C-terminal region is variable in size and less conserved. Group 4 LEA proteins contain five conserved motifs, with the absence or presence of motifs 4 and 5 distinguishing two subcategories, 4A and 4B. Group 4A proteins have a length range of 80-124, while group 4B proteins have a length range of 108-180.

The accumulation of group 4 LEA proteins is the earliest known instance of protein accumulation in dry embryos. The developing wheat seed coleorhizae contain transcripts of group 4 LEA proteins and also accumulate these proteins in coleoptiles under abiotic stress conditions. The scattered accumulation of group 4 LEA proteins in different tissues of other plant seeds may be triggered by ABA pathways or stress, and these proteins are also homogeneously distributed in all embryo tissues.

Research shows that group 4 LEA proteins are induced by drought, salinity, or temperature stresses, and histone deacetylation during germination suppresses their transcript formation. These proteins play a crucial role in plant stress tolerance by preventing LDH inactivation, even after losing 99% of their water due to dehydration. It is possible that the metal binding ability of these proteins plays a role in their ability to detoxify during periods of stress, particularly in situations where the production of reactive oxygen species (ROS) is associated with metal toxicity (Ali *et al.*, 2020).

LEA proteins Group 5

In this group, those LEA proteins are categorized which have high quantity of hydrophobic contents and lacks hydrophilic properties. Thus, most of members of this LEA proteins group are non-homologous in nature. Owing to this, the structurally related hydrophobic proteins are further divided into three subgroups i.e., 5A,5B, and 5C groups. Till date, very less in known about these LEA proteins. Few studies have reported about the accumulation of these proteins' transcripts during the later stage of seed development. Drought, salinity, cold, UV light and wounds are the few abiotic stress factors that triggers the accumulation of these proteins. These proteins implement the globular-like shape during stress condition, which makes them insoluble after boiling (Ali *et al*., 2020).

LEA proteins Group 6 (PvLEA-18)

The first protein from LEA group 5 to be identified in bean was PvLEA-18. Since then, approximately 36 proteins have been identified, mostly from vascular plants. These proteins are typically 7-14 kDa in size and are characterized by their conserved structure. They contain four distinct motifs with highly conserved motif 1 and motif 2. The presence of "Pro" and "Thr" amino acid residues at positions 6 and 7, respectively, and the sequence LEDYK are conserved with 100% accuracy in motif 1. Additionally, LEA proteins belonging to group 5 possess an inherent lack of structure. These proteins are typically hydrophilic in nature and do not contain "Cys" and "Trp" residues. One of the characteristic features of the LEA proteins of group 6, do not get coagulated at extremely high temperature. In fact, during SDS-PAGE, the proteins of this group migrate to higher molecular mass instead of near to the predicted mass determined on the basis of amino acid sequence. The accumulation of these proteins of group 6 is accorded in embryo radical during pollen formation and earlier seed germination phase. Moreover, elevation in level of these proteins is recorded after the water stress conditions and ABA treatment, indicating about the protective role in plants. On contrary to other LEA proteins group, the group 6 LEA proteins does not prevent the inactivation of enzymes during dehydration condition. Furthermore, the target molecules of these proteins are different in comparison to the other LEA proteins, which signifies that proteins of this group exhibit protective activity due to different factor than hydrophilicity (Ali *et al.*, 2020).

LEA proteins Group 7 (ASR1)

As other group, this group of LEA proteins encompasses hydrophilic residues in large number and that are classified into multiple sub-families. ASR1, belonging to group 7 LEA proteins are generally small, intrinsically unstructured and heat stable. These proteins are known to contain three consensus sequences i.e., motif 1, 2, and 3. In this protein, motif 3 is localized at C-terminal region and contains an NLS. Other than this, motif 4 conserved sequence is also found in these proteins. Interestingly, all the motifs found in these proteins has regions rich in Histamine. Moreover, physiochemical properties of this group proteins are linked with the other groups of LEA proteins. Accumulation of these proteins is also accorded in water-deficit stress conditions and during late embryogenesis stage. In fact, different species of plants

shows varied expression of these proteins. During ripening of fruit, seed maturation, senescence, pollen maturation or when exposed to stresses like cold, drought, salinity or limited light the accumulation of transcripts of group 7 LEA proteins has been recorded. Besides this, the alteration in the sugar or ABA level also affects gene expression. Apricot seeds, grape, melon and tomato have been reported to contain the transcripts of LEA proteins of group 7. Moreover, these proteins display a random structure in aqueous solution as with other LEA proteins, but will become ordered when Zn ions are bound or desiccation stress is applied (Ali *et al.*, 2020; Battaglia *et al.*, 2008).

LEA proteins in wheat

Various studies categorized the physical properties of polypeptides that accumulate during cold acclimation to determine their function. Their study showed that the *cor* genes of *Arabidopsis thaliana* and *Triticum aestivum* encode boiling stable proteins which do not precipitate upon boiling in aqueous conditions. Poly (A^+) RNA which was isolated from non-acclimated and cold-acclimated wheat and Arabidopsis plants was translated under *in vitro* conditions. Polypeptide products were fractionated either directly or were first boiled and then separated by SDS-PAGE. Imperilling *in-vitro* translated products from both the acclimated and non-acclimated samples were boiled which resulted in precipitation of many of polypeptides. However, *cor* mRNAs encoding some boiling-soluble polypeptides gathered in both the cold acclimated wheat and Arabidopsis plants. A cDNA clone specific to boiling stable COR polypeptide (47 kDa protein, designated as pH7.2) was identified in Arabidopsis by the hybrid arrest translation experiments carried under the *in-vitro* conditions. After establishing that the cor mRNA accumulation was a response common to both Arabidopsis and wheat, they also determined whether the *cor* genes were conserved between these two plants. For this they constructed cDNA library from the RNA which was extracted from cold acclimated wheat cultivar Winoka and was examined for clones hybridizing with *cor47* of Arabidopsis. Then Southern and Northern blot analyses was done which indicated the presence of *cor* gene in wheat that was related to a 'boiling-stable' COR 47 kDa polypeptide of Arabidopsis. They recommended the likely fundamental role of boiling-

stable COR polypeptides to act as cryoprotectants as well in acclimation to cold temperatures (Lin *et al.,* 1990).

Morris *et al.,* 1990 explained the expression regulation of early methionine (*Em*) labelled gene in wheat embryos cultured on agar added with Murashige-Skoog nutrient medium throughout their development and upon ABA and osmotic stress imposition. The *Em* polypeptide was found to be a cytosolic protein of mature, dry wheat embryo characterized by high hydration potential due to large number of hydrophilic residues present within the random-coil conformation. *Em* mRNA accumulation was described to be persuaded by the exogeneous application of ABA and osmotic stress in the cultivated wheat embryos. Thus, they proposed that the accumulation of this hydrophilic *Em*-gene expression could be considered a protective function under osmotic stress (Morris *et al.,* 1990).

In 1991, Curry *et al.,* cDNA sequence encoding a group 3 LEA mRNA in wheat was analysed, and it was found to be inducible by various environmental stress conditions, such as ABA or dehydration. Morris *et al.,* 1991 constructed cDNA library from the embryos of wheat cultivar and treated with ABA and the cDNA clone pMA2005 was obtained as result and was further sequenced. The length of cDNA clone (designated as pMA2005) was 934 bp which encoded a 23 kDa hydrophilic protein of 224 amino acids. The deduced protein sequence showed great homology with the LEA proteins of group 3, with maximum being to clone pHVA1 clone of barley (*Hordeum vulgare*). Ten tandem repeats of 11 amino acids tract, representative of LEA proteins belonging to group 3, was found in the wheat clone pMA2005. Northern analysis was done using the cDNA clone pMA2005 which showed that dehydrating conditions in wheat shoots also induced high levels of pMA2005 that correlated well with an increase in endogenous ABA level. Under dehydrating conditions, western blotting was performed which detected a protein band that strongly cross- reacted with an antibody produced against cDNA clone pMA2005. Thus, it was hypothesized that pMA2005 protein product might be a part of drought tolerance in wheat (Curry *et al.,* 1991).

Guo *et al.,*1992 isolated a cDNA clone pWG1 representing *cor39* gene from wheat cultivar Winoka, and related to *cor47* gene of *Arabidopsis thaliana*. *In vitro*

studies of poly (A^+) RNA translation studies discovered that the wheat *cor39* gene, encoded a 39 kDa hydrophilic polypeptide and was therefore designated as COR39. Analysis of the DNA sequence revealed that pWG1 cDNA clone had 1303 bp insert and contained an ORF of 1232-bp, that encoded a 391 amino acid polypeptide. COR39 sequence investigation indicated the majority of glycine (27%), threonine (16%), histidine (11%) and absence of both tryptophan and cysteine residues. The hydropathic index was found out to be -1.1, which indicated that it was a hydrophilic protein. Further analysis indicated the presence of two repeating units in the polypeptide-one glycine rich designated GR and the other lysine rich unit designated KR and these units repeated six times in the polypeptide. Southern blot analysis was done which specified that wheat has several loci related to *cor39*. Total RNA was extracted from different plant tissues (crown, root and leaf) of non-acclimated plants and plants were acclimated for 2 weeks at 2°C. Transcripts of around 0.8, 1.5, and 3.3 kb hybridizing with *cor39* were accumulated in different tissues of cold-acclimated plants. Contrasting hybridization pattern for RNA samples from root, crown and leaf of cold-acclimated plants, transcripts in seeds were about 1.3 kb. The 3.3- and 1.5- kb transcripts hybridizing with *cor39* also accumulated in response to water stress and upon ABA application besides cold acclimation. The resemblances in wheat and Arabidopsis cor genes expression and the likely functional relationships among COR39, COR47, and LEA proteins suggested their likely biochemical role in freezing and desiccation tolerance(Guo *et al.,* 1992).

Houde *et al.,* 1992 isolated, sequenced and expressed a wheat cold specific (wcs120) cDNA clone hybridizing to a major 1650 nucleotides mRNA species from the cold-acclimated wheat. They detected that wcs120 mRNA peaked to its maximum level at 4°C after 24 h of cold acclimation period and persevered at great levels all through 36 days of cold-acclimation period but was insensitive or unresponsive to drought, heat shock, or ABA treatments. The mRNA abundance during period of acclimation was dependent upon genotype because the expression of *wcs120* was momentary and dropped sharply during the later stages of cold acclimation in the less freezing tolerant genotype Glen LEA. The wcs120 cDNA was having an ORF which encoded about 390 amino acid long boiling stable polypeptide. It exhibited abundance of histidine, glycine and threonine residues, but phenylalanine, cysteine and tryptophan

were absent. The WCS120 protein was having two repeated domains, unique to dehydrins of barley and ABA induced protein families of rice. For the identification of encoded protein, ORF of *wcs120* was expressed in *E. coli* using T7 RNA Polymerase promoter. After purifying protein from *E. coli*, the *in-vitro* translated RNA products were isolated from both the non-acclimated and cold-acclimated plants. The protein which was expressed in bacteria was found to be a boiling stable, hydrophilic protein and this product co-migrated with a major protein formed *in vitro* and *in vivo* during cold acclimation in plants. Based upon their major findings, they hypothesized that the highly hydrophilic nature of these proteins might be significant in trapping water inside the cell to inhibit local dehydration occurring during stress conditions (Houde *et al.*, 2004).

Ohno *et al.,* 2003 conducted an experiment to assess the kinetics of induction of *Wdhn13* (wheat gene). This gene encodes a 12.8 kDa protein (designated WDHN13) containing three lysine-rich segments and exhibiting hydrophilic cold-responsive and boiling stable. The evaluation by polyclonal antibody i.e., anti-WDHN13-antibody raised against Wdhn13 cDNA revealed the presence of large number of cross-reacting proteins in seedling leaves, mature embryos and endosperms at 25°C. In contrast to that, single major protein was detected at low temperature of 4°C in leaves. Moreover, transient rise in the amount of mRNA was recorded and rise in peak was only observed between 3rd to 5th day of the low temperature treatment, whereas a significant time lag (day 10) was observed for the protein accumulation. Furthermore, in low temperature treated seedlings, steady-state protein and transcript levels were greater in the leaves in contrast to roots, which also gets altered by light /dark conditions. Besides that, Southern blot analysis unveiled that *Wdhn13* was present on the homoeologous group 7 chromosomes, which is different from other wheat dehydrin genes that are generally present on the group 6 chromosomes (Ohno *et al.*, 2003).

Takumi *et al.,*2003 isolated and characterized a member of cold responsive gene family in wheat i.e., *Wcor15*. The molecular weight of *Wcor15* was determined to be 14kda and showed the high similarity with identified barley and wheat proteins. Further, the southern blotting analysis conducted on the diploids, haploid, hexaploidy

and tetraploid of wheat and Aegilops species revealed that wheat and its closely related wild species have multiple copies of *Wcor15* homologous. The utilization of nulli-tetrasonic analysis in the study allowed the assigning of five copies to homologous group 2 chromosomes. Whereas, northern blot analysis revealed that low temperature was responsible for inducing the expression of Wcor15 and **homologous transcripts accumulation led the increase in the steady-state level of leaves. Further, expurgated spiderwort leaves were subjected to Bombardment-mediated transient expression analysis, which showed protein targeting to epidermal guard cells chloroplasts. Furthermore, the promoter of *Wcor15* showed the presence of three CRT/DRE-like sequence motifs that are generally found in *Arabidopsis* Cor genes and influenced the expression of reporter GUS gene in transgenic tobacco plants leaves under conditions such as low light and low temperature. The result obtained from this study revealed that functional cor gene system having CRT/DRE cis-element are conserved in both dicotyledon and monocotyledon plants (Takumi *et al.*, 2003).**

Goyal *et al.,* 2005 conducted a study on Em (group 1 LEA protein) from Wheat and AavLEA1 (a group 3 LEA protein) from *Aphelenchus avenae* (nematode) to assess the chaperonic activity of LEA proteins. Citrate synthase was selected for heat stress due to its vulnerability to high temperature. Rather than acting like a classical molecular chaperone, LEA proteins revealed a protective and synergistic effect in the presence of trehalose (chemical chaperone) at high temperature. Both the LEA proteins could autonomously safeguard citrate synthase from accumulation under freezing and desiccation conditions. Thus, the first testament of LEA proteins for anti-aggregation activity during water stress was recognized. LEA and trehalose, a non-reducing disaccharide, documented a protective collaborative effect under conditions of dryness and freezing. Based upon their results, a model for LEA proteins was anticipated whereby, they might take the unusual form of molecular chaperone that aid in preventing the aggregation of damaging protein during water stress condition(Goyal *et al.,* 2005).

Nakaminami *et al.,* 2005 proved that purified recombinant WCSP1 belonging to the family of cold shock domain (CSD) proteins was boiling soluble and was shown to bind single stranded /double stranded DNA and mRNA. The 3D structure of WCSP1 along with its nucleic acid binding activity was sustained even after the boiling treatment. In contrast to LEA proteins, WCSP1 was found to possess five-β-stranded structure containing two consensus RNA recognition domains. Based upon its melting temperature (Tm) of 45.8°C, it was concluded that boiling treatment followed by a cooling process resulted in the conversion from an unfolded to a folded state. A deletion mutant of WCSP1, with only a CSD part, lost its representative nucleic acid-binding activity; whereas another WCSP1 mutant with first glycine-rich region and CSD of WCSP1 revealed the activity. Their findings specified that the first glycine-rich region of WCSP1 is vital only for the nucleic acid binding activity but not for the heat stability of the protein (Nakaminami *et al*., 2005).

Brini *et al.,* 2007 recognized a dehydrin protein named as DHN-5 in wheat that was found to be strongly linked to maize RAB17. The Dhn-5 cDNA length was determined to encode for 227 amino acid long hydrophilic protein rich in glycine residues. The protein contained two repeats of lysine rich conserved K- segment (EKKGIMDKIKEKLPG) having the stretch of eight serine residues on the head side, which is a typical characteristic of group 2 LEA proteins. Further, strong *Dhn-5* transcript accumulation was accorded in mature wheat embryos, while in salt and ABA-treated seedlings, transcript accumulation was accorded to be less. Comparative examination revealed the accumulation of DHN-5 protein in the mature embryos of two *Tunisian durum* wheat varieties, differing in drought and salt tolerance unveiled differential accumulation of DHN-5 protein in the two varieties. Immunoblot studies was then done using polyclonal antibody raised against maize RAB17, revealed the accretion of multiple bands but with an intense accumulation of the predicted 26 kDa DHN-5. A differential DHN-5 phosphorylation pattern was obtained with the help of immunoblot analyses in resistant and sensitive variety using the maize RAB17 antibody. In resistant variety, several acidic spots were detected besides a single basic protein spot, while in the sensitive variety, spots were barely detected. They suggested an extensive phosphorylation of DHN-5 protein, with greater phosphorylation in the

resistant variety. Phosphorylated DHN-5 accumulation mainly in the resistant variety suggested its possible implication in dehydration tolerance mechanism. In addition, the DHN-5: GFP fusion protein was found to be primarily localized in the nucleus, which suggested a nuclear role of this protein in osmotic stress response (Brini *et al*., 2007).

Vitamvas *et al.,* 2007 conducted a study on two winter wheat cultivars i.e., *Bezostaya 1* and *Mironovskaya 808* and assess their frost tolerance ability based on the accumulation of WCS120 family proteins. For the assessment of difference in the protein patterns before and after 3 weeks of cold acclimation was identified and quantified by two-dimensional gel electrophoresis (2-DE) and western blot analysis using WCS120 antibodies followed by mass spectrometry (MS). The *WCS120* gene family was determined to be encoding for a cluster of boiling soluble, hydrophilic wheat proteins, which are rich in threonine and glycine and share sequence homology with the D11 dehydrin family. Whereas, in the non-acclimated leaves, only *WCS120* protein from *WCS120* family proteins showed the evident result whereas in the cold acclimated leaves, all five *WCS120* family proteins were evident. In comparison to *Bezostaya 1*, *Mironovskaya 808* exhibited greater *WCS40, WCS66* and *WCS120* proteins accumulation. MS analysis of total soluble proteins from *Mironovskaya 808* (MIR) cultivar showed seven and three COR proteins in the cold-acclimated and non-acclimated samples respectively. In conclusion, on the basis of presence of *WCS40, WCS66* and *WCS120* proteins, the two frost-tolerant winter wheat cultivars were distinguished (Vítámvás *et al*., 2007).

Sun *et al.,* 2009 examined the transcription of cor genes in ABA-dependent and independent pathways regulated by molybdenum (Mo) application in winter wheat under cold condition, to establish the profundity of the molecular mechanisms involved in the process. In the study, '97003'(Mo-efficient) and '97014'(Mo-inefficient) winter wheat cultivars were used and grown for 40 days after germination in control (without Mo) and Mo fertilizer (with Mo) treatments, where temperature in day was set at 15°C and in night it was set as 12°C. Further, low temperature stress was induced by reducing the (day / night) temperature to 5/2°C. The first completely expanded leaves from both the control and Mo- treated plants were collected at different hours of cold stress

imposition (0, 3, 6 and 48 h). On analysis, it was found that Indole-3-Acetic Acid (IAA) synthesis increased tremendously under Mo application thereby resulting in an increased ABA concentration in the leaves of winter wheat. Moreover, the Mo-treated winter wheat at 0, 3, 6, and 48 h at low-temperature showed the increase level of Wlip19 and Wabi5 (basic leucine zipper (bZIP) type of Transcription factor (TF) genes) and Wrab15, Wrab17, Wrab18, and Wrab19 (hydrophilic, ABA-dependent cor genes). Thus, concentration of ABA, expression level of bZIP-type TF genes and variation in hydrophilic, ABA-dependent cor genes in concomitant manner indicates that Mo controls the cor genes in winter wheat (Sun *et al*., 2009).

Vaseva *et al.,* 2010 conducted a study to evaluate the drought response of opposing Bulgarian wheat cultivars i.e., *Pobedo, Katya* and *Sadovo* by assessing the dehydrin expression at early vegetation stage during advancing soil water stress and recovery. The result obtained from the study revealed about the variation in endogenous ABA content impelled by the continuous water stress in winter wheat. In the drought-stressed leaves of tolerant Katya variety, early immunodetection of dehydrins, elevated ABA content and noticeable rise in transcript levels of *wzy2* gene were determined. one step Reverse Transcriptase PCR analysis reported about the increased expression level of *WCOR410b* and *TADHN* (acidic dehydrin genes) under drought stress during early vegetative development phase. Whereas, in water conditions, increase level and accumulation of *TaLEA2* and *TaLEA3* (neutral *wzy2* dehydrin gene) was recorded. On contrary, least drought tolerant cultivar Sadovo showed the poor expression of *TaLEA2* and *TaLEA3* gene. Further, two apparent *wzy2* isoforms resulting from alternative gene splicing were observed under normal and stress conditions. Thus, the initial expression of LEAII (WZY2) and LEAIII (TaLEA2 and TaLEA3) proteins along with elevated ABA content in unstressed wheat plants leaves were associated based on their response to continuous drought stress (Vaseva *et al*., 2010).

Brini *et al*. 2011 conducted worldwide transcriptome profiling on transgenic plants (line DH-4) of Arabidopsis that overexpress wheat DHN-5 cDNA, and elucidate the mechanisms by which dehydrin DHN-5 of wheat show augmented tolerance against osmotic stress. The reformed expression of several number of genes implicated largely

in cellular metabolism, transcriptional regulation, stress signaling and stress tolerance was observed. In DH-4 line, about 50% of 26 genes encoding the stress related proteins like LEA, ABA / stress-related RD29B, Pathogenesis related (PR) were found to be upregulated. Generally, these genes showed a two- to three- fold increase but the one encoding a putative LEA protein (AT1G52690), designated LEA4 showed a 34-fold increase. Positive correlation was also established between the upregulated monodehydroascorbate reductase (MDAR) gene playing significant role in ascorbate biosynthetic pathway and unveiling the presence of ascorbate content in higher amount improves the tolerance to oxidative stress imposed by H2O2 in transgenic lines. In contrast, diverse kinds of transcription factors exhibited the down-regulated expression. In comparison to wild-type plants, jai3-1 (jasmonate insensitive mutant) and the dehydrin-overexpressing lines were found to be impervious to jasmonate, signifying the functional link between DHN5 and jasmonate (Brini *et al*., 2011).

Morran *et al.,* 2011 investigated the prospect of inflecting transcriptional regulation of cold and drought responses in wheat and barley by the constitutive overexpression of two wheat dehydration-responsive element-binding proteins (DREB) factors. These factors are known to cohere specific cis-elements in the promoters of drought-regulated genes like *lea*. Transgenic wheat and barley plants were created displaying double 35S (constitutive) and drought-inducible (maize *rab*17) expression of TaDREB and TaDREB3 transcription factors from wheat grain. Transgenically, constructed populations showed negative characteristics like slow growth, delayed flowering and decreased yield in grain compared to the non-transgenic population owing to the constitutive over-expression of DREB factors. However, TaDREB3 and TaDREB2 transgenic population depicted improved survival rate under extreme drought and frost conditions comparative to the non-transgenic population. This was possible because heightened expression of TaDREB3 and TaDREB2 factors lead to an elevated expression of a wide array of genes encoding stress responsive LEA / COR / DHN proteins (Brini *et al*., 2011).

Sasaki *et al.,* 2014 characterized a novel winter wheat hydrophilic WCI16 protein and provided evidence that WC116 and LEA had certain similar features.

Amino acid sequence of WC116 suggested it to be distinctly hyper-hydrophilic and boiling soluble in nature just like LEA proteins but without any significant sequence similarity to them. Even the ^{1}H-nuclear magnetic resonance (NMR) spectroscopy reasserted that the WCI16 structure was random and had no hydrophobic regions. There was an induction in *WCI16* expression during cold acclimation in winter wheat. The proof of its protection ability during environmental stress conditions was provided by the double-stranded DNA binding activity and the *in vitro* cryoprotection of the freeze-labile enzyme lactate dehydrogenase in the presence of WCI16, signifying that WCI16 might protect cellular biomolecules like DNA and proteins during environmental stresses. Besides, heterologous expression of WCI16 conferred enhanced freezing tolerance in *Arabidopsis thaliana*. The results indicated that WCI16 epitomizes an unusual class of LEA proteins involved in tolerance towards freezing conditions (Sasaki *et al*., 2014).

Drira *et al.*, 2015 conducted a study on E. coli to assess the significance of lysine-rich K segment under different stress conditions by generating a succession of truncated derivatives of a hydrophilic DHN-5 recombinant. On assessing the result, the E. coli cells under different stress treatments (PEG, NaCl) showed the overexpression of DHN-5, K1ΦK2, and YS. Further, results of this study reported no significant variation in the growth of E. coli. However, under different stress conditions, higher growth rates were observed in cells transformed with K1-Φ-K2 or DHN-5 recombinant plasmids. Again, there was up to five-fold and ten-fold increase of Colony Forming Units under freezing and heat shock treatments in cells transformed with K1-Φ-K2 or DHN-5 recombinant plasmids. Further, it was determined whether wheat DHN-5 improved the stress tolerance of E. coli by preventing aggregation. A variety of stress treatments were used to induce the aggregate behaviour of protein extracts containing one of the DHN-5 recombinant forms from E. coli and the antiaggregating potential was assessed by light scattering. The result obtained revealed that the E. coli strains containing full-length DHN-5 and the truncated K1ΦK2 had a potent anti-aggregation effect under different stress treatments. The ability of different dehydrins to take an amphipathic, α-helical form is believed to impart the antimicrobial activity to different dehydrins. For this, antimicrobial potential of DHN-5 (purified form as well as its truncated derivatives)

against different microbial pathogens was investigated. It was confirmed on assessment that the K-segments present in dehydrin both exhibited the antifungal and antibacterial potential against Staphylococcus aureus, E. coli, Agrobacterium tumefaciens and fungi. Based upon their findings, it was proposed that their study offer a firm base for understanding the action mechanism at molecular level of DHN-5 (Drira *et al*., 2015).

Chen *et al*., 2016 cloned a new hydrophilic LEA group 3 gene, named as *TaDlea3*, from Shaanhe 6 (winter wheat). The ORF of TaDlea3 was found to be 492 bp long, encoding a 163- amino- acid protein and showed high similarity with LEA proteins from barley and crested wheat grass (*Agropyron cristatum*). The protein TaDlea3 is a hydrophilic LEA protein with a GRAVY score of -1.026. Subcellular localization analysis revealed that fusion of TaDlea3-GFP protein started their accumulation in the cytoplasm. Further, to determine the TaDlea3-expression patterns at different development phases like flowering, tillering, jointing and seedling under drought stress was done by using qRT-PCR. The result from the study revealed the high induction of expression of TaDlea3 at all the four-development phase under drought stress. Furthermore, Western-blot result also showed the similar result as qRT-PCR under drought stress condition but, showed the diminishing result after recovery at all the four developmental stages. The study also showed that under drought treatment, in contrast to the wild-type plants, antioxidant activities increased in the transgenic plants and hence might be implicated in improving the tolerance capability of overexpressing plants showing the expression of TaDlea3 protein against drought stress (Chen *et al*., 2016).

Wang *et al*., 2016 isolated AREB (ABA response element binding) transcription factors from wheat and named it as TaAREB3. The AREB transcription factors mostly encode hydrophilic LEA proteins (COR, RAB, RD and ERD). The sequence analysis revealed TaAREB3 is a 936 bp long sequence, encoding a 311 amino acids polypeptide with 34 kDa (predicted molecular mass). Further, TaAREB3 protein was determined to be composed of three parts: 145 amino acid conserved N-terminal with threonine /serine like protein kinase phosphorylation sites, a variable M region containing 146-257 amino acids and a conserved C-terminal of 258-311 amino acids with a bZIP domain. The multiple sequence alignment (Clustal-W) and phylogenetic tree analysis

using Molecular Evolutionary Genetics Analysis (MEGA) 5 software revealed that TaAREB3 is similar to an AREB- AtAREB3 subgroup member belonging to the bZIP family A group. TaAREB3 showed constitutive expression in different parts of the plant like seeds, leaves, florets, pistils, anthers, roots and also showed the induction during ABA stress and low temperature stress. Using fluorescence confocal microscope, subcellular localization of TaAREB3-GFP was predicted to be in epidermal leaf cells nuclei of tobacco and in the root cells nuclei of T3 transgenic Arabidopsis lines. Additionally, TaAREB3 protein was found to bound with AREB cis-element in vitro condition via Electrophoretic mobility shift assays (EMSA). Functional analysis showed that TaAREB3 also activated hydrophilic COR15A, COR47, RD29A, RD29B proteins by binding promoter regions, thereby activating their expression and leading to drought and freezing tolerance (Wang *et al*., 2016) .

Rakhra *et al*., 2017, conducted a study on seedlings exposed to drought-stress of wheat cultivar (PBW 175; drought-tolerant), where they were analysed, sequenced as well as cloned to encode a stress-controlled boiling stable protein (titled as WZYb, which is a boiling stable stress responsive protein of wheat). The result obtained from qRT-PCR reported the high expression levels of WZYb in tolerant cultivar during cold and drought condition. The PBW 175 showed the adaptive response to stress, whereas, sensitive cultivator (PBW 343) showed the significant low response to stress. They also performed in-silico analysis for characterizing and molecular modelling of WZYb via bioinformatic tools such as 3D structure analysis, active site prediction, secondary structure prediction, homology search and motif analysis. Moreover, the theoretical data obtained and physio-chemical analysis of WZYb confirmed that it belongs to group 2 LEA protein. Furthermore, they expressed the recombinant WZYb protein in E. coli and determined it size to be around 11 kDa via SDS- PAGE. The functional assessment of the WZYb in *E. coli* unveiled that it is important for maintaining the bacterial growth and survival of E. coli under different stress conditions (Rakhra *et al*., 2017).

Artur *et al*., 2019 in his studies identified six LEA proteins and worked upon there structure and function of these proteins in Xerophyta schlechteri during water

limiting condition. In silico assessment of XsLEAs revealed about their hydrophilic and disordered nature. Further, circular dichroism (CD) analysis revealed that these proteins remain in unstructured confirmation in water and acquires secondary structure in solution of hydrophobic nature, which signifies these structural dynamics plays vital role in functions of these proteins in subcellular environment. Further to show protective properties of XsLEAs its ability was tested to preserve the enzyme activity of lactate dehydrogenase during drought, oxidative and heat stress and expression analysis was assessed by growing E. coli exposed to salinity stress. Furthermore, these identified proteins i.e., XsLEA1-8 were determined that they belong to group 1 LEA protein family, and exhibit noteworthy protective and disorder-to-order propensity potential in both in vitro and in vivo analysis (Artur *et al*., 2019).

Zan *et al*., 2020 conducted a study in which LEA genes were identified in *Aegilops tauschii*, barley, *Brachypodium distachyon*, *Triticum aestivum*, *Triticum dicoccoides* and *Triticum urartu*. Further, based on the presence of conserved domains TaLEA (wheat LEA gene family) genes was categorized into eight sub-families. Interestingly, all the TaLEA genes showed the presence of a smaller number of introns i.e., < 3 on the 21 chromosomes in uneven manner. Furthermore, 9 pairs of segmental duplication gene and 39 pairs of tandem duplication genes were identified in LEA gene family of wheat. The extensive investigation on segmental duplication and tandem duplication revealed that they both play significant role in the TaLEA gene family expansion. Additionally, on performing the qRT-PCR and transcriptome data analysis, the TaLEA genes showed the diverse expression patterns on tissues and also reported about their association with different abiotic stresses, particularly, cold and salt stress. This study foundation information and deep insight about the wheat LEA gene family (Zan *et al*., 2020a).

Ali *et al*.,2020 conducted a study in which associated of LEA proteins have been done with drought stress, and LEA family has been sub-divided into seven groups. In this study, CD spectrum and Fourier transform infrared spectrum (FTIR) techniques were used for determining the structural and molecular properties of LEA proteins. Further, vesicles and artificial lipid granules were used to assess their mechanism of action with the membrane. Correspondingly, they used different genetic engineering

techniques like green fluorescent protein (GFP) tagging technique, overexpression, RNA interference to get better insight about the localization and function of these proteins in plants. Role of LEA proteins with respect to drought stress in wheat have been explained and evident result have been gathered justifying the activation of specific protein under extreme water loss condition. Further, elucidation of safety mechanism adopted through natural mechanism by various plant to overcome the different kind of stresses, in wheat plant, the LEA proteins were determined to serve the same purpose (Ali *et al*., 2020).

Koubaa and Brini in their studies done the functional analysis of wheat LEA proteins of group 3 in Arabidopsis thaliana under biotic and abiotic stress conditions and isolated three genes TdLEA3. There findings revealed the highly disordered nature of TdLEA3 in fully hydrated condition and found that it has ability to inactivate dehydrogenase under stress conditions. Also, they overexpressed TdLEA3 in particular plant to investigate its role by assessing their pattern of expression in abiotic stress conditions in 2 wheat genotypes. Resulting in generation of high tolerance to salinity and oxidative stress in genetically modified Arabidopsis in contrast to wild type (Koubaa & Brini, 2020).

Zayed *et al*., 2020 conducted a study to identify the cold-responsive gene important for developing cold-tolerant crop, to increase productivity of crops throughout temperate regions. In this study, *in-silico* characterization of novel ABA responsive and water deficit gene present in wheat was done. The analysis showed the sequence similarity these gene with the known abiotic stress related genes found in different plants, for instance, *Arabidopsis thaliana* having RD29B, *Craterostigma plantagineum* having CDeT11 and Spinacia oleracea having CAP160.

Further, Homology studies revealed that gene of interest share close homology with these genes and functional analysis revealed that the specific genes might share at least two functions which are associated with abiotic stress conditions, out of one perform the similar function as that of LEA protein (Zayed *et al*, 2020).

Sharma *et al*., 2020 isolated water stress responsive cDNA and sequenced followed by molecular 3D modelling which encodes for the dehydrin-like boiling soluble protein *TaBsSRP1* and *TaBsSRP2* by exposing seedlings to water stress from

wheat PW175 i.e., drought tolerant cultivar. In silico analysis of TaBsSRP1 was done with help of bioinformatic tools which explained it physio-chemical properties, stated that it belongs to LEA group 3. This study suggests possible mechanism of boiling soluble proteins in drought conditions (Sharma *et al*., 2020).

Liu *et al*., 2020 isolated novel transcription factor 'bHLH' related to drought stress from wheat cDNA library i.e., TabHLH49, and treated it with cold and drought stress via one yeast hybrid system. Further, Real time PCR was used to determine tissue-specific as well as drought-stress responsive expression in wheat of isolated stress responsive gene TabHLH49.

Also, the verification in Y1H and electrophoretic mobility shift assay was done to elucidate that TabHLH49 protein bound and interact with the promoter region of WZY2 dehydrin protein of the wheat. Moreover, dual-luciferase assays results revealed that it can positively control the WZY2 dehydrin expression. It can help to improve stress tolerance ability in wheat(Liu *et al*., 2020).

Tiwari *et al*., 2021 studied details of dehydrin and its gene family; providing complex, progressive, interlinked and comprehensive understanding of dehydrin family. They stated that dehydrin plays important role in plants to overcome the stress conditions. They act as chaperons, chelators and cryo-protectants. Also, transcriptional regulation in cells is affected by dehydrins during stress conditions this is because they regulate the stress responsive genes. This study also provides the evidence about the participation of dehydrins in histone modification and indirect relation with epigenetic process, as it known to that histone modification has positive impact on the dehydrin as well as other drought responsive genes expression. This change in epigenetic regulation further impacts the genome expression by histone mutation, DNA methylation and post-translational histone modifications. These differences in histones and DNA play vital role in regulating gene expression and crop growth exposed to different environmental stress conditions (Tiwari & Chakrabarty, 2021).

Kamara *et al*., 2022 studied highly genetic variations among the parent variety and their F1 phenotype hybrids of wheat under abiotic stress conditions, other optimal conditions and heat stress. Parental genotypes as well as their cross groupings were exposed to stress tolerance gene i.e., DREB (dehydration-responsive element binding

2 gene), which shows its expression as a response to abiotic stresses. Further cluster analysis was done and it classified the parent plants as well as their crosses into 4 categorizes differing from heat tolerance to heat sensitive based on the standard heat indices out of which P2 and P4 (parental genotypes) were found out to be good source of valuable alleles that are accountable for high yield even under heat stress. In addition, DNA sequence analysis was also performed on DREB transcription factors and determined that heat tolerant wheat varieties had the highest homologies with the dehydrin gene sequence (Kamara *et al*., 2022).

Dehydrins (member of LEA group-2 family)

Dehydrins are ubiquitously distributed among the different group of plants. Additionally, immunological evidences are available which reports about the presence of dehydrins in algae, cyanobacteria, ferns and liverworts (Close & Lammers, 1993; Eui Cheol Kim *et al*., 2012). Dehydrins have also been characterized from moss *Physcomitrella patens* (Agarwal *et al*., 2017). Dehydrins are not only expressed during the late stage of embryogenesis in mature seeds but also found to be accumulated in abscisic acid treated plants (Talanova and Titov, 1994), as well as plant exposed to salt (Info *et al*., 2020; Nylander *et al*., 2001), drought (Wei *et al*., 2014), and temperature stress conditions (Nylander *et al*., 2001).

Sequence and Architecture of Dehydrin

Dehydrins sequence architecture has been reported to contain interspersed motifs (conserved) along with inadequately conserved regions and is categorized on the basis of variable number of conserved motifs. The size of these proteins is accorded to vary from 9.6-70 kDa (Eui Cheol Kim *et al*., 2012; Labhilili *et al*., 1995). Though molecular weight of 200 kDa has also been reported earlier for dehydrin, but this range of molecular weight is evident for dehydrin proteins as determined by SDS-PAGE. Due to high net negative charge and comparatively low binding with the SDS the dehydrin migrates very slowly in the SDS-PAGE; therefore, exhibit a comparably larger size in the gel. The distinguishing feature of all the dehydrins is the presence of K-segment (EKKGIMDKIKEKLPG), a 15-amino acid long lysine-rich conserved domain. This domain is generally known to be present near the C-terminus of the dehydrin protein

(Campbell & Close, 1997). Even though, the position and number of K-segments may vary in dehydrin but all dehydrins are known to contain K- segment. Other characteristic features of dehydrin involves the presence of S-segment (a track of Serine residues); Y-segment, (T/VDEYGNP, a consensus motif) present near the N-terminus; and Φ-segments (less conserved regions, generally rich in polar amino acids) (Campbell & Close, 1997).

K-Segment

Early analysis of six LEA proteins by (Stacy & Aalen, 1998) proposed that 4 of the proteins encompasses stretches of amino acid that could occur as an amphipathic helices. These stretches were later identified as K-segments. The K-segment is the conserved segment found in dehydrins from all species. Though a number of variations also occurs in the K-segment of dehydrins therefore the conservation is not absolute. The most conserved residues are Lysine-Isoleucine-Lysine-Glutamic acid which occurs in central portion of this segment. The residues flanking this central portion vary to some extent (Graether & Boddington, 2014). The sequence of the K-segments shows the similarity with class A2 lipid-binding amphipathic α-helical segment found in α-synucleins and apolipoproteins (Zhang *et al*., 2006). Extensive studies on class A amphipathic helices have unveiled that they contain well defined polar as well as non-polar faces with residues having negative charge and opposite to hydrophobic faces, whereas, the residue with positive charge were found at the polar/non-polar interface (Rorat, 2006). Many studies stated that the K-segments proficiently forms the α-helices of amphipathic nature. In aqueous form, K-segments generally show randomly-coiled structure.

Additionally, it has been observed that the formation of α-helix structure occurs in the presence of membrane mimics and membranes (Candat *et al*., 2014). An example of a dehydrin that undergoes a structural change in the presence of membranes is purified cowpea dehydrin. When analysed in free aqueous solution, the dehydrin is typically unstructured, as indicated by spectra of circular dichroism (CD). However, when analysed in sodium dodecyl sulphate (SDS) micelles, it exhibits the spectrum of typical α-helices (Ismail *et al*., 1999). Removal of Y and S-segments from maize DHN1

still induced α-helicity in presence of micelle as depicted via CD spectrum (Koag *et al*., 2003a). In addition, it has been observed that the α-helix transition is not observed in the same vesicles when either one or both K-segments are removed from wheat DHN1 (Koag *et al*., 2009).

Y- Segments

The Y-segment is composed of amino acid sequence i.e., (V/T) D(E/Q) YGNP, which contains highly conserved aspartic acid and glycine-asparagine-proline residues. This sequence is also stated to share similarity with binding site of bacterial nucleotide (Campbell & Close, 1997)(Close, 1997) though there is no published report, in support of this idea.

ϕ-Segments

The Φ-segments are flexible, glycine-rich domains that are interspersed among the other segments in dehydrins. These domains are highly variable in sequence and are thought to confer flexibility to the protein, allowing it to adopt different conformations under different environmental conditions (Close, 1997). The commonly found amino acids in ϕ -segments are Glycine, Glutamine, and Threonine, while, Phenylalanine, Cysteine, and Tryptophan presence is recorded ≤1%(Graether & Boddington, 2014).

S-Segment

The S-segment contains 5-7 serine residues in a row in amino acid stretch and it starts with Serine-Aspartic acid (Graether & Boddington, 2014). The S-segment is involved in nuclear localization of the protein upon phosphorylation. For example, maize Rab17 (DHN1) first gets phosphorylated before it binds with a signal peptide during nuclear localization (Mehta *et al*., 2009). ERD14 is stated to have calcium ion binding potential which is phosphorylation- dependent and increase in S-segment phosphorylation is according during stress induced by cold (Alsheikh *et al*., 2003). ERD10 and COR47 have also been reported to showing similar binding potential. The S-segment ion binding potential have been found to have crucial importance for dehydrin protection in plants during induced by salinity and drought. Phosphorylation

of two *Thellungiella* dehydrins (TsDHNs) induced in lab conditions is reported to show very slight structural variation. On contrary, they have been reported to show shift in structure to conformation of α-helical on binding with LUVs, indicating the S-segment phosphorylation improves the localization of membrane (Rahman *et al*., 2011).

On the basis of the number as well as order of the Y, K and S -segments, the dehydrin are further subdivided into five subclasses i.e., YnKn, YnSKn, KnS, Kn and SKn**.**

Table 1: Allagulova *et al*. (2003a) identified different sub-families of dehydrins and characterized their distinct features.

Y_nSK_n	The most typical dehydrin architecture is considered to be caused by ABA or drought but not by extreme cold. The YSKn architecture is shared by all barley dehydrins.
K_n	This include 2 to 9 K-segments and are brought on by ABA, dehydration, and freezing temperatures. When wheat cultivars that are robust to freezing are subjected to 4°C for 24 hours, the WCS120 concentration increases by 20 times compared to untreated plants (Houde et al., 1992).
SK_n	Usually have one S-segment and two to three K-segments, and they are most often brought on by cold weather. In direct reaction to field temperatures, two SKn type dehydrins from *Cichorium intybus* (Chicory) accumulated more in wild trees (Mingeot et al., 2009).
K_nS	The K-segment of this family begins with an (H/Q) KEG segment, which is unique to the family. Example, PtrDHN-6 from poplar and DHN13 from barley.
Y_nK_n	This class is linked to seed embryos' ability to withstand cold temperatures. An YnKn type dehydrin from *Vigna unguiculata* (cowpea) has been discovered and sequenced (Ismail & Hall, 1999).

Distribution of dehydrin in plant tissues

In different plant species, the tissue specific distribution of dehydrin has been assessed using immunohistochemical methods. Dehydrins has been found to be present almost in every tissue in plants. Different studies conducted on dehydrins have demonstrated that they do not only accumulate during the later stages of embryogenesis and under water deficit conditions (Goday *et al*., 1994), but their presence has also been accorded in different plants during normal growth and development of plants (Rorat,

2006). However, the level of expression of the dehydrins were found to be higher under stressed conditions such as drought, salinity, and temperature stress (Allagulova *et al*., 2003b; Ingram & Bartels, 1996). The dehydrin p-80 was determined to be localized in epidermis of shoots and vascular tissue in case of cold-acclimated barley, whereas in case of non-acclimated leaves, the presence of dehydrin P-80 was determined to be in vascular bundles (Bravo *et al*., 1999). Another dehydrin PCA60 from peach was found to localize in all tissue of shoots collected in the month of January (Wisniewski *et al*., 1999). Accumulation of TAS14 dehydrin was recorded in the vascular tissue of the shoot, in cortical cells (differentiated) of the leaves and stems and in developing adventitious root primordia in salt-stressed tomato via immunolocalization method(Goday *et al*., 1994). Another study reported about localization of RAB17 dehydrin of maize in mature embryos (Goday *et al*., 1994). Another study reported about accumulation of WCOR410 dehydrin in wheat and strawberry in the vascular transition area (Danyluk *et al*., 1998b; Houde *et al*., 2004). A carrot dehydrin i.e., ECP40 localization was determined to be in zygotic embryos and endosperm in mature seeds (Kiyosue *et al*., 1993).

Moreover, during normal growth localization of different types of DHNs have been reported in different tissues. However, there are some dehydrins which showed tissue specific expression when subjected to stress conditions than under normal growth conditions. For instance, ERD14 and ERD10 (Lti29) from *Arabidopsis* were found to be present in cells of all tissue under cold stress condition, while primarily they were present in vascular tissues and root tips during normal growth conditions (Nylander *et al*., 2001). Another two dehydrins P-80 from Hordeum vulgare and DHN24 from *Solanum sogarandinum* showed exactly similar distribution pattern like ERD10 and ERD14 (Rorat, 2006)S.

Subcellular localization of dehydrins

Furthermore, sub-cellular localization of dehydrins data has revealed about their localization in different cellular compartments such as cytoplasm, chloroplast, vicinity of the plasma membrane, mitochondria, vacuoles and nucleus (Danyluk *et al*., 1998a; Godoy *et al*., 1994; Heyen *et al*., 2002; Rinne *et al*., 1999). Though, the dehydrins are

primarily known to localize in nucleus and cytoplasm. For example, OpsDHN1 present in *Opuntia streptacantha* are found to be accumulated in the nucleus and cytosol (Hernández-Sánchez *et al*., 2015). PpDHNA and PpDHNB are two dehydrins identified in moss *Physcomitrella patens* and their localization was determined to be in nucleus and cytosol (Liu *et al*., 2017). However, the DSP16 dehydrin in *C. plantagineum* has been reported to localized in the cytosol (Rorat, 2006). RAB21 dehydrin of rice has been reported to be localized in the cytosol Mundy and Chua, 1988). Another wheat dehydrin, WCOR410 was reported to be localized in the vicinity of the plasma membrane of cells in the vascular transition area (Danyluk *et al*., 1998a). HbDHN1 dehydrin from *Hevea brasiliensis* has been reported to be accumulated in the plasma membrane (Cao *et al*., 2017). PCA60, a peach dehydrin has been reported to be associated with cell organelles, as in this case the associated organelle was chloroplasts along with nucleus and cytoplasm (Wisniewski *et al*., 1999). CuCor19 from *Citrus unshiu* was found to localize in mitochondria (M. Hara *et al*., 2003). Further, fractionation study conducted on CAP85, a spinach dehydrin was found to be associated with endoplasmic reticulum (Neven *et al*., 1993). Furthermore, the dehydrin from maize showed their association with lipid bodies and proteins in addition to nucleus and cytosol (Battaglia *et al*., 2008).

Table 2:Tissue-specific localization of different dehydrins

Group	Organism	Protein	Localization	References
YnSKn	*Daucus carota*	ECP40	Endosperm and zygotic embryos in mature seeds	(Kiyosue et al., 1993)
	Zea mays	RAB17/DHN1	Parts of embryos and in endosperm of mature seeds	(Goday et al., 1994; Lång & Palva, 1992)
	Arabidopsis Thaliana	RAB18	embryos, endosperms of mature seeds and stomatal guard cells	(Goday et al., 1994; Lång & Palva, 1992; Nylander et al., 2001)
SKn	*Triticum aestivum*	WCOR410	Vascular transition is of roots, leaves and crown	(Danyluk et al., 1998a)
	Pisum sativum	DHN-COG	Developing cotyledons and dehydration- stressed seedlings	(Rorat, 2006)
	Arabidopsis thaliana	ERD10 ERD14	Root tips, vascular tissues of roots, stems, leaves and flowers	(Nylander et al., 2001)
YnKn	*Glycine max*	MAT1	Seeds	(Cheng et al., 2013)
	Glycine max	MAT9	Seeds	(Momma et al., 2003)
	Prunus persica	PCA60	Cells of all tissues of shoots	(Wisniewski et al., 1999)

Functional studies for dehydrins

Dehydrins are known to be the stress responsive proteins present in plants and are speculated to protect plants cells from damage caused by dehydration (Ingram & Bartels, 1996). In normal conditions, wide distribution of dehydrins is accorded in

vegetative parts of the plants indicating that dehydrins might have imperative role in normal growth and development of plants. Diverse role of dehydrins have been comprehended till date. Moreover, diverse functional role of dehydrins in plants have been extensively studied, which encompasses antioxidant activity, cryoprotective activity, metal chelation and lipid binding property.

The experiments conducted in labs have unveiled that most YSKn type DHNs binds to lipid vesicles having phospholipids (acidic) (Koag *et al*., 2003a), while most KnS-type DHNs tends to bind with metals (Hara, 2010b). Other than this, DHNs are known to scavenge hydroxyl radicals (Hara *et al*., 2004), exhibit cryoprotective potential towards freezing-sensitive enzymes (Wisniewski *et al*., 1999) and protect lipid membranes from peroxidation (M. Hara *et al*., 2003). The extensive studies in this direction have uncovered that the SKn and Kn type dehydrins are primarily involved in drought resistance and cold acclimatization.

The ability of dehydrins to bind lipids and proteins

Usually, dehydrins are generally found to be in intrinsically unstructured form in aqueous solution, but its transforms itself into secondary structure when it binds to a target molecule (Garay-Arroyo *et al*., 2000). The extensive studies conducted on dehydrins have disclosed that all dehydrins have K-segment resembling to lipid-binding class A2 amphipathic α-helical segment, generally found in -synucleins and apolipoproteins (Davidson *et al*., 1998). Previously published literature has provided the evidence that α-synuclein protein has lipid binding ability and is known to bind with phospholipids (acidic) and vesicles via small diameters. Moreover, this type of binding is stated as the α-helicity (Davidson *et al*., 1998). These finding signifies that K-segment might interact with denatured proteins and membranes. Experiments conducted on dehydrins have reported their interaction with lipid molecules, which indicates the involvement of K-segment in such interactions (Close, 1996). Furthermore, numerous in-vitro experiments have been conducted to study the membrane binding potential of dehydrins using different membrane systems like membrane-mimicking detergent micelles (M. Hara *et al*., 2003; Ismail *et al*., 1999; Koag *et al*., 2003a).

The first study to determine the membrane binding potential of dehydrin was done using Y2K type *Vigna unguiculata* (cowpea) dehydrin of 26.5 kDa molecular weight. In this study, dehydrin CD spectrum was analyzed in the presence of SDS micelles (Ismail *et al*., 1999). The result obtained from the study reported the high number of negative peaks near the 200 nm declining towards the negative intensity in the presence of micelles and weak negative minimum at approximately 220 nm was also accorded showing elevation in negative intensity. These variations in data indicates the loss of random coil structure and formation of helical structure in dehydrin in presence of micelles (Ismail *et al*., 1999). Another study reported about the K3S type dehydrin CuCOR19 from *Citrus unshiu* to form α-helical structure in the presence of SDS (Hara *et al*., 2003). The CD spectrum analysis showed minimum signal at 197 nm in the absence of SDS, which signifies the random coil nature of the dehydrin. Whereas, on the addition of SDS the signal minimum at 197 nm showed the strong negative signal within the range of 205-235 nm signifying the formation of α-helices in CuCOR19 (T. Hara *et al*., 2001).

Studies conducted on YSK2-type Zea mays (Maize) dehydrin, also known as ZmDHN1 or RAB17 isolated from mature seeds has provided the evidence about the lipid binding potential of dehydrins (Koag *et al.*, 2003). In *in-vitro* condition, ZmDHN1 binds to lipid vesicles containing acidic phospholipids. Further, intensive investigation showed the favourable binding of this dehydrin with vesicles having small diameter and fabricated from negatively charged phospholipids encompassing phosphatidylglycerol (PG), phosphatidyl-Ser (PS) and phosphatidic acid (PA) (Koag *et al*., 2003b). More direct evidence about the binding of dehydrins with PA-derived vesicles have reported the apparent increase of 9% in the α-helicity of the protein. Additionally, increase in α-helicity of dehydrin on binding with phospholipid vesicles in laboratory conditions suggests that dehydrins might adopt α-helical structures on associating with vesicles in *in-vivo* condition and two K-segments present in dehydrin protein might be involved in membrane binding (Koag *et al*., 2003b). Another report on ERD10 and ERD14 dehydrin of *Arabidopsis* and its interaction with liposomes fabricated using (1:1) PC:PS lipids was reported. Moreover, addition of 800nM NaCl during interaction of dehydrins with liposome was reported to substantially reduce their binding. Besides that, the CD

spectra showed the no gain of α-helicity in ERD10 and ERD14 dehydrin protein indicating different mode of binding (Kovacs *et al*., 2008). Further binding of dehydrins with functional groups on the head of lipid (negatively charged) indicates the involvement of positively charged K-segments in the interaction. In 2009, Koag and his colleagues conducted a study on maize protein ZmDHN1 by generating mutants by deletion in which one mutant contains first K-segment (ΔK1), second contains the second K-segment (ΔK2) and one mutant which does not contains both the K-segment (Koag *et al*., 2009). On evaluating the interaction of these proteins with lipid vesicles showed an increase in α-helicity in mutants containing either of ΔK1 or ΔK2 segments, whereas, no structural change was accorded in ΔK3 mutant. This experiment provided the clear indication that the K-segment are involved during membrane binding shown by ZmDHN1 protein. Another study proposed that the flanking sequence of the K-segment might be playing imperative role during lipid binding. In support, membrane binding of LTI30 dehydrin (K6 type) from *Arabidopsis* was modified by histidine residues present on the either side of the K-segment (Eriksson & Harryson, 2011). On evaluation, modification in LTI30 resulted in induced aggregation of lipid vesicle in a pH-dependent manner. Though, these histidine residues were not considered to be crucial for membrane binding in case of other dehydrins.

Enzyme protection activity

The published literature has unveiled that dehydrins involved in cryoprotection of enzyme lactate dehydrogenase (LDH) (T. Hara *et al*., 2001; Momma *et al*., 2003) ((T. Hara *et al*., 2001; Momma *et al*., 2003). For instance, during the repeated thawing and freezing cycle, LDH completely losses its potential because of aggregation and denaturation (Hughes and Graether, 2011). Whereas, when dehydrins or other cryoprotective proteins were added in the enzyme solution resulted in the sustainability of enzyme activity due to their protective action. CuCOR19 dehydrin obtained from *Citrus unshiu* has been reported to exhibit protective activity against lactate dehydrogenase, which got inactive due to freezing (Hara *et al*., 2001). Additionally, Dehydrins obtained from *Betula pubescens* has been reported to showed cryoprotection of α-amylase enzyme subjected to cold-induced stress (Rinne *et al*., 1999). PCA60

dehydrin from *Prunus persica* has been reported to exhibit anti-freeze and cryoprotective activity (Wisniewski *et al*., 1999). Moreover, this dehydrin has also been reported protect the LDH enzymatic activity even after repeated thaw-freeze cycles (Wisniewski *et al*., 1999). Lately, published literature has provided the evidence that few dehydrins have the potential to protect they enzyme exposed to high temperature stress. Furthermore, supporting literature is available for DHN-5 (Wheat dehydrin) in protecting ß-Glucosidase, glucose oxidase and LDH subjected to high-temperature stress (Brini *et al*., 2011). Extensive research on DHN-5 (Wheat dehydrin) uncovered that their K-segments play vital role in exhibiting protective activity. Beside this, truncated forms of these DHN-5 having only one or two K-segments showed the protection of β-glucosidase and LDH enzyme activity even after the exposure to stresses in *in-vitro* conditions (Drira *et al*., 2015).

Radical-scavenging ability

High temperature, salinity, and drought are the primary stress factors that can cause oxidative stress and cellular damage (Mahajan & Tuteja, 2005; Mittler, 2002). The formation of reactive oxygen species (ROS) in cells, such as superoxide radical (O2), singlet oxygen (1O2), hydroxyl radicals (·HO), and hydrogen peroxide (H2O2), can lead to oxidative stress. Effective scavenging mechanisms that regulate the excessive accumulation of ROS are necessary to overcome these damaging conditions and develop tolerance to these stresses. Peroxisomes and chloroplasts are the two major cellular organelles that regulate the oxidative load in plants under stress. In addition, a variety of mechanisms work together to protect plants from oxidative damage, such as the production of radical scavengers and antioxidants and increased enzyme synthesis in response to the dismutation of free radicals. However, an imbalance between scavenging mechanisms and reactive oxygen species can trigger unregulated oxidative cascades.

Recent studies have proposed that dehydrins also possess radical scavenging activity. The radical scavenging potential of dehydrins has been reported in some recent research. For example, CuCOR19, a K3S-type dehydrin found in Citrus unshiu, has been observed to prevent the peroxidation of liposomes in in-vitro conditions and

scavenge the hydroxyl radical (·HO) synthesized via the Fe2+/H2O2 system, as well as the peroxyl radical generated from 2,2′-azobis(2-amidinopropane) (Hara *et al*., 2003, 2004). Lipid peroxidation is a process in which polyunsaturated fatty acids are degraded by free radicals, resulting in the production of lipid radicals. Studies on the CuCOR19 dehydrin have shown that the radical scavenging activity of this protein is attributed to the histidine, glycine, and lysine residues present in its amino acid sequence (Hara *et al*., 2004). Previous literature has also demonstrated that dehydrins play a protective role against oxidative damage caused by metal ions and reactive oxygen species (ROS) (Zhang *et al*., 2006). Earlier reports on KnS-type dehydrins have also demonstrated their ability to bind metal ions (Hara *et al.*, 2005). Furthermore, the sequestration of metal ions inhibits the production of hydroxyl radicals (·OH) via the Fenton's reaction (Hermes-Lima *et al*., 2015). The presence of the K-segment in dehydrins also provides protection against oxidative damage by exhibiting a shielding effect (Y. *et al*., 2017). Furthermore, the composition and number of amino acids present in dehydrins also contribute to their preservation ability, as they contain a large number of histidine, glycine, and lysine residues that can sequester transition metal ions, making them inaccessible for the Fenton reaction.

Metal-binding activity

Few dehydrins have been reported to have metal binding potential depending on their structural confirmation. In 2001, Hara and his colleagues have reported about CuCOR15, a KnS-type citrus dehydrin, which exhibits metal binding potential. In this study, metal binding potential of CuCOR15 dehydrin was determined via immobilized metal ion affinity chromatography (IMAC). The result obtained from the study reported the binding of CuCOR15 with Co^{2+}, Cu^{2+}, Fe^{3+}, Ni^{2+} and Zn^{2+}, while metal ions like Ca^{2+}, Mg^{2+} and Mn^{2+} did not show any binding activity. Further, Cu^{2+} showed the highest metal binding affinity on detection. Moreover, extensive investigation unfolded the involvement of Histidine-rich sequence i.e., HKGEHHSGDHH in Cu^{2+} binding with dehydrin, which is located near the N-terminal end in CuCOR15(Hara *et al*., 2001).

Furthermore, ERD14 and VBA45 from *Arabidopsis* and celery have been reported to involve in phosphorylation-dependent calcium binding (Alsheikh *et al*., 2003; Heyen *et al*., 2002). Another study reported about ERD10 and COR47 (two acidic subclass dehydrin), which also showed phosphorylation-dependent calcium binding via casein kinase II (CKII) (Alsheikh *et al*., 2003). AtHIRD is another dehydrin obtained from *Arabidopsis thaliana*, which showed high copper binding potential because of the presence of histidine-rich domain, which aid proteins in preventing the hydroxyl and hydrogen peroxide radical's generation in the Cu–ascorbate system (Hara, 2010b).

Contribution to stress tolerance

Extensive literature has been published in past which have shown positive association between the dehydrin accumulation and their role in tolerance against drought, freezing and salinity stress (Houde *et al*., 2004; Lång & Palva, 1992; Nylander *et al*., 2001; Rorat, 2006). Additionally, heterologous expression in yeast and transgenic plants have reported about the overexpression of dehydrin genes, which explains about the imperative role of dehydrins in generating tolerance against abiotic stress.

For instance, CuCOR19 i.e., (K3S-type) citrus dehydrin gene, overexpression in transgenic tobacco plant has been reported to slight decrease the ion leakage while exposing it to freezing and chilling stress (Hara, 2003). Another study reported about overexpression of ERD10 (SK3-type) or LTI29 and LTI30 (K6-type), that are they multiple dehydrin genes found in *Arabidopsis* and have been reported to improve their tolerance to freezing and enhance their survival rate in low temperature, signifying the role of dehydrins in generating tolerance against freezing (Puhakainen *et al*., 2004). Another study reported about musaDHN1 i.e., SK3-type dehydrin of banana, whose overexpression increase their tolerance against salt and drought stress. Moreover, TAS14 dehydrin has been isolated and well-characterized in tomato, which primarily induce due to action of abscisic acid (ABA) and osmotic stress (mannitol and NaCl). The overexpression of tas14 dehydrin gene has been reported to improve the tolerance against osmotic stress imposed by salinity and drought in tomato (Godoy *et al*., 1994). Further, genetically modified tomato plant overexpressing tas14 gene regulated by

CaMV35S promoter exhibit the improved tolerance against salinity and drought, without affecting the growth of plant (Muñoz-Mayor *et al*., 2012). Kumar and his colleagues reported about OsDhn1 (rice dehydrin gene), which showed their involvement in improving the tolerance against salt and drought stress via scavenging of reactive oxygen species during overexpression of OsDhn1 gene. SK3-type DHN (ShDHN) gene, a cold-induced gene has been isolated from *Solanum habrochaites* (wild tomato species) and reported to improve tolerance against drought and cold stress. Furthermore, it showed enhanced growth of seedling under osmotic and salt stress in cultivated tomato (H. Liu *et al*., 2015). The results of these experiments indicate that dehydrin can respond to stress and improve tolerance levels in transgenic plants when ectopically expressed.

Genome-wide analysis of stress responsive genes using *in-silico* tools

In *C. sinensis* (tea plant),48 LEA2 family genes were identified using Hidden Markov Model(HMM) profiles.It was found that all these 48 genes contain full open reading frames and they were further distributed in seven groups based upon domain analysis using PFAM. Physiochemical analysis carried out using compute_pi software revealed that isoelectric point ranged from 10.16 to 4.72 while the molecular weight ranged from 7.55 to 29.47kDa. GRAVY (grand average of hydropathy) index was also predicted using ProtParam tool which showed that out of 48 proteins, 26 were basic while 12 were acidic in nature. Subcellular location was predicted using WoLF PSORT tool which indicated that majority of CsLEA proteins were primarily localised in cytoplasm, nucleus and chloroplast. Phylogenetic relationships were built using CLUSTALX 2.1 software and MEGA 7 with 1000 bootstraps and based upon this, 48 CsLEA genes were clustered into seven distinct subfamilies. Gene structure was analysed using GSDS web server in which it was found that majority of CsLEA proteins contained no intron or only one intron. Only 5 were found to have 2 or 3 introns. Motif analysis using MEME web server was done which revealed that all the members of CsLEA proteins possess several group specific conserved motifs like lysine rich k segment. (Jin *et al*., 2019).

In *Brachypodium distachyon*, L. 36 LEA genes were identified and the data was used for functional analysis especially under water deficient conditions using *in silico* tools. Using PFAM database, proteins which contain atleast one conserved repeat of LEA were identified and classified as LEA family member. The typical LEA2 repeat was found among the 28 members out of 36 LEA proteins. Also, physiochemical analysis was done in which pI value (4.40-11.1) and GRAVY index (0.48 to -1.423) was observed and it also revealed that, 19 out of 36 genes were hydrophobic in nature while others were found to be hydrophilic in nature. The segmental and tandem duplications revealed the duplication in only the 8 genes. Phylogenetic studies was done using ClustalW and MEGA 5.0 software which revealed that BdLEA proteins are further sub divided into eight groups (Filiz *et al*., 2013).

In silico studies of wheat cyclophilins was done using various *in silico* tools, which resulted in identification of 83 stress responsive cyclophilins genes. Genomic characterization of these cyclophilins was done which revealed most of the genes were localized in chloroplast using LOCTREE3 software. Also, gene structure analysis was performed using GSDS web server in which intron exon organization was predicted which indicated the functional divergence of cyclophilins. (Singh *et al*., 2019).

Genome-wide analysis and abiotic stress responsive pattern of heat shock proteins in *Triticum aestivum L*. (*TaHsfs*) was done using HMM which resulted in identification of 82 *TaHsfs* located on 21 chromosomes. Phylogenetic tree was generated with neighbour joining method with bootstraps 1000 using MEGA 5.0, which indicated the prevalence of various homologues of these proteins. Intron exon organization (gene structure analysis) was generated using GSDS web server which indicated a highly conserved organization for these genes. Among these heat shock genes, 62 genes contained 2 exons while 10 hsf genes were found to contain three exons. Also, nine genes were found out to be intron less which point out towards their role in stress response in plants. Motif analysis was also done using MEME software in which lysine rich conserved motifs were identified in majority of the proteins (Duan *et al*., 2019).

Another study on wheat family was done in which LEA genes were identified in *Triticum aestivum (281), Hordeum vulgare (53), Triticum urartu (151), Triticum dicoccoides (89), Aegilops tauschii (99),* and *Brachypodium distachyon* (99). The wheat LEA gene family (TaLEA) was divided into 8 sub-groups on basis of domain analysis using PFAM and SMART web tool. Multiple sequence alignment and phylogenetic analysis was conducted for LEA proteins in all the four members and the results showed that the wheat, *T. urartu* and *T. dicoccoides* LEA proteins clustered together. On basis of gene annotation information available in online database, intron exon organization was performed for gene structure analysis using online GSDS web server and it revealed that 37% of *TaLEA* genes contained one intron, 1% has two introns whereas the remaining 62% of genes were having no introns. Conserved motifs were predicted using MEME web tool. Synteny analysis was carried out using McScanX tool which resulted in identification of 39 pairs of tandem repeats and 9 pairs of duplicate collinear genes playing an important role in expansion of the *TaLEA genes*. Chromosome locations were predicted using phytozomev 12.1 tool (Zan *et al*., 2020b).

In *Sorghum bicolor* L., 68 LEA genes, belonging to 8 families were identified. The Gramene database predicted them to be evenly distributed on all the 10 chromosomes with majority of them present on 1,2 and 3 chromosomes. In gene structure analysis performed using GSDS web server, most of the genes were found to be intron less. Synteny analysis was done to predict the segmental duplications and majority of paralogues were found to be segmental duplicates. In promoter analysis, cis elements like DRE, MYB, MYC and GT1which are abiotic stress responsive were identified using PAL2NAL software. Along with this biotic stress related and hormone responsive cis elements were also identified. This revealed that LEA proteins plays an important role during stress conditions as well as in developmental processes in the plant(Nagaraju *et al*., 2019).

Hypothesis

Wheat is the most common rabi crop which is consumed all over the world. Wheat is majorly grown in North India and various challenges are faced during the growth for example, drought, cold stress, heat stress etcetera. Drought, common abiotic environmental stress, which is affecting productivity of the crops. Naturally, plants have developed a mechanism in which some specific proteins get accumulated in cells which protect them from the surrounding stress. such proteins are known as stress responsive proteins and dehydrins are one of the well-known stress responsive proteins.

This study is an attempt to identify and characterize dehydrin proteins belonging to WZYb family, which is dehydrin family in wheat. This current study is first attempt to characterized the novel proteins in WZYb family *in-silico. In-silico* will provide a deep insight into the functional and structural versality of dehydrins. Additionally, expression analysis studies will be carried out to support the functional and expressional aspect of identified dehydrins under drought stress.

Research Objectives

- Identification and genomic characterization of the wheat boiling soluble WZYb.
- Expression analysis of the identified proteins under different abiotic stress conditions using qRT-PCR.
- Understanding the role of stress responsive cis elements in the abiotic stress responsiveness of different WZYb genes.

Chapter-3

Materials and methods

3.1 Genome-wide identification of dehydrin genes in wheat

The wheat dehydrin-like protein WZYb was utilized as a reference to conduct a homology search for dehydrin genes within the genome of wheat (*Triticum aestivum*). The WZYb gene is responsible for encoding the dehydrin-like protein WZYb (Rakhra *et al*., 2017). The sequence for WZYb(>LNTYGQQGHTAGMAGTGGTYGQPGHTGMAGTGTLGTDGTGEKKG IMDKIKEKLPGQH) was taken from Uniport, where data is available in the name of A0A0F7WA67 (Figure 5).

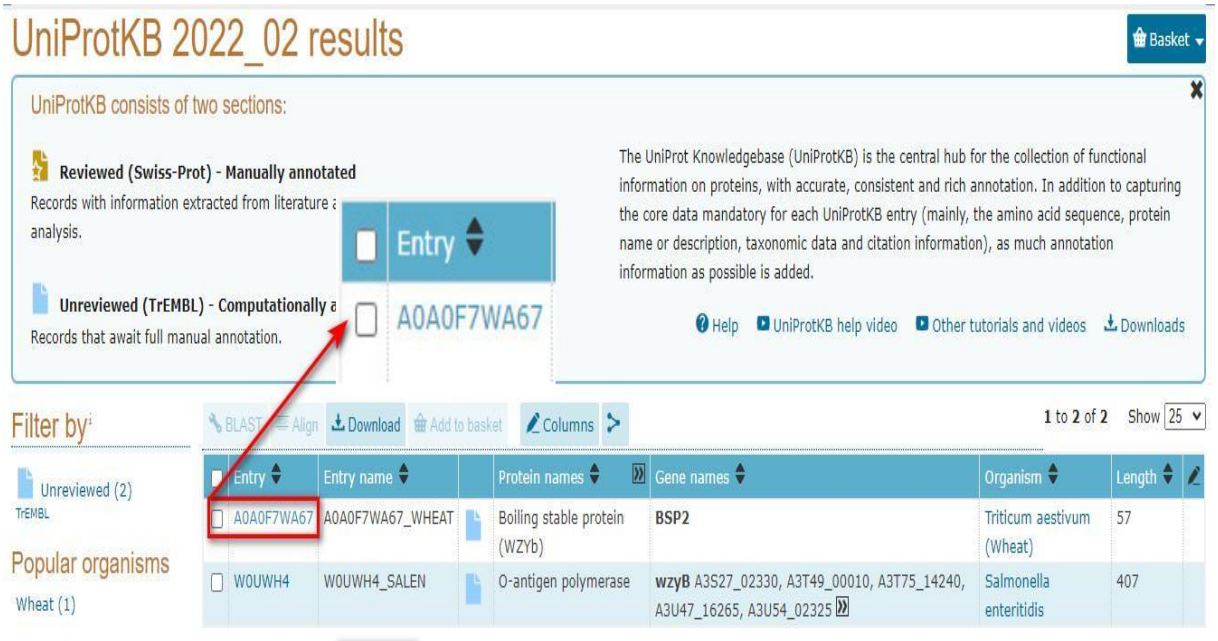


Figure 6:: The search for WZYb protein in the UniProt database

The sequence of WZYb was BLAST searched against the *Triticum aestivum* in the Ensembl plants' database, IWGSC RefSeq v1.1 gene annotation, which is the latest wheat assembly with an E-value threshold of 1.0. The homology search was done 2-3 times to identify all the significant matches. After the query search, the genome-wide data for wheat, *T. aestivum* was exported from the Ensembl Plants database (Bolser *et al*., 2017) to construct a local database based on BLAST performed and the gene hits showing similarity index (>90%) were selected for further analysis (>90%). The search identified 48 hits which were downloaded and saved as a text file with the name of

transcript IDs. The text file obtained contains all the information about identified genes like CDS sequence, cDNA sequence, exons, introns, peptide sequence, untranslated regions and genomic sequence (Figure 6).

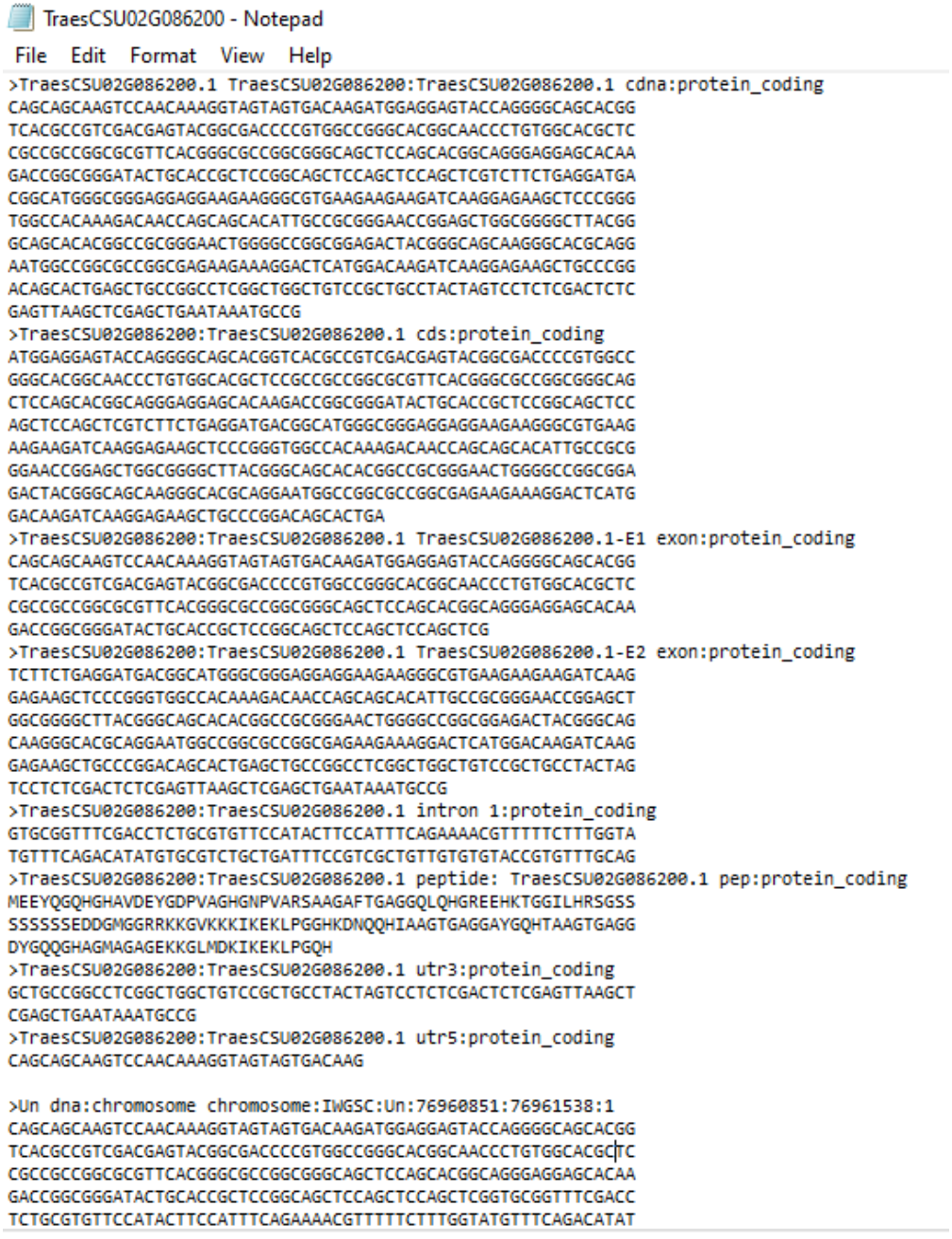


Figure 7: Text file containing genomic sequence, CDS, UTRs, introns, exons, cDNA and peptide sequences

3.2 Structure analysis of wheat dehydrin genes

For the gene structure analysis, we studied the various characteristic properties of genes like their distribution on different chromosomes, arrangement and distribution of exons, introns and untranslated regions (UTRs).

3.3 Chromosome Mapping of wheat dehydrins

The DNA sequences obtained from the wheat assembly IWGSC RefSeq version 1.1 were utilized to determine the chromosome distribution. MapChart2.32 web database was used for chromosome mapping (Voorrips, 2002). The genes were mapped to their respective chromosomes based on their coordinates, and the chromosomes were depicted proportionally. Homologous genes were identified and marked using identical colours. Representation of chromosome mapping was done using Ink Space (Inkscape (RRID: SCR_014479).

3.4 Gene structure analysis of wheat dehydrins

Various components of gene structure: introns, exons, 3'UTR and 5'UTR of each dehydrin gene were displayed using their coordinates which were retrieved from IWGSC RefSeq v1.1 using Ensemble plant database in GFF format. Gene structure was displayed and analysed using the GSDS2.0 server (Hu *et al*., 2015). The editing was done with the help of custom colours, resolution and shapes accordingly.

3.5 Physiochemical properties and analysis of conserved regions of wheat dehydrins

The compute_pi was used to determine the molecular weight and pI (isoelectric point) (Bjellqvist *et al*., 1993).

Analysis of domain was done using PFAM and Prosite (Sigrist *et al*., 2013) databases. The Prosite database was used to cross-verify the PFAM results. The amino acid sequences of dehydrin domains of each dehydrin were mined using EMBOSS extractseq web server (Olson, 2002). The presence of conserved motifs in dehydrin domains was analysed by MEME motif analysis from the MEME Suite4.12.0 web server (Bailey *et al*., 2015).

I.B.S 1.0, which is a freely available online tool was used for graphical representation of domains (Liu *et al*., 2015). The Cj-Chen Tbtools software was used for the graphical representation of motifs (Chen *et al*., 2018).

3.6 Prediction of subcellular localization of dehydrins in wheat

To predict the intracellular localization of different wheat dehydrins, LOCTREE3 (Goldberg *et al*., 2014) database was used and the peptide sequence was given as an input command. The presence of signal peptides was checked using SignalP 4.1 (Petersen *et al*., 2011) and cross-checked using TargetP2.0 server (Emanuelsson *et al*., 2007).

3.7 Disorder nature and secondary structure prediction of dehydrin in wheat

To predict the degree of disordered nature of dehydrin proteins, PONDR (Dyson & Wright, 2005b) was used with default parameters.

To predict the secondary structure of identified dehydrin proteins, the GOR database (Sen *et al*., 2005) was used which revealed the organization of alpha helix, turn, sheets and random coils in dehydrins.

3.8 Multiple sequence alignment

Multiple alignments are one of the most common bioinformatic tools used for homology i.e., to check similarity or identity, evolutionary relationships, predicting functions and structure modelling etc. The multiple sequence alignments of all proteins, coding sequences (CDS) and domains amino acid sequences were carried out using Clustal omega (Sievers *et al*., 2011) to analyse similarity, identity and differences among the sequences. The identity matrix for 48 dehydrin protein sequences indicating percentage identity and similarity was downloaded in text form and was further modified using the Matrix Global Alignment tool (MatGat v 2.02) along with the BLOSUM50 scoring matrix v(Campanella *et al*., 2003).

3.9 Active sites analysis, gene ontology and protein-protein interaction of dehydrins in wheat

The alignment of dehydrin domain sequences obtained in the previous step of multiple sequence alignment was manually edited and submitted to ESPript3.0 (Robert & Gouet, 2014) for highlighting active site residues and elements of secondary structure along with Cys residues. The results were compared to reference dehydrin protein in *Arabidopsis thaliana* (1YYC)(Banerjee & Roychoudhury, 2016).

Gene ontology was done for enrichment analysis to check the involved functional and molecular pathways using ShinyGO web server (Ge *et al*., 2020).

Protein-protein interaction was done to analyse the functional partners of dehydrins using STRING database (Szklarczyk *et al*., 2021).

3.10 Phylogenetic analysis of dehydrins in wheat

Phylogenetic analysis reveals the study of evolutionary relationships between different groups of proteins, genes or organisms (Choudhuri, 2014). Phylogenetic analysis is done in two steps: firstly, homologues are identified (with the help of multiple sequence alignment) and in the second step these homologues are compared using different phylogenetic tree construction methods to analyse the phylogenetic relationship between the species. In this study, the multiple sequence alignment of different dehydrins was used to create a phylogenetic tree via MEGA X employing neighbour-joining (NJ) method having condition of 1000 bootstrap replicates. The output obtained from MEGA X was saved in Newick format, which was further submitted to interactive tree of life (Letunic & Bork, 2019) for additional editing as well as annotations.

3.11 Synteny analysis of wheat dehydrins

The analysis of synteny between *Triticum aestivum*, *Triticum dicoccoides*, *Triticum Urartu*, and *Aegilops tauschii* was conducted using MCScanX with default settings, except for the matching size which was changed to 2 from 5 that was used for collinear and tandem gene studies (Y. Wang *et al*., 2012). Further, the synteny plots were drawn using ShinyCircos web server (Krzywinski *et al*., 2009).

3.12 Expression analysis of wheat dehydrin genes

To analyse the expression of dehydrin genes in wheat under drought conditions qRT-PCR and western blotting were done.

3.12.1 The plant material and grown under specific growth and stress conditions

The seeds of *Triticum aestivum*, PBW175 (drought tolerant) were procured from Punjab Agricultural University (PAU), Ludhiana, Punjab, India. For analysis, seeds were soaked in double distilled water (sterile) for overnight and seeds were surface sterilized via Tween 20. The washing step was repeated thrice and after washing, the seeds in equal number were placed on petri plates holding moistened sterile filter paper sheets. After this, these petri plates were kept in seed germinator and incubated for 5 days at 25 ± 2 °C. After 5 days of growth, seedlings were exposed to drought stress in which the water supply was withdrawn for 3 days. The control was set up against the stressed petri plates, to which the water supply was given normally. After 3 days of stress, samples were collected from both controlled and stressed plants and preserved at -80°C for RNA isolation (Rakhra *et al*., 2017).

3.12.2 RNA isolation

The total RNA was extracted from drought-stressed seedlings of PBW175 using the Qiagen RNeasy mini kit, which comprises 2 columns (Qia-Shredder and mini-spin), following the manufacturer's protocol. Briefly, a small centrifuge vial was taken and 480µl of RLT buffer was added to it along with a small part of plant tissue sample (drought stressed and control seedlings). The samples were homogenised using a waring blender to obtain the lysate containing the RNA along with other nucleic acids and proteins. After this, the lysate was transferred to upper purple section of the Qia Shredder column containing the cartridge and this upper section was then placed above the lower collection tube of the Qia-shredder column. The column containing the lysate was centrifuged at 13000 rpm for 1 minute. Now equal volume of 100% ethanol (350 µl) was added to the centrifugate, mixed well by pipetting and then transferred to the mini-spin column. The next step involved centrifugation at 13000 rpm for 30 seconds. Flow through liquid was discarded, RNA was present in the column. 700 µl of RW1 buffer was added to column followed by the centrifugation for 30 seconds at 13000rpm.

Washing was done 2 times with 500µl RPE buffer along with centrifugation at 13000 rpm for 30 seconds. Flow through liquid was discarded and column was placed in fresh 2ml tube and centrifuged at 13000rpm for 1 minute. Again, changed the tube to 1.5ml and added 30-50µl of RNase free water onto the membrane of the column followed by centrifugation at 1 minute at 13000rpm. The RNA was extracted at bottom of the column and the quality was checked by running the agarose gel electrophoresis(Gehrig *et al*., 2012).

3.12.3 cDNA extraction from isolated RNA

Down streaming of RNA involves purification of RNA and cDNA isolation. iScript^{TM} cDNA synthesis kit was used to isolate cDNA. First, 1µl of RNA sample was mixed with 5X iScript RT buffer mix and then placed at 42°C for 26 minutes. The reaction was assembled on ice. The obtained cDNA was diluted by a factor of two using sterile water and stored at a temperature of -20°C for future use (Kolenda *et al*., 2021).

3.12.4 Quantitative real-time (qRT)- PCR analysis of dehydrin gene

Homolog genes for the hits obtained through Ensembl plants were identified through Clustal Omega and qRT-PCR primers for such homolog genes pairs were designed using PRIMER-BLAST (Table 3) (Ye *et al*., 2012). Primer's specificity was checked by semi-quantitative RT-PCR (data not shown). Further, qRT-PCR analysis was done according to the manufacturer's protocol using a qPCR BioRad CFX384 machine with a Brilliant III ultra-Fast SYBR green qPCR master mix (Agilent Technologies, USA). The qRT-PCR reaction mixture of 10µl was prepared by combining the following: 5µl of 2× SYBR green QPCR master mix, 3.5µl of molecular grade water, 0.5µl of diluted cDNA (1/100), and 0.5µl of each forward and reverse primer at a concentration of 100nM.

Further, PCR amplification was done by first incubating the reaction mix at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 10 s; annealing at 60 °C for 30 s and extension at 72 °C for 30s (Rakhra *et al*., 2017). To confirm the amplification specificity, a melt curve analysis was conducted. To check for genomic

DNA contamination, dimer formation, and other potential sources of contamination, both a no template control and a no enzyme control (lacking reverse transcriptase) were included during PCR amplification. Furthermore, the Ct values were processed via $2^{-\Delta\Delta Ct}$ method for calculating the relative mRNA level of different genes, by taking actin as a reference (Livak & Schmittgen, 2001). The resulted analysis was done using two-way ANOVA.

Table 3:The homolog genes identified using Clustal Omega along with their respective primers designed using PRIMER-BLAST

S.No.	Homologs	Primer1 Primer2
1	TaDHN28-1-6D TaDHN28-2-6A TaDHN27-4-6B	F1-TGACGCGAAGGAGAAGAAGG R1-TGGGCTTGTGCTCGCC F2-GCCCGAGGAGGAGAAGAAAG R2-GGAGCAGCGTGCGTGA
2	TaDHN14-8-U TaDHN15-4-U TaDHN15-6-6A	F1- AGGGGCAGCACGGTCA R1- GTCTTGTGCTCCTCCCTGC F2- GGGGCAGCACGGTCAC R2-GGTCTTGTGCTCCTCCCTG
3	TaDHN11-1-7D TaDHN12-1-7B TaDHN12-2-7A	F1- CACCGATGGCAACTACGG R1- CTGTCCAGGCAGCTTGTC F2- CACCGATGGCAACTACGGG R2- CTGTCCAGGCAGCTTGTCC
4	TaDHN9-1-6D TaDHN9-2-6A TaDHN19-1-6A	F1- GGCGAGAAGAAGGGCATCA R1- CCAGTGCCAGTCGTTCCG F2- CGGCGAGAAGAAGGGCAT R2- CCGGTGGCCGTGGTG

5	TaDHN101-1-6A TaDHN40-1-6B	F1- AAGGAGAAGCTCCCTGGTGG R1- CGTGCCGGTCATTCCAGT F3- GAGAAGCTCCCTGGTGGC R3- CGTGCCGGTCATTCCAGTG
6	TaDHN22-2-3D TaDHN22-3-3B	F2- GTGCCGTTTCCCCGTCC R2- TCTTTGGTGGGCTGGATCTG F3- CACGAGAGCGTGGTAGGC R3- GACCATGCCCTCATAAGCGT
7	TaDHN15-1-5B TaDHN15-2-5A TaDHN15-3-5B TaDHN15-5-5D TaDHN15-8-5D	F1- ATCAAGGAGAAGCTCCCCG R1- GCCTTGCTGCCCGTAGG F2- GAGGACGACGGCATGGG R2- CCCGTAGGCTCCTCCAGT
8	TaDHN41-1-4D TaDHN43-1-4A TaDHN43-2-4B	F1- AAGACTCGTGGCATCCTCCA R1- TGGTCTGCTCCTTGTTACCG F3- GACTCGTGGCATCCTCCAC R3- GTGGTCTGCTCCTTGTTACC
9	TaDHN27-1-3A TaDHN27-2-3D TaDHN27-3-3B	F1- ATGGGTGGACGGAGGAAGAA R1- GGGCTGCACGTAGTAAGGG F2- CCCTTACTACGTGCAGCCC R2- TCATGCCCTTCTTCTCACCAG

10	TaDHN20-1-6A	F1- ACGGACAGCAAGGTCATACG R1- GTCGGTCATTCCGGTGTGT F2- GACAGCAAGGTCATACGGCA R2- GTGTCGGTCATTCCGGTGT
11	TaDHN13-1-5D TaDHN14-1-5B TaDHN14-2-5A TaDHN14-3-5B TaDHN14-4-5D TaDHN14-6-5A	F1- GAGCCCACAAGGACGCC R1- CCTTCTTCTCGCCGGTGG F2- GGGATGAAGGAGAAGATCAAGGA R2- CTTCTTCTCGCCGGTGGC
12	TaDHN22-1-6A TaDHN23-2-6D TaDHN23-3-6B	F1- CATGAGGGACGAGCACCAG R1- ATGCCCTTCTTCCTCCTCC F2- GGGCATTTCCAGCCCATGA R2- TGCCCTTCTTCCTCCTCCC
13	TaDHN16-2-6D	F1- ACGGACAGCAAGGTCATACG R1- TGTCCATGACGCCCTTCTTC F2- GACAGCAAGGTCATACGGCA R2- TTGTCCATGACGCCCTTCTT
14	TaDHN14-5-6D TaDHN14-7-6A	F1- GCTCACAAGACCGGAGGG R1- CTTGATGCCCTTCTTCCTCCT F2- GGAGGAGGAAGAAGGGCATC R2- GTCATTCCGGTGTGTCCCTG

15	TaDHN16-3-6A TaDHN15-9-6D TaDHN15-10-6B TaDHN15-7-6A	F2- GCGACCAGCAGCAGACC R2- GGGCAGCTTCTCCTTGATCT F3- GGCGACCAGCAGCAGAC R3- GGCAGCTTCTCCTTGATCTTG
16	TaDHN16-5-6D TaDHN16-1-6D TaDHN16-4-6B	F1- GCACGGCCAGGCGAC R1- CCGGCCTTGTGCTCCTC F2- CTCCAGCTCGTCTGAGGATG R2- TGCTGCTGGTCACCGT
17	TaDHN17-1-6D	F1- CATGGACACGCTGGAGTGAT R1- CTCTTCTTCTCGCCGATCCC F2- TGGAGAACCAGGCACACATC R2- GTACCAGCGGTCTCCTTGTG

3.12.5 SDS-PAGE and Western blotting

3.12.5.1 Plant growth and stress conditions

The five-day-old seedlings from both the control and stressed samples of PBW 175 were taken for the western blot analysis. After five days of growth, stress was given by holding the water supply to stressed samples for 3 days and samples were collected at the same time on all the days of stress. On other hands, control samples plates of PBW175 were prepared by giving regular water supply. After this, the stressed samples were re-watered and the samples were again collected after 24 hours. Simultaneously, the control samples were also collected for the comparative analysis of dehydrin expression (Rakhra *et al*., 2017).

3.12.5.2 Preparation of protein extracts

From each sample, 1g of fresh plant tissue was cut finely using razor blade and was placed into pre-chilled mortar and pestle. It was then homogenized in liquid nitrogen with 1ml of cold RIPA buffer containing protease inhibitor (1X). Homogenised products were transferred to 1.5ml centrifuge tubes and ccentrifuged at 14,000 g for 30 minutes at 4℃. Supernatants were collected and stored in a fresh tube at -80℃ for further use in SDS-PAGE and western blotting.

3.12.5.3 SDS-PAGE

10% SDS-PAGE was carried out to separate proteins using 50µg of protein samples. The electrophoretic glass plates were cleaned properly, dried in an oven, and sealed with adhesive tape. The plates were then clamped in a gel casting device, and the plates were subsequently sealed with a plug gel solution to prevent leakage. Thirty ml of 10% separating gel solution was poured at the level of 2cm under the top edge of the notched plate. 1 ml of n- butanol which was water saturated was poured over the separating gel solution. After 20-30 minutes when the gel sets, n-butanol was drained off. 4% stacking gel solution was poured over the separating gel. The comb was then inserted into the gel solution avoiding any bubble formation. After the polymerization of the stacking gel, the comb was removed carefully and tank buffer

was poured into the electrophoresis tank reservoir. Protein samples were mixed equally with loading dye buffer and loaded for the Coomassie blue staining of the gel. Electrophoresis was then done at 100V till the dye reaches the bottom. The gel was removed carefully and was kept over the rocker for 12 hours for staining in CBB-R250 dye. The next day, the gel was de-stained for 4 hrs in the de-staining solution and the protein bands were analysed (Ceccardi *et al*., 1994).

3.12.5.4 Western blotting

3.12.5.4.1 Electro-elusion

10% SDS-PAGE gel was equilibrated in a transfer buffer for western blotting analysis. The process of transferring protein components from gel to the nitrocellulose membrane is called electro elusion (Figure 8). The wet transfer was done for 16 hrs overnight in the cold room. Nitrocellulose membrane and Whatman paper were cut down in the same size as a gel. A sandwich-type layer was prepared which contains three Whatman paper, nitrocellulose membrane, gel and 3 Whatman paper pieces aligned exactly. The gel side was put on the catholic side and the membrane was placed on an anodic side so that proteins get transferred from gel to membrane as current runs from cathode to anode. A blotting cassette was used for the transfer procedure, in which stacked gel is placed to avoid any bubble formation. A blotting buffer was filled in the apparatus and blotting was done at a constant 100V current for around 2 hours (Rakhra *et al*., 2017) (Figure 8).

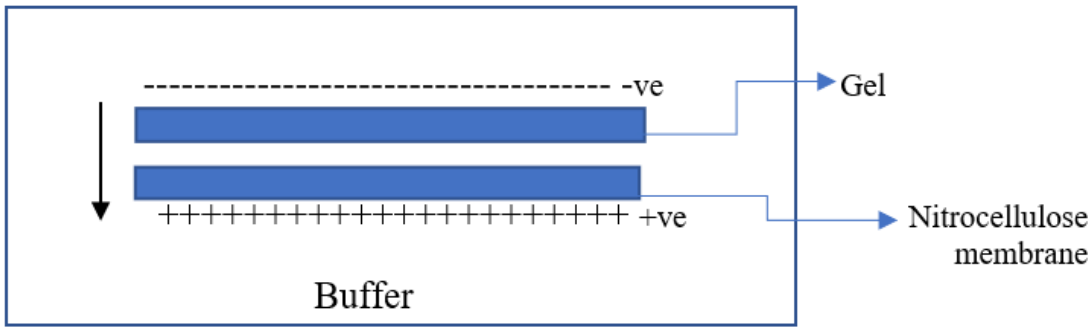


Figure 8: Schematic diagram of electro elusion, where proteins are transferred from polyacrylamide gel to nitrocellulose membrane

3.12.5.4.2 Hybridisation

Hybridisation was done at room temperature. After the transfer, the membrane was removed carefully and dried at room temperature (Rakhra *et al*., 2017).

3.12.5.4.3 Blocking

Dry milk was used as a blocking agent, this dry milk was dissolved in 20ml of TBS. The membrane was incubated in a blocking solution for 1 hour, followed by 5 minutes of washing with Tris buffer. This is important to reduce false results as the blocking solution covers almost all the area of the membrane except the target protein, and also helps in reducing background signals (Rakhra *et al*., 2017).

3.12.5.4.4 Antibody incubation (primary and secondary antibody)

To check the quality of the transfer, blot was thoroughly rinsed with water and stained with Ponceau S solution. Ponceau S stain was removed by washing 2-3 times with TBST. Kept the blot in 3% BSA in TBST for 1 hour at room temperature. The primary antibody (dehydrin-specific antibody-ADI-PLA-100 dehydrin plant polyclonal antibody) was diluted in a blocking buffer and the membrane was incubated overnight in the cold room at 4°C. The next day, the blot was rinsed 3-5 times for 5 minutes with TBST. After this, the HRP-conjugated IgG rabbit secondary antibody was diluted with 5 % skimmed milk in TBST and incubation was carried out for 1 hr at room temperature. Then, the blot was rinsed for 5 minutes with TBST.

3.12.5.4.5 Imaging and data analysis

A chemiluminescent substrate (luminol) was applied to the blot (Rakhra *et al*., 2017). When the HRP enzyme and luminol comes in contact, light signals are generated as a by- product of the reaction and these were observed using an X-Ray film. Grey faded lines are considered to be the protein bands and these are easily seen as X-ray film is darker in shade.

3.12.5.4.6 Quantitation

Image analysis software was used to read the band intensity of the targeted dehydrin proteins.

3.12.5.4.7 Statistical analysis

The paired T-test (P<0.1) was performed to compare the significant expression levels of dehydrin proteins under stress conditions (Linnet, 1999) .

3.13 Identification of cis-regulatory elements in the promoter regions of the wheat dehydrin genes

For identification of various *cis*-regulatory elements, the genomic sequences up to 2000bp upstream of the transcription start sites of the dehydrin genes were mined from the genomic sequence assemblies IWGSC RefSeq v 1.0. The WheatExp database, which is based on homology, was used to examine how dehydrin genes are expressed across various tissues of wheat. This analysis helped to identify patterns of expression for these genes in the polyploid wheat species. These extracted flanking sequences were submitted to the PlantCARE database (Lescot *et al*., 2002) for prediction and analysis of different *cis*-regulatory elements.

3.14 Digital expression analysis of wheat dehydrin

The WheatExp database, which is based on homology, was used to examine how dehydrin genes are expressed across various tissues of wheat. This analysis helped to identify patterns of expression for these genes in the polyploid wheat species (Pearce *et al*., 2015).

Using the tblastn tool, the protein sequence for wheat dehydrins was subjected as a query counter to the wheat expression database. For every best-matched transcript, the expression values were compiled in form of a text file. This text file dataset was submitted to the heatmapper web database for the generation of the heatmap. To cluster the data, the Average Linkage method was used whereas the Euclidean distance method was used for measuring distance.

Chapter-4

Results and discussion

Dehydrins belong to the LEA group 2 class, which is primarily involved in drought stress tolerance(Rorat, 2006). Dehydrins play important roles in cellular osmolarity maintenance, and stabilization of the cellular proteins and membranes preventing their peroxidation without altering plant metabolism under different environmental cues (P. K. Singh *et al*., 2022).

4.1 Identification of dehydrin genes and genome-wide analysis of identified dehydrins in wheat

Members of the dehydrin gene family in wheat were identified by homology search using WZYb dehydrin-like protein sequences from wheat as a query against IWGSC RefSeq v1.0 sequences assembly (Table 5.1).

The hits obtained were further used as a query sequence in the BLAST search to find out significant matches. Genome-wide analysis of wheat dehydrins resulted in a total of 48 dehydrin genes which encode for 48 different dehydrin proteins (Table 5.1) and these identified proteins are comparable to 31 DHN genes in tetraploid durum wheat *Triticum dicoccoides*, 15 in diploid *Triticum urartu* and 16 in diploid *Aegilops tauschii* (Hao *et al*., 2022a). Similar results were found in various plants like in rye (*Sorghum bicolor* L.) where 68 LEA- 2 genes were identified (Ding *et al*., 2021), 99 were identified in *barley* and *Brachypodium distachyon* (Zan *et al*., 2020a), 7 DHNs were identified in *Pyrus pyrifolia* (Asian pear)(Hussain *et al*., 2015), 5 DHN genes namely (*St*LEA6, *St*LEA7, *St*LEA13, *St*LEA18 and *St*LEA27) were identified in the potato which belong to 29 identified LEA genes under salt and drought stress(Charfeddine *et al*., 2015). The same criteria as stated by previous researchers were followed for identifying the wheat orthologs by limiting the sequence identity to >90% (Hanhart *et al*., 2017).

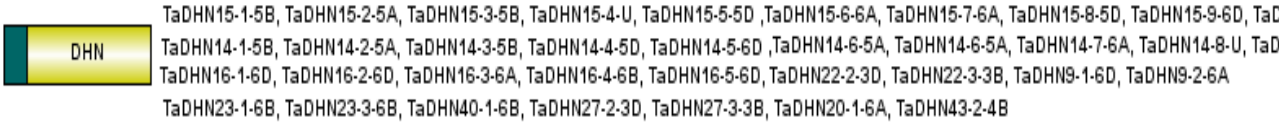
4.1.1 *In-silico* analysis of dehydrin proteins in wheat

The various dehydrin proteins show inconsistency in size and sequence, with the smallest predicted proteins (TaDHN9-1-6D and TaDHN9-2-6A) consisting of 93 amino acid residues each with molecular weight 9.65kDa and 9.66kDa respectively as compared to largest identified dehydrin protein TaDHN101-1-6A with 991 amino acid residues and molecular weight 101.60kDa. The pI values ranged from 5.19 to 10.7

(Table 5.1). It has been known that dehydrins are characterized by molecular masses ranging from 9kDa-200kDa(Hanin *et al*., 2011). Individual amino acid sequences for each protein are given in Appendix-I.

4.1.2 Domain analysis of dehydrins proteins in wheat

The characteristic feature of all the identified proteins was that they were having a dehydrin domain. In this study, 35 proteins were having a single domain while the remaining 13 were found to have multiple domains (Figure 9). This was found to be similar to *Arabidopsis thaliana* stress-responsive proteins EDR14, COR47 and LTI 29 (Nylander *et al*., 2001), which are also having dehydrins as their functional domain. Also, in *Populus*, 53 stress-responsive genes were identified, out of which 14 were found to be having LEA conserved domain(Lan *et al*., 2013). The presence of dehydrin domains suggest functional diversity of gene in plant growth and development as it acts as a stress-responsive, antioxidant, radical scavenging agent and cryoprotectant for plants (Tiwari & Chakrabarty, 2021)



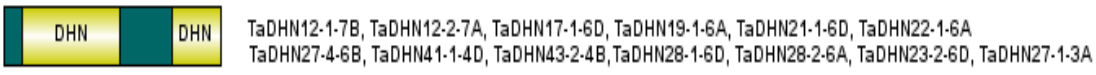


Figure 9: Single and multiple domains of TaDHNs. The green portion indicates the protein and the yellow parts show the dehydrin domain present in the protein. Protein names are given in front of protein strands.

Table 4: *In silico* genome-wide analysis of wheat dehydrins proteins.

S.No.	Protein Name	Gene ID (IWGSC V1.0)	Amino acid (AA)	Coding sequences (CDS) (bp)	Molecular weight (MW) (kDa)	Isoelectric point (pI)	Subcellular Localization	Domain Analysis	Exons
1	TaDHN9-1-6D	TraesCS6D02G332600.1	93	282	9.65	6.79	Cytoplasm	SD	1
2	TaDHN9-2-6A	TraesCS6A02G350200.1	93	282	9.66	6.79	Cytoplasm	SD	1
3	TaDHN11-1-7D	TraesCS7D02G549900.1	112	339	11.52	6.28	Cytoplasm	SD	1
4	TaDHN12-1-7B	TraesCS7B02G484900.1	125	378	12.67	6.49	Cytoplasm	MD	1
5	TaDHN12-2-7A	TraesCS7A02G560000.1	124	375	12.82	7.20	Cytoplasm	MD	1
6	TaDHN13-1-5D	TraesCS5D02G379300.1	133	402	13.93	8.81	Cytoplasm	SD	2
7	TaDHN14-1-5B	TraesCS5B02G372200.1	138	417	14.21	8.01	Cytoplasm	SD	2
8	TaDHN14-2-5A	TraesCS5A02G369900.1	140	423	14.24	8.01	Cytoplasm	SD	2
9	TaDHN14-3-5B	TraesCS5B02G372100.1	143	432	14.42	8.00	Cytoplasm	SD	2
10	TaDHN14-4-5D	TraesCS5D02G379200.1	143	432	14.51	7.11	Cytoplasm	SD	2
11	TaDHN14-5-6D	TraesCS6D02G333100.1	144	435	14.51	9.19	Cytoplasm	SD	2
12	TaDHN14-6-5A	TraesCS5A02G369800.1	143	432	14.57	8.00	Cytoplasm	SD	2

13	TaDHN14-7-6A	TraesCS6A02G350800.1	143	432	14.81	9.22	Cytoplasm	SD	2
14	TaDHN14-8-U	TraesCSU02G122200.1	149	450	14.85	6.86	Cytoplasm	SD	2
15	TaDHN15-1-5B	TraesCS5B02G426700.1	150	453	15.17	9.52	Cytoplasm	SD	2
16	TaDHN15-2-5A	TraesCS5A02G424800.1	149	450	15.21	9.33	Cytoplasm	SD	2
17	TaDHN15-3-5B	TraesCS5B02G426800.1	150	453	15.22	9.36	Cytoplasm	SD	2
18	TaDHN15-4-U	TraesCSU02G086200.1	151	456	15.28	9.10	Cytoplasm	SD	2
19	TaDHN15-5-5D	TraesCS5D02G433200.1	152	459	15.34	9.52	Cytoplasm	SD	2
20	TaDHN15-6-6A	TraesCS6A02G059800.1	153	462	15.51	8.84	Cytoplasm	SD	2
21	TaDHN15-7-6A	TraesCS6A02G350700.1	152	459	15.52	7.17	Cytoplasm	SD	2
22	TaDHN15-8-5D	TraesCS5D02G433300.1	154	465	15.58	9.52	Cytoplasm	SD	2
23	TaDHN15-9-6D	TraesCS6D02G333200.1	155	696	15.72	8.83	Cytoplasm	SD	2
24	TaDHN15-10-6B	TraesCS6B02G383600.1	158	477	15.83	9.13	Cytoplasm	SD	2
25	TaDHN16-1-6D	TraesCS6D02G333000.1	162	489	16.19	8.05	Cytoplasm	SD	2
26	TaDHN16-2-6D	TraesCS6D02G333300.1	160	483	16.25	8.07	Cytoplasm	SD	2
27	TaDHN16-3-6A	TraesCS6A02G350600.1	162	489	16.28	9.22	Cytoplasm	SD	2
28	TaDHN16-4-6B	TraesCS6B02G383800.1	166	501	16.70	8.05	Cytoplasm	SD	2

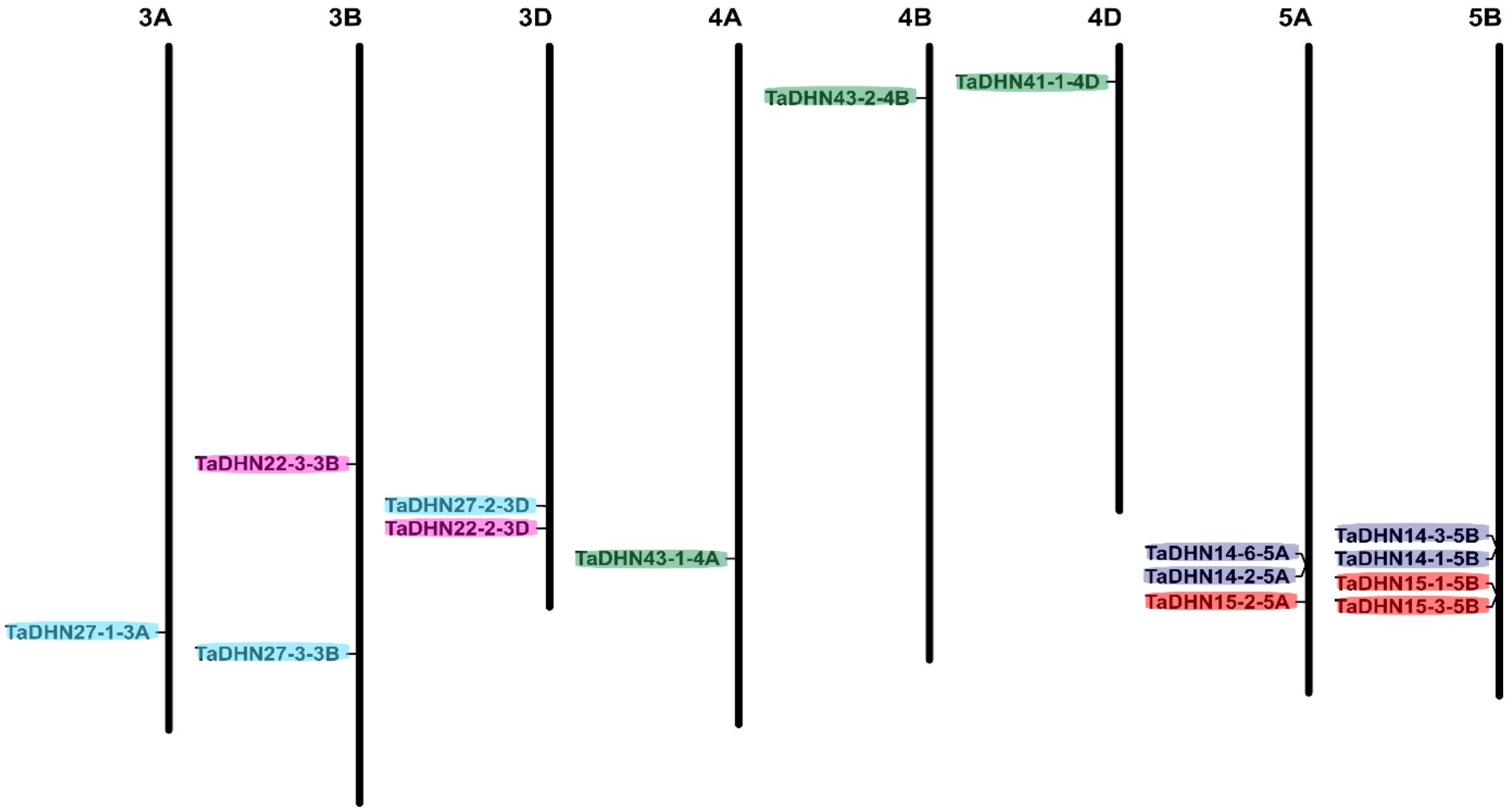
29	TaDHN16-5-6D	TraesCS6D02G333600.1	167	504	16.71	7.17	Cytoplasm	SD	2
30	TaDHN17-1-6D	TraesCS6D02G332500.1	179	540	17.80	7.41	Secreted	MD	2
31	TaDHN19-1-6A	TraesCS6A02G350100.1	190	573	19.23	7.08	Secreted	MD	2
32	TaDHN20-1-6A	TraesCS6A02G350900.1	190	573	20.14	10.7	Cytoplasm	SD	2
33	TaDHN22-1-6A	TraesCS6A02G350500.1	221	666	22.04	9.05	Secreted	MD	2
34	TaDHN22-2-3D	TraesCS3D02G255500.1	215	648	22.24	6.63	Cytoplasm	SD	2
35	TaDHN22-3-3B	TraesCS3B02G286600.1	217	654	22.29	6.87	Cytoplasm	SD	2
36	TaDHN23-2-6D	TraesCS6D02G332900.2	231	696	23.02	9.05	Secreted	MD	2
37	TaDHN23-3-6B	TraesCS6B02G383500.2	231	696	23.22	9.22	Secreted	SD	2
38	TaDHN27-1-3A	TraesCS3A02G396200.1	275	828	27.02	9.60	Cytoplasm	MD	2
39	TaDHN27-2-3D	TraesCS3D02G390200.1	275	828	27.15	9.70	Cytoplasm	SD	2
40	TaDHN27-3-3B	TraesCS3B02G428200.1	274	825	27.19	9.74	Cytoplasm	SD	2
41	TaDHN27-4-6B	TraesCS6B02G273400.1	259	789	27.97	5.20	Nucleus	MD	2
42	TaDHN28-1-6D	TraesCS6D02G234700.1	262	432	28.15	5.19	Nucleus	MD	2
43	TaDHN28-2-6A	TraesCS6A02G253300.1	268	807	28.82	5.25	Nucleus	MD	2
44	TaDHN40-1-6B	TraesCS6B02G383200.1	405	501	40.29	6.83	Secreted	SD	2

45	TaDHN41-1-4D	TraesCS4D02G063100.1	430	1293	41.22	9.04	Nucleus	MD	2
46	TaDHN43-1-4A	TraesCS4A02G250900.1	455	1368	43.74	8.84	Nucleus	SD	2
47	TaDHN43-2-4B	TraesCS4B02G064200.1	457	1374	43.89	8.84	Secreted	MD	2
48	TaDHN101-1-6A	TraesCS6A02G350300.1	991	2976	101.60	5.86	Secreted	MD	2

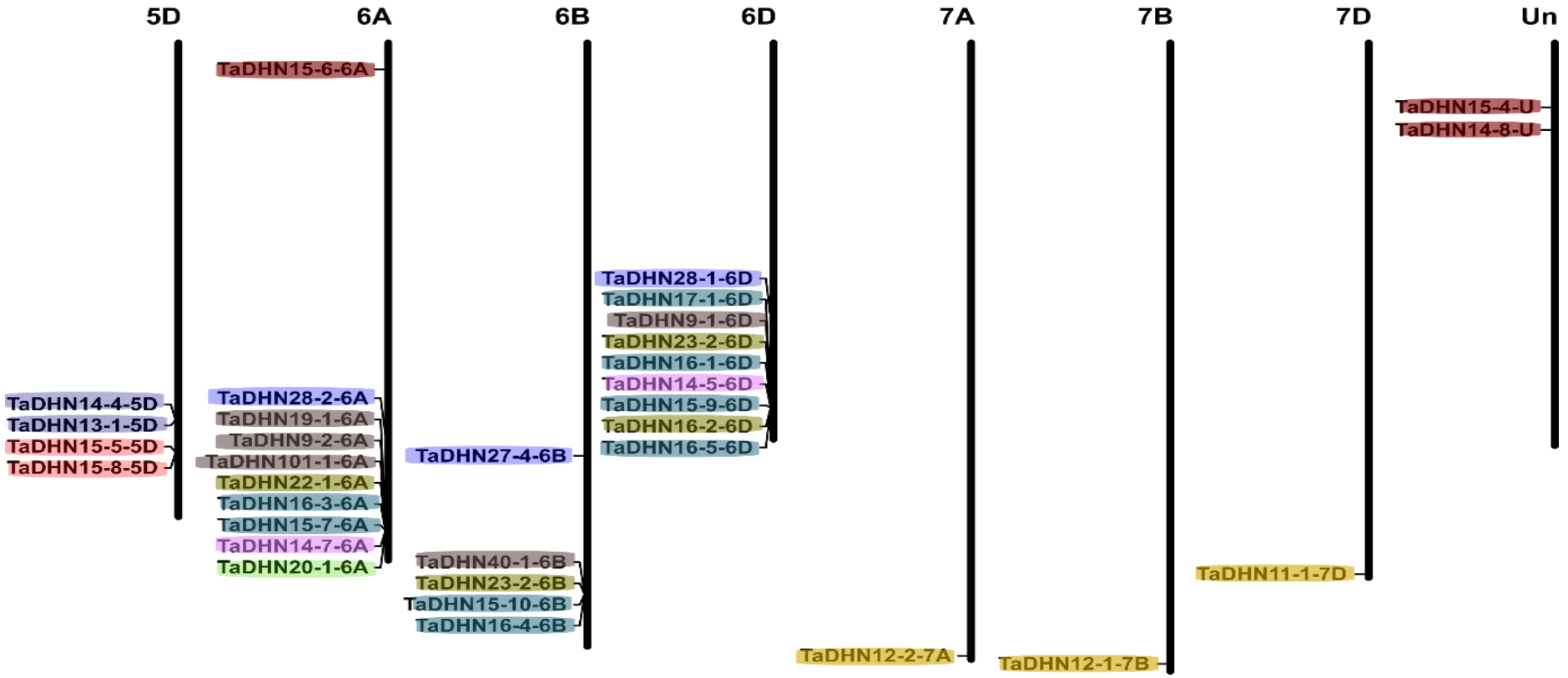
kDa-kilo Dalton, SD-single domain, MD-multiple domain

4.1.3 Chromosome distribution

A chromosome map was generated to determine the organization of chromosomes and distribution of chromosome. Chromosome distribution showed that dehydrins are distributed on 17 different chromosomes but with uneven density (Figure 10). Two proteins i.e., TaDHN14-8-U and TaDHN15-4-U didn't show the precise chromosomal location, they are still at their outset. Chromosome 6A presented the highest number of dehydrins (ten), followed by nine proteins on 6D whereas the lowest number of dehydrins was found to be present on 4A,4B,4D and 7D (one each). Together, the D chromosomes show the presence of the highest number of dehydrin genes (17) followed by A chromosomes (16) and B chromosomes (13), whereas 2 are unidentified. There are three sets of progenitor chromosomes in wheat, due to which various TaDHNs show the presence of high sequence homology (homologous dehydrins). Homologous proteins are highlighted by their identical colors (Figure 10). For example, TaDHN22-2 and TaDHN22-3 are present on 3B and 3D but not on the 3A chromosome. There was no gene observed on chromosome 1 (1A,1B and 1D) and chromosome 2 (2A,2B and 2D). The two unidentified genes TaDHN15-5 and TaDHN14-8 on the U-chromosome were found to be homologues of TaDHN15-6 which is present on the 6A chromosome. Almost all the proteins are found to be homologs in triplets (some of them are more than three) and are present on the same chromosomes (Figure 10). Similarly, the same genomic organization was observed in the *Populus* Lea family, where 53 genes were localized on 12 chromosomes (Lan *et al*., 2013). Also, in *Brassica napus*, 108 BnLEA genes were seen to be distributed on 19 chromosomes and were appeared as clusters on chromosomes C5, C4, A9 and C2 in genes (Liang *et al*., 2016). In wheat, *TaLEA* genes were found to be distributed unevenly on all 21 chromosomes(Zan *et al*., 2020a).


3A
3B
3D
4A
4B
4D
5A
5B
TaDHN43-2-4B
TaDHN41-1-4D
TaDHN22-3-3B
TaDHN27-2-3D
TaDHN22-2-3D
TaDHN43-1-4A
TaDHN14-3-5B
TaDHN14-6-5A
TaDHN14-1-5B
TaDHN14-2-5A
TaDHN15-1-5B
TaDHN15-2-5A
TaDHN15-3-5B
TaDHN27-1-3A
TaDHN27-3-3B

(a)


5D
6A
6B
6D
7A
7B
7D
Un
TaDHN15-6-6A
TaDHN15-4-U
TaDHN14-8-U
TaDHN28-1-6D
TaDHN17-1-6D
TaDHN9-1-6D
TaDHN23-2-6D
TaDHN16-1-6D
TaDHN14-5-6D
TaDHN15-9-6D
TaDHN16-2-6D
TaDHN16-5-6D
TaDHN14-4-5D
TaDHN13-1-5D
TaDHN15-5-5D
TaDHN15-8-5D
TaDHN28-2-6A
TaDHN19-1-6A
TaDHN9-2-6A
TaDHN101-1-6A
TaDHN22-1-6A
TaDHN16-3-6A
TaDHN15-7-6A
TaDHN14-7-6A
TaDHN20-1-6A
TaDHN27-4-6B
TaDHN40-1-6B
TaDHN23-2-6B
TaDHN15-10-6B
TaDHN16-4-6B
TaDHN11-1-7D
TaDHN12-2-7A
TaDHN12-1-7B

(b)

Figure 10: (a and b) The distribution of dehydrin genes in *Triticum aestivum* (common wheat) was analyzed by mapping them onto the chromosomes using the IWGSC1.0 assembly. The resulting map, which was drawn to scale, indicates the location of each dehydrin gene on its respective chromosome. The dehydrin genes, which encode homologs of the dehydrin protein, are color-coded to make it easier to distinguish them from other genes. The chromosome number is provided at the top of each strand.

4.1.4 Structure of dehydrin genes in wheat

Analysis using the GSDS2.0 database revealed considerable variance in the structure of exon and intron structure of TaDHNs (Figure 11). Out of 48 dehydrin genes, five genes *TaDHN 9-1-6D, TaDHN9-2-6A, TaDHN11-1-7D, TaDHN12-1-7B* and *TaDHN12-2-7A* lack introns in their ORFs (open reading frames), while in other genes only one intron is present (Figure 11). Also, homologous genes are having the same number of introns. The size of introns varies among the TaDHNs, with the smallest (79bp) observed in *TaDHN15-3-5B*, and the largest (1082bp) in *TaDHN101-1-6A* followed by (525bp) in *TaDHN17-1-6D*. Other dehydrin genes in wheat such as; *TaDHN14-8-U, TaDHN15-4-U, TaDHN16-5-6D, TaDHN16-2-6D, TaDHN15-9-6D, TaDHN14-5-6D, TaDHN16-1-6D, TaDHN16-4-6B, TaDHN27-4-6B, TaDHN20-1-6A, TaDHN15-7-6A, TaDHN16-3-6A, TaDHN19-1-6A, TaDHN28-2-6A, TaDHN27-1-3A, TaDHN27-3-3B, TaDHN22-2-3D, TaDHN27-2-3D* and *TaDHN40-1-6B* are also having large sized introns (more than100bp).

Diversity is also observed in untranslated regions of wheat dehydrins genes. Out of forty-eight dehydrin genes, seven (*TaDHN12-1-7B, TaDHN9-1-6D, TaDHN17-1-6D, TaDHN14-7-6A, TaDHN101-1-6A, TaDHN19-1-6A,* and *TaDHN22-3-3B)* lacked 3' as well as 5' UTR regions. Two genes (*TaDHN12-1-7B* and *TaDHN9-1-6D*) lacked both the UTR regions, as well as introns. In *TaDHN20-1-6A*, 5' UTR is present whereas 3' is absent.

Further, gene structure organization analysis showed the extensive variation in the exons and introns structure of TaDHNs. In current study, 44 out of 48 genes showed the presence of one intron while 4 dehydrins (TaDHN9-1-6D, TaDHN9-2-6A, TaDHN12-1-7B and TaDHN12-1-7B) lack introns, which indicate the function of the identified dehydrins in response to drought stress. Loss or gain of introns is an important characteristic of gene structure variation and is important for the gene evolution as it works as enhancers, repressors and promoters of gene transcription (Fu *et al*., 2005). Gene structure analysis consists of intron-exon organization with most of the stress-responsive genes generally containing a small number of introns (Lan *et al*., 2013). Similar results were observed in *Sorghum bicolor L*, where about 55% of genes

belonging to LEA group 2 were lacking introns, while 27.94% were having a single intron(Nagaraju *et al*., 2019). Also, in *Brassica napus*, out of 108 *Bn*LEA, 92 were not having more than 2 introns (Liang *et al*., 2016). This confirmed that the number of introns affects gene expression by delaying transcript production, and this has a significant impact on the length of the transcript. (Jeffares *et al*., 2008). Exons were found to be present in all genes. In accordance with these findings, seed maturation proteins (SMPs), which are known to be stress-responsive were identified in the rye and it was observed that they were having only one intron and 2 exons.

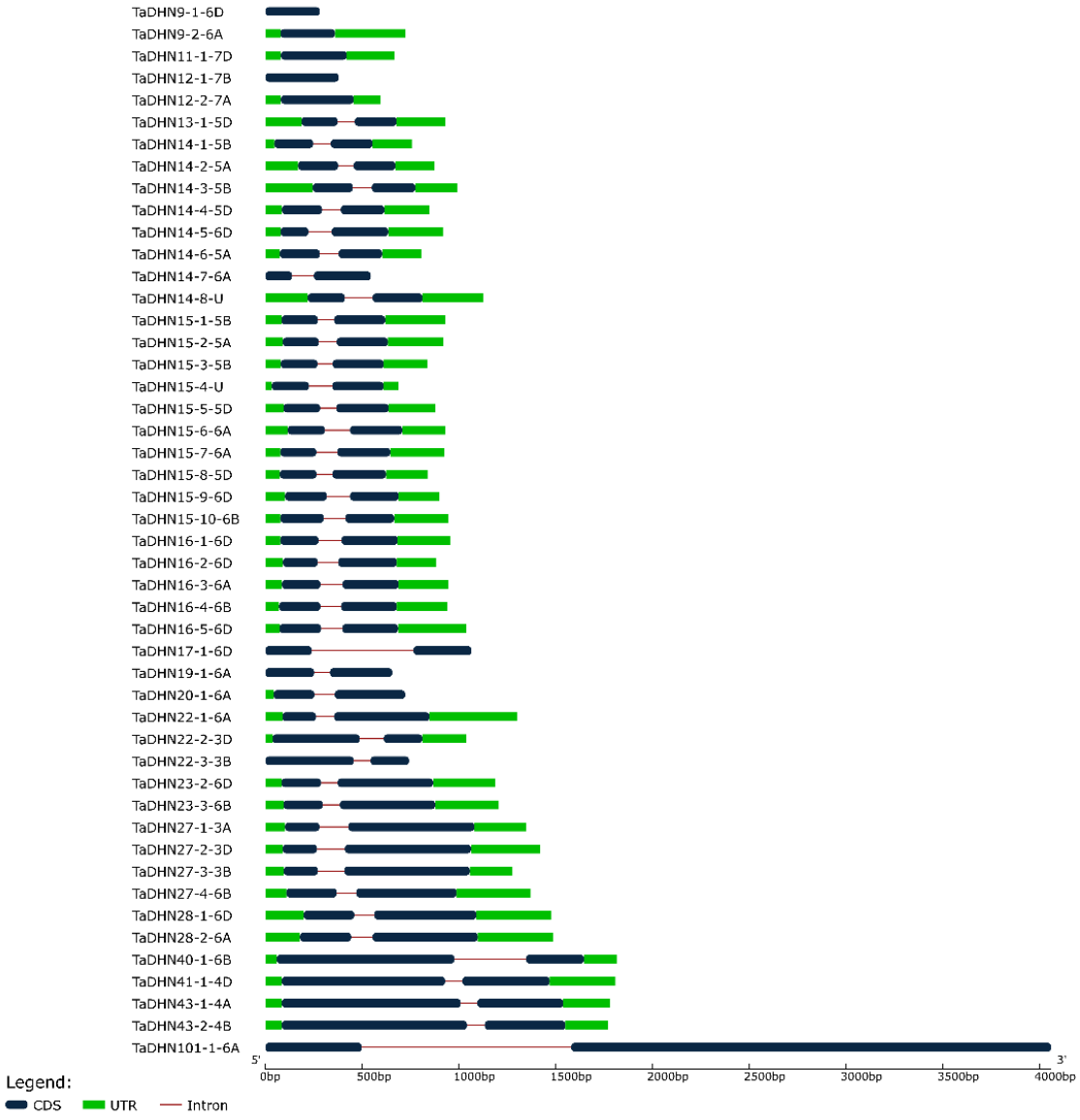


Figure 11: Pictorial depiction of organization of exon and intron of different wheat dehydrins. Blue coloured boxes and red coloured boxes indicate the presence of the exons and introns respectively. Untranslated regions are represented by green coloured boxes on either side of genes

4.1.5 Subcellular localization of dehydrins

Localization of specific proteins plays a significant role to understand the function of proteins as well as cell organization (Scott *et al*., 2005). In the present study, *in silico* prediction revealed that wheat dehydrins were localized in intracellular organelles like cytoplasm (35), and nucleus (5) while some of them are secreted (8) (Table 5.1). None of the dehydrins showed the presence of signal peptide, hence these proteins stay in the cytosol for the rest of the translation. Most of the LEA proteins are found to be localized in the cytosol (Candat *et al*., 2014) and cytoplasm (NDong *et al*., 2002). TaDHN27-4-6B, TaDHN28-1-6D, TaDHN28-2-6A, TaDHN41-1-4D and TaDHN43-1-4A were predicted to be nuclei proteins consisting of nuclear protein fraction and act as a barrier between the cytoplasm and nuclear membrane. Out of these 48 proteins, 8 dehydrins (TaDHN17-1-6D, TaDHN19-1-6A, TaDHN22-2-6A, TaDHN23-2-6D, TaDHN23-3-6B, TaDHN40-1-6B, TaDHN43-2-4B and TaDHN101-1-6A) were found to be secreted, and none of the wheat dehydrins were appeared to be localized in golgi apparatus, mitochondria, ER or plasma membrane. Earlier studies revealed that most LEA proteins were found to be localized in chloroplast, followed by cytoplasm, nucleus, mitochondria, plastids and in extracellular matrix; cytoplasm-nucleus, chloroplast-nucleus, chloroplast mitochondria and chloroplast-cytoplasm(Nagaraju *et al*., 2019). LEA proteins are known to be mostly located in cellular compartments, though some of them have been localized experimentally in various cellular compartments including cytoplasm, plastids etc (Tunnacliffe & Wise, 2007).

4.1.6 Phylogenetic analysis of wheat dehydrins

To explore the evolutionary relationship amongst the wheat dehydrins, phylogenetic analysis was performed with the help of their predicted amino acid sequences. The findings of this study revealed several clusters of proteins with high

sequence similarity. Phylogenetic analysis has revealed clustering patterns of TaDHNs in wheat, which are influenced by the presence of additional functional domains and subcellular localization (Hao *et al*., 2022), as observed in other plant species such as Arabidopsis, rice, Brassica napus, and soybean, as reported by previous studies (Hanhart *et al*., 2017). Evolutionary relation analysis revealed three major clades of TaDHNs in wheat. Clade 1 contains most of the dehydrins which are subdivided into various branches further (Figure 12). WZYb dehydrin-like protein is falling in clade 1 and is localized in cytoplasm while ten proteins formed clade 2. TaDHNs localized in the cytoplasm with multiple domains were grouped in the same clades.

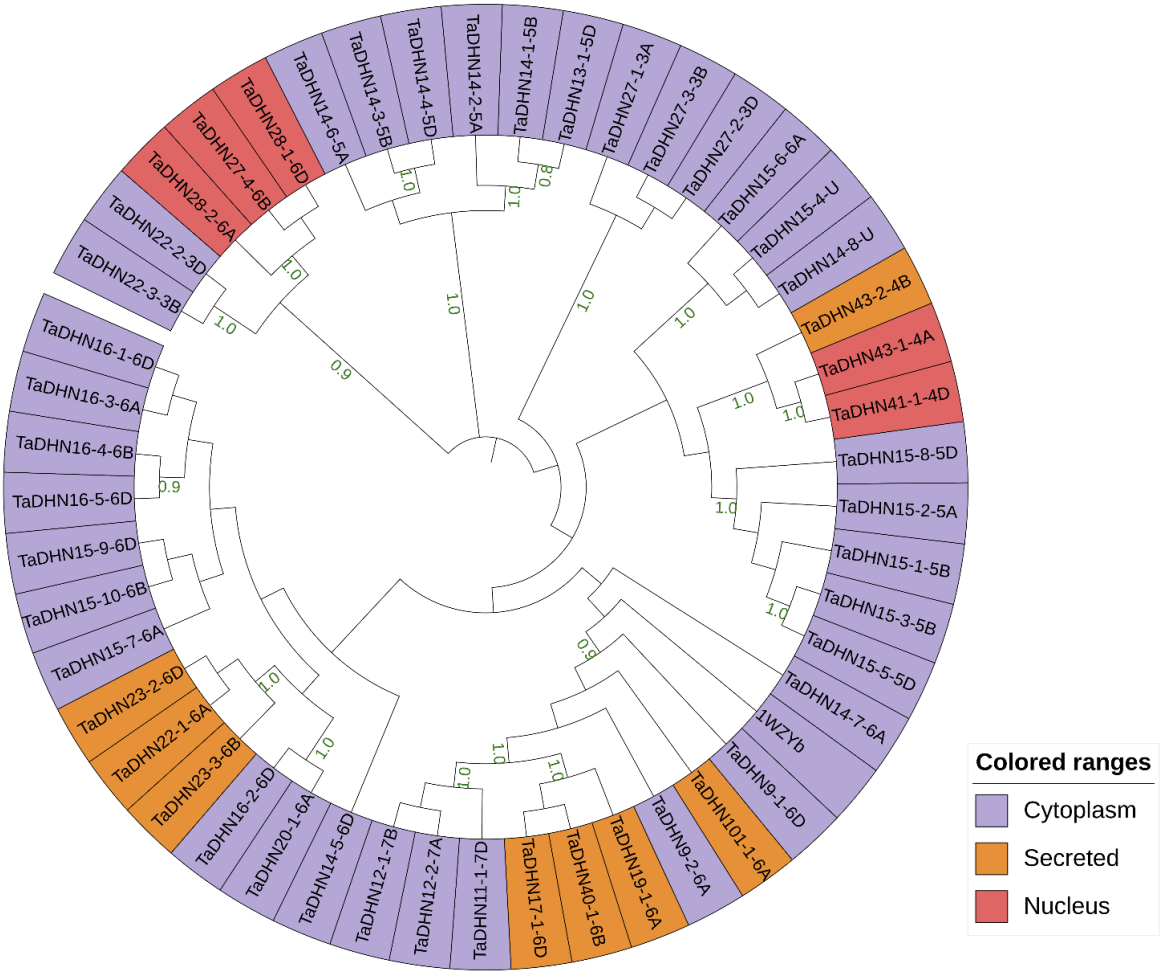


Figure 12: The evolutionary relationships among the dehydrin proteins (TaDHNs) in wheat were analyzed using MEGA-X software. A neighbour-joining tree was generated by aligning the peptide sequences of 48 dehydrins obtained from Clustal omega. This

analysis allowed for the identification of phylogenetic relationships among the TaDHNs and provided insights into their evolutionary history

4.1.7 Motif analysis of wheat dehydrins

The conserved motifs of wheat dehydrins were predicted using the MEME suite 4.12.0 server (http://meme-suite.org/tools/meme) (Bailey *et al*., 2009). The 15 different motifs that were identified by *in silico* analysis varied in length from 6 to 80bp (Table 5.2) (Figure 13). Analysis of these motifs was done using PFAM and PROSITE databases which revealed that none of the motifs covered the conserved dehydrin domain completely. The complete dehydrin domain consisted of different combinations of motifs 1,2,3, 13 and 14. The majority of TaDHNs contain motifs 1 (48) and motif 2 (47) followed by motif 3 (39) and motif 4 (38). Other motifs 5,7,10,11,12,13,14 and 15 are also observed in different dehydrins of wheat.

The identified dehydrin proteins contain the unique segments of dehydrin like K-segment, Y and S as their motifs. The K segment is typified by a lysine-rich region, Y- segment is known to be N-terminal conserved sequence and also known to be a homolog of nucleotide binding chaperon in bacteria and plants (Martin *et al*., 1993). In addition, the S-segment motif is located at N-terminal or C-terminal. Moreover, a few motifs showed the association with other functional domains like MYRISTYL (N-*myristylation* site), protein kinase C phosphorylation site and carboxy-terminal domain (CTD) which helps in protein-protein interaction and cell signalling.

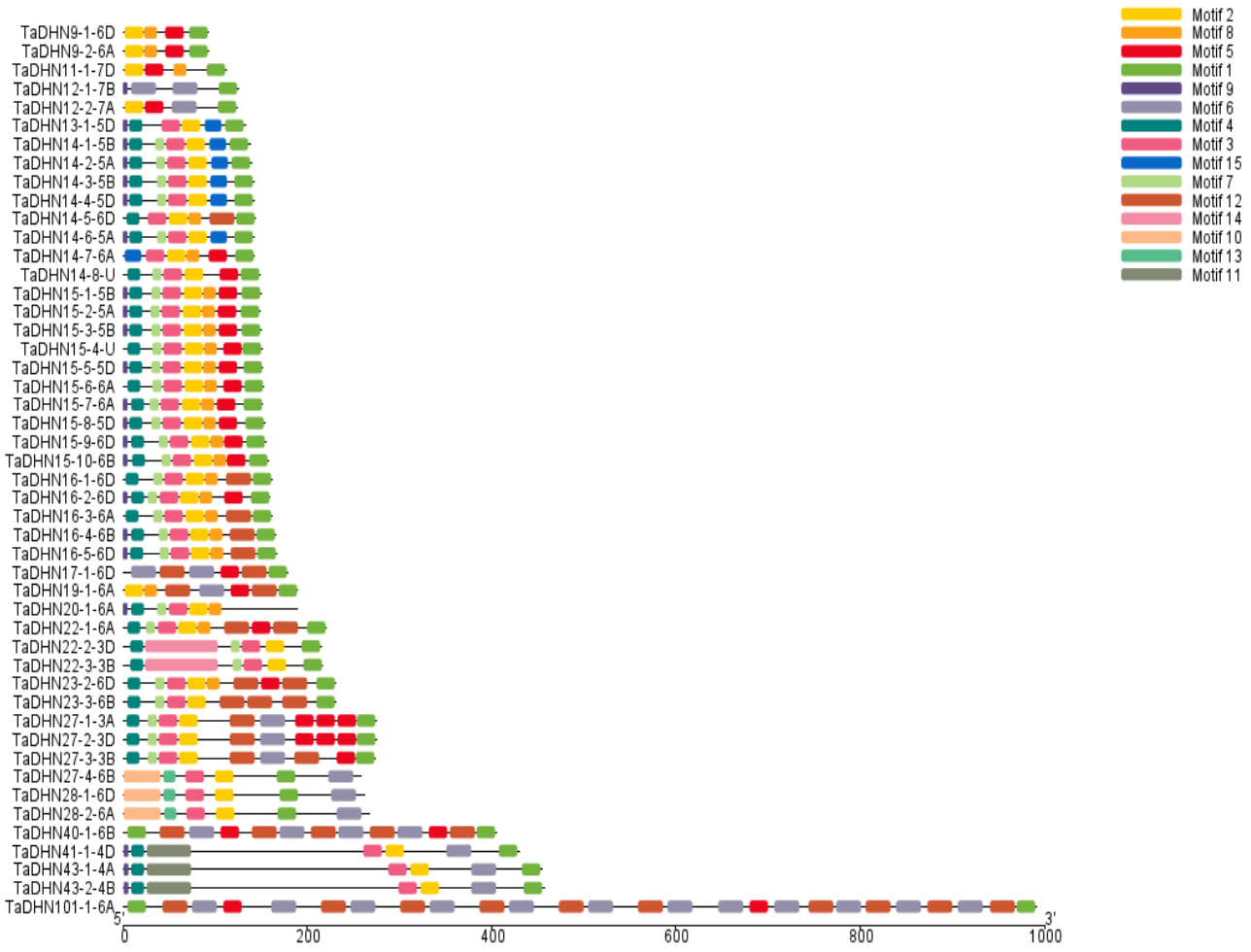


Figure 13: Distribution of conserved motifs in the wheat dehydrins proteins. 15 different putative motifs are indicated in different coloured boxes and patterns

4.1.8 Interaction Analysis of wheat Dehydrins: Protein-Protein Interaction (PPI)

To understand the type of interactions in which the multi-domain TaDHNs may take part, we analysed PPI networks for each of the TaDHN containing specific functional domains using the STRING database.

Since data related to interaction networks for proteins is very less, the interaction networks for 48 wheat dehydrin proteins were created with the advanced setting of network type to full STRING network, required score to medium confidence (0.400) and FDR stringency to medium i.e., 5%. The details of functional interacting partners are given in Table 5.3. All the proteins showed interaction with five members and out of these best matches on basis of score and identity were taken. Most of the

functional partners are loosely arranged at the periphery while only a few of them are present at the centre of the network (Figure 14). Some of the partners are also connected by one or more lines which represent the interactions derived from more than one source of information. The yellow lines represent the text-mining evidence and the black line represents the co-expression evidence. Figure 15 shows gene co-occurrence, the appearance and non-appearance of the linked functional partner proteins. In the subsequent grid (Figure 15), the occurrence of the protein in a *Triticum aestivum* is denoted with the red square and white space represents protein absence. The red square colour intensity depicts the portion of conservation of proteins which are homologous in the species. Most of the interacting partners are found to be uncharacterized proteins but they belong to the plant dehydrin family. Furthermore, TaDHN23-2-6D, TaDHN23-3-6B and TaDHN 27-3-6B are having a functional partner DHN3, which is already identified as a salt-induced YSK2 dehydrin 3 in pepper (*Capsicum annuum L*.) and known to play a quintessential role under various stress conditions (Meng *et al*., 2021). Similarly, CS120, a cold-shock protein known to reduce (Sarhan *et al*., 1997) intracellular freezing damage during winter due to hydrogen-bonding to the lattice of the nascent ice crystals is found to be an interaction partner for four dehydrin proteins (TaDHN101-1-6A, TaDHN9-2-6A, TaDHN19-1-6A and TaDHN40-1-6B). Another cold-induced protein (COR410) reported by Houde *et al*., 2004 was found to be an associated partner for the TaDHN28-2-6A and TaDHN27-4-6B proteins.

Table 5:List of Putative motifs predicted in different wheat dehydrins the motifs were predicted using Pfam and Prosite database

Motif	Motif sequence	Motif width	Putative function (Pfam/Prosite)	TaDHNs having this motif
1	DGTGEKKGIMDKIKEKLPGQH	21	Dehydrin	48
2	DDGMGGRRKKGIKEKIKEKLP	21	Dehydrin	47
3	REEHKTGGILHRSGSSSSSSS	21	Dehydrin	39
4	GQATNRVDEYGNPVA	15	-	38
5	TGAHGTTATGGTYGQQGHTGM	21	MYRISTYL (N-*myristylation* site)	32
6	EKKGVMEKIMEKLPGGHKBHQZTGGAAG	28	Dehydrin	12
7	TGAAAGGQFQP	11	MYRISTYL (N-*myristylation* site)	30
8	GGHGDQQQTAGTYGQ	15	-	27
9	MEYQGQ	6	-	24

10	EDERSTQSYQGGEAAEQVEVTDRGL LGNLLGKKKAEEDKEK	41	MYRISTYL (N-*myristylation* site) PKC_PHOSPHO_SITE AMIDATION	3
2z11	GVTGTEGLGHFQGQGQQHGHPTTRL DEYGNPVTAGHGVGLGSTGTGVH	48	MYRISTYL (N-*myristylation* site)	3
12	TGTHGTTATGGTYGQQGHAGMTGTGTHG	28	MYRISTYL (N-*myristylation* site)	10
13	EKKGVMEKISEKLP	14	Dehydrin Protein kinase C phosphorylation site	8
14	VDQYGNPIPREPGQVPAY SSGGAAPSYGSAGA VTSADYGAGVTPGYGQRGA	80	CTD, Dehydrin, CK2_PHOSPH-SITE	2

	VHPHESVVGGAVSPSGVAHT HEGALSGGL			
15	KDAATGQQHTAAAGEYAGT	19	MYRISTYL (N-*myristylation* site)	7

Table 6:Putative Functional partners of Wheat Dehydrins

S.No.	Dehydrin proteins	STRING Id	Identity%	Score	Preferred Name od functional partners
1	TaDHN14-8-U	Traes_6AS_453F82CC3.1	82.4	129	Traes_6AS_453F82CC3.1
2	TaDHN15-4-U	Traes_6AS_453F82CC3.1	79.1	122.1	Traes_6AS_453F82CC3.1
6	TaDHN16-5-6D	Traes_6DL_B28B5501C.1	100	120.6	Traes_6DL_B28B5501C.1
7	TaDHN16-2-6D	Traes_6AL_E2BFEA5F4.2	74	92.8	Traes_6AL_E2BFEA5F4.2
8	TaDHN15-9-6D	Traes_6DL_42DF4F0A4.2	84.4	116.3	Traes_6DL_42DF4F0A4.2
9	TaDHN14-5-6D	Traes_6DL_340B35395.1	100	165.2	Traes_6DL_340B35395.1
10	TaDHN16-1-6D	Traes_6DL_42DF4F0A4.2	100	171.4	Traes_6DL_42DF4F0A4.2
11	TaDHN23-2-6D	Traes_6DL_518F5BCCC.1	100	198.4	DHN3
12	TaDHN9-1-6D	Traes_6DL_42DF4F0A4.2	77.1	65.5	Traes_6DL_42DF4F0A4.2
13	TaDHN17-1-6D	Traes_6DL_134445958.2	97.8	131	CS120

14	TaDHN28-1-6D	Traes_6AL_033D6C680.1	93.4	248.4	COR410
15	TaDHN16-4-6B	Traes_6DL_B28B5501C.1	86.9	108.2	Traes_6DL_B28B5501C.1
16	TaDHN15-10-6B	Traes_6DL_42DF4F0A4.2	79.1	87.8	Traes_6DL_42DF4F0A4.2
17	TaDHN23-3-6B	Traes_6DL_518F5BCCC.1	94.4	179.9	DHN3
18	TaDHN40-1-6B	Traes_6DL_134445958.2	79.3	150.6	CS120
19	TaDHN27-4-6B	Traes_6AL_033D6C680.1	90.7	253.8	COR410
20	TaDHN20-1-6A	Traes_6AL_DAE444F2F.1	100	236.5	Traes_6AL_DAE444F2F.1
21	TaDHN14-7-6A	Traes_6DL_42DF4F0A4.2	78.6	90.9	Traes_6DL_42DF4F0A4.2
22	TaDHN15-7-6A	Traes_6AL_E2BFEA5F4.2	74	93.2	Traes_6AL_E2BFEA5F4.2
23	TaDHN16-3-6A	Traes_6DL_42DF4F0A4.2	94.4	155.2	Traes_6DL_42DF4F0A4.2
24	TaDHN22-1-6A	Traes_6AL_E2BFEA5F4.2	100	164.5	Traes_6AL_E2BFEA5F4.2
25	TaDHN101-1-6A	Traes_6DL_134445958.2	60.7	76.6	CS120

26	TaDHN9-2-6A	Traes_6DL_134445958.2	62.4	59.3	CS120
27	TaDHN19-1-6A	Traes_6DL_134445958.2	82.4	69.3	CS120
28	TaDHN28-2-6A	Traes_6AL_033D6C680.1	100	271.9	COR410
29	TaDHN15-6-6A	Traes_6AS_453F82CC3.1	100	160.2	Traes_6AS_453F82CC3.1
30	TaDHN15-8-5D	Traes_5DL_D5836CCE6.1	99.4	173.7	Traes_5DL_D5836CCE6.1
31	TaDHN15-5-5D	Traes_5BL_3B390119D.1	92.1	172.6	Traes_5BL_3B390119D.1
32	TaDHN13-1-5D	Traes_5DL_134F29727.1	84.1	146.4	Traes_5DL_134F29727.1
33	TaDHN14-4-5D	Traes_5DL_134F29727.1	100	199.1	Traes_5DL_134F29727.1
34	TaDHN15-3-5B	Traes_5BL_3B390119D.1	100	202.6	Traes_5BL_3B390119D.1
35	TaDHN15-1-5B	Traes_5BL_EC1881F75.1	100	196.4	Traes_5BL_EC1881F75.1
36	TaDHN14-1-5B	Traes_5DL_134F29727.1	86.6	149.1	Traes_5DL_134F29727.1
37	TaDHN14-3-5B	Traes_5DL_134F29727.1	98.4	191.4	Traes_5DL_134F29727.1

38	TaDHN15-2-5A	Traes_5BL_3B390119D.1	89.3	158.7	Traes_5BL_3B390119D.1
39	TaDHN14-2-5A	Traes_5DL_134F29727.1	81.9	136.7	Traes_5DL_134F29727.1
40	TaDHN14-6-5A	Traes_5DL_134F29727.1	92.7	178.3	Traes_5DL_134F29727.1
41	TaDHN41-1-4D	Traes_4DS_993693839.1	100	202.6	Traes_4DS_993693839.1
42	TaDHN43-2-4B	Traes_4DS_993693839.1	94.6	105.9	Traes_4DS_993693839.1
43	TaDHN43-1-4A	Traes_4DS_993693839.1	77.2	114	Traes_4DS_993693839.1
44	TaDHN27-2-3D	Traes_6AL_E2BFEA5F4.2	62.6	65.5	Traes_6AL_E2BFEA5F4.2
45	TaDHN22-2-3D	Traes_3B_EC4229279.2	86.7	150.2	Traes_3B_EC4229279.2
46	TaDHN27-3-3B	Traes_6DL_518F5BCCC.1	62.3	71.2	DHN3
47	TaDHN22-3-3B	Traes_3B_EC4229279.2	100	198	Traes_3B_EC4229279.2
48	TaDHN27-1-3A	Traes_6AL_E2BFEA5F4.2	67.7	69.3	Traes_6AL_E2BFEA5F4.2

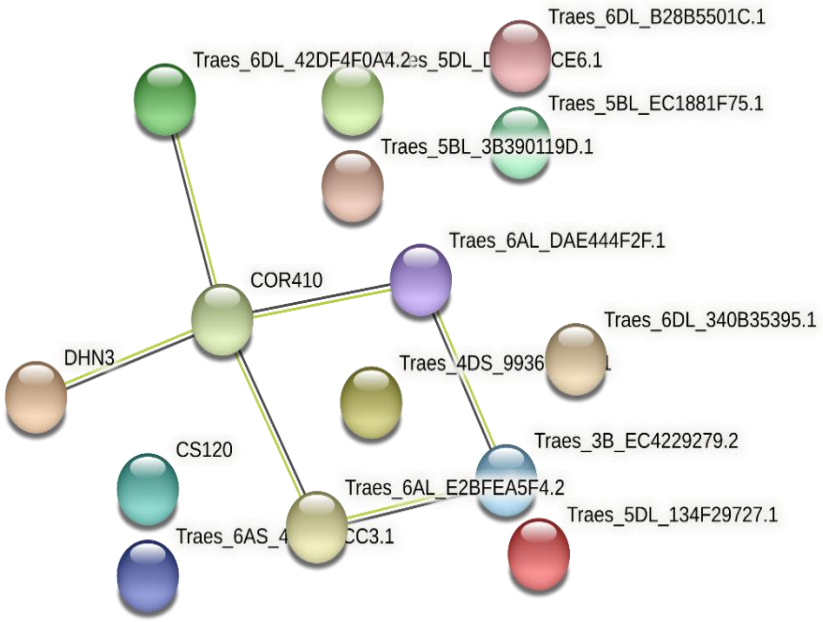


Figure 14: The STRING network view of dehydrin proteins in wheat.

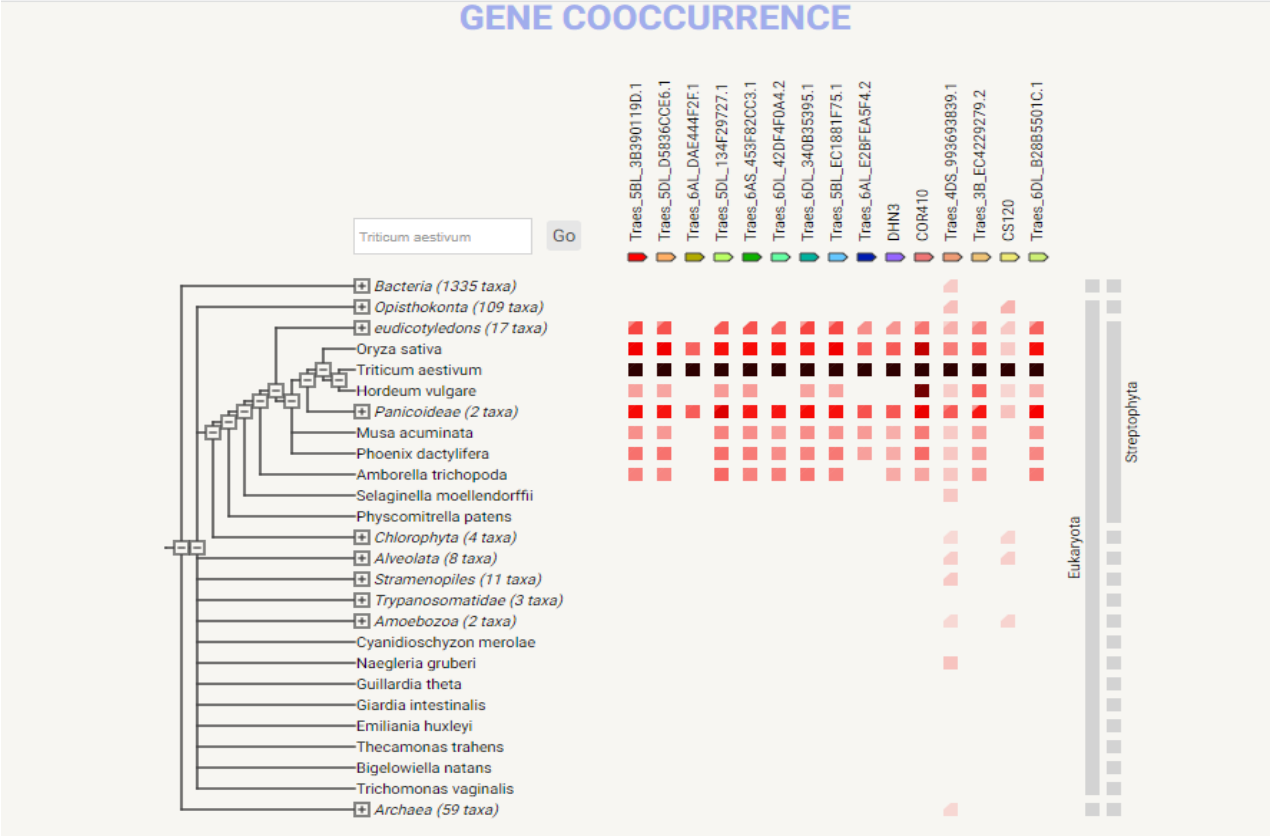


Figure 15: The presence or absence of linked proteins across species is indicated by gene-occurrence. The top of the picture has a list of proteins, and down a list of species names is given.

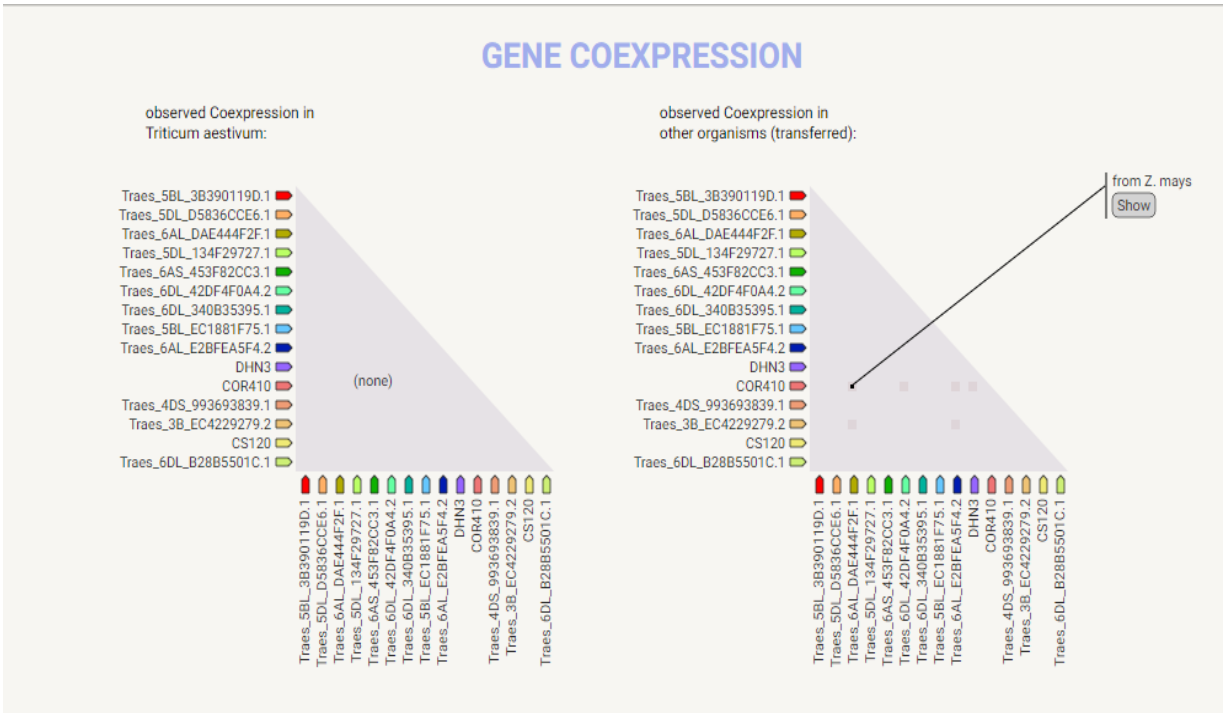


Figure 16: The genes that co-express with genes from other species in the same origin are displayed. Given the information on the total expression of the proteins in the organism, the intensity of the colour represents the degree of certainty that two proteins are functionally related. The red square indicates co-expression; the more vivid the colour, the higher the expression data's association score.

4.1.9 Gene Ontology analysis of wheat dehydrins

The putative genes were subjected to gene ontology (GO) analysis to represent their functionality on biological, cellular and molecular level. Annotation of these genes via ShinyGO 0.76 web server resulted in the identification of 14 different processes (Figure 17).

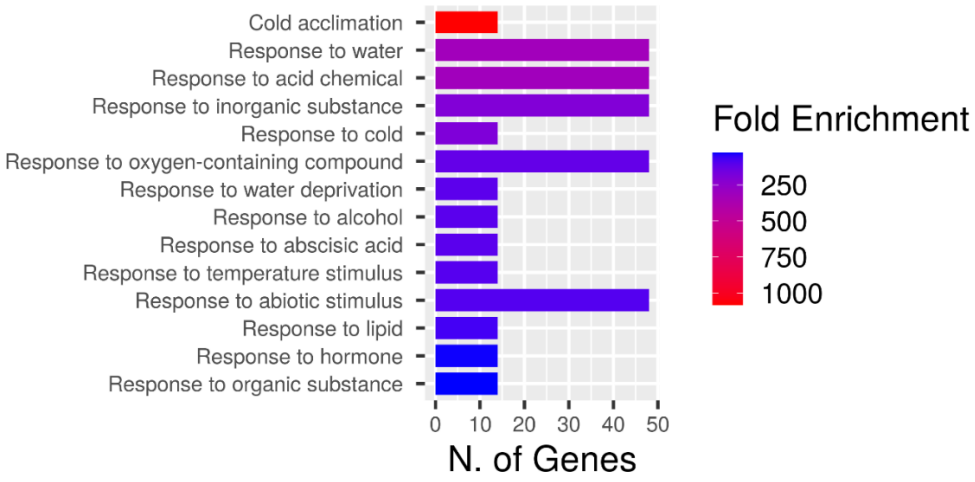


Figure 17: Gene ontology terms showing the number of genes (x-axis) in different pathways involved in v/s fold enrichment (y-axis)

All the 48 identified genes were found to involve in response to abiotic stimulus, O_2 containing compound, water and acid chemical. In addition, genes are also implicated in cold acclimation, inorganic substrate, alcohol, ABA, temperature, hormone and organic substrate. Fold enrichment is inversely proportional to the number of genes present in functional pathways lesser the genes, the more the fold enrichment. The relationship between enriched pathways is shown by interactive plots (Figure 18). Two pathways (nodes) are connected if they share 20% (default) or more genes. Darker nodes are more significantly enriched gene sets. Bigger nodes represent larger gene sets. Thicker edges represent more overlapped genes.

The results of the present study corroborate the previous reports (Eren *et al*., 2014) showing the involvement of DHN genes in various biological, molecular and

cellular functions.

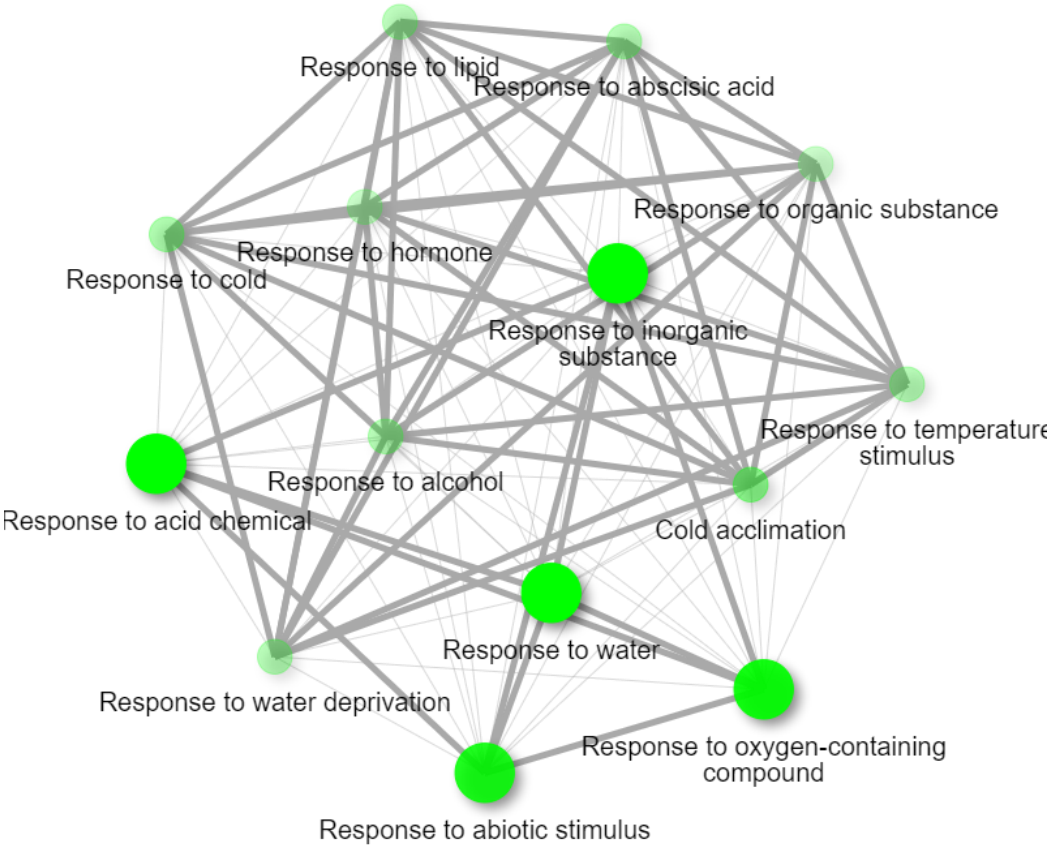


Figure 18: Interactive plot showing nodes and edges connecting two or more functional pathways. More significant gene sets are represented by darker nodes. larger gene sets are represented by bigger nodes and thicker nodes indicates the overlapped genes.

4.1.10 Disordered nature of wheat dehydrins

The dehydrin protein was found to be highly disordered by using the PONDR web server (Table 5.4). Dehydrins are well known to be intrinsically unstructured proteins(M. Hara, 2010b), which allows them to act without any structural limitation under various physiochemical environments. TaDHN14-6-5A was found to be highly disordered among all the identified proteins with 88.81% disorder nature while TaDHN28-1-6D was having the least percentage of disorder i.e., 57.25%. All the proteins are having disordered regions ranging from 3-21, which shows a good sign of

the presence of binding sites to common partners. According to intrinsic disorder's flexibility, different disordered regions can bind to the same binding site on a common partner. Binding diversity plays an essential role in protein-protein, protein-DNA interaction networks and, most likely, gene regulatory networks and efficient repair systems. Dehydrins show specific roles by modifying their secondary structure from a disordered state to an ordered state when they come in contact with their partner molecules during the encounter of the plant cell with the stress(Dyson & Wright, 2005a), (Tompa, 2005)(Z. Yu *et al*., 2018). The intrinsically disordered nature is highly advantageous to dehydrins and renders them incapable to denature during drought or low temperatures since they don't have any significant structure that can be lost and have very rare hydrophobic residues that can cause aggregation (Graether & Boddington, 2014). Globular proteins are subjected to hydrophobic residues in an aqueous environment during denaturation, where they might interact with the exposed hydrophobic residues and begin to congregate.

4.1.11 Secondary structure prediction of wheat dehydrins

The GOR tool was used to predict the secondary structure of the identified dehydrin proteins, revealing the presence of both random coils and alpha helices. The secondary structure prediction also showed that all proteins lacked ordered secondary structure and were intrinsically disordered due to the presence of random coils. The number of coils present varied, with TaDHN101-1-6A having the highest number of coils (216) and TaDHN9-2-6A having the lowest number (17). Analysis of the amino acid composition revealed that TaDHN101-1-6A had the highest glycine content (51.9%), which is thought to allow the protein to form intra-molecular hydrogen bonds and adopt a random coil conformation. This property enables the protein to stretch, bend, and expand in all possible orientations, allowing it to bind water molecules and protect cellular structures and lipid membranes from dehydration under dehydrating conditions (Rorat *et al*., 2006).

Amphipathic α-helices are mainly formed by the K-segment which associates with the membrane surfaces(Rorat *et al*., 2006)(Allagulova *et al*., 2003). In secondary structure prediction, it has been found that α-helix is present in all the identified

proteins, which means that this would stabilize the proteins and membrane structure during abiotic stress (L. N. Rahman *et al*., 2010). TaDHN28-1-6D is showing the maximum number of α-helixes i.e. 154, which assists more binding of dehydrins and water cells and therefore helps to stabilize the cell structure and its organelles to prevent loss of water during environmental stress(S. Eriksson *et al*., 2016).

Table 7: Percentage of disordered nature of dehydrin proteins predicted by PONDR database. Ta indicates *Triticum aestivum*, DHN indicates dehydrin followed by the molecular weight of protein and chromosome number

S.No.	Protein Name	Predicted residues	No. of residues disordered	% disordered	No. of disordered regions	Longest disordered region	Average prediction score
1	TaDHN14-8-U	149	121	81.21	5	41	0.7176
2	TaDHN15-4-U	151	103	68.21	5	44	0.6583
3	TaDHN11-1-7D	112	73	65.18	4	30	0.6095
4	TaDHN12-1-7B	125	86	68.80	3	49	0.6398
5	TaDHN12-2-7A	124	82	66.13	3	47	0.5879
6	TaDHN16-5-6D	167	143	85.63	3	93	0.7692
7	TaDHN16-2-6D	160	120	75	4	44	0.7235
8	TaDHN15-9-6D	155	132	85.16	3	93	0.7647

9	TaDHN14-5-6D	144	118	81.94	4	59	0.7725
10	TaDHN16-1-6D	162	135	83.33	5	51	0.7532
11	TaDHN23-2-6D	231	180	77.92	5	109	0.7575
12	TaDHN9-1-6D	93	63	67.74	3	45	0.6452
13	TaDHN17-1-6D	179	103	57.74	4	63	0.5578
14	TaDHN28-1-6D	262	150	57.25	7	44	0.5633
15	TaDHN16-4-6B	166	140	84.34	3	92	0.7511
16	TaDHN15-10-6B	158	134	84.81	3	95	0.7562
17	TaDHN23-3-6B	231	183	79.22	4	110	0.7630
18	TaDHN40-1-6B	405	255	62.96	7	73	0.6229
19	TaDHN27-4-6B	259	164	63.32	6	45	0.5940
20	TaDHN20-1-6A	190	144	75.79	7	45	0.7047

21	TaDHN14-7-6A	143	124	86.71	3	66	0.7979
22	TaDHN15-7-6A	152	124	81.58	4	42	0.7349
23	TaDHN16-3-6A	162	124	76.54	5	47	0.7198
24	TaDHN22-1-6A	221	174	78.73	4	110	0.7649
25	TaDHN101-1-6A	991	686	69.22	21	67	0.6340
26	TaDHN9-2-6A	93	60	64.52	3	42	0.5934
27	TaDHN19-1-6A	190	122	64.21	4	62	0.6254
28	TaDHN28-2-6A	268	163	60.82	5	44	0.5738
29	TaDHN15-6-6A	153	115	75.16	4	42	0.7044
30	TaDHN15-8-5D	154	104	67.53	5	39	0.6527
31	TaDHN15-5-5D	152	98	64.47	5	38	0.6486
32	TaDHN13-1-5D	133	79	59.40	5	37	0.5463

33	TaDHN14-4-5D	143	118	82.52	3	90	0.6880
34	TaDHN15-3-5B	150	93	62.00	5	34	0.6254
35	TaDHN15-1-5B	150	115	76.67	4	66	0.7072
36	TaDHN14-1-5B	138	96	69.57	6	38	0.5979
37	TaDHN14-3-5B	143	118	82.52	3	90	0.6922
38	TaDHN15-2-5A	149	102	68.46	7	38	0.6565
39	TaDHN14-2-5A	140	110	78.57	3	90	0.6604
40	TaDHN14-6-5A	143	127	88.81	2	119	0.7298
41	TaDHN41-1-4D	430	312	72.56	7	122	0.7069
42	TaDHN43-2-4B	457	334	73.09	8	156	0.6923
43	TaDHN43-1-4A	455	341	74.95	7	147	0.7168
44	TaDHN27-2-3D	275	230	83.64	4	134	0.8008

45	TaDHN22-2-3D	215	166	77.21	3	156	0.7533
46	TaDHN27-3-3B	274	212	77.37	4	144	0.7315
47	TaDHN22-3-3B	217	169	77.88	3	159	0.7432
48	TaDHN27-1-3A	275	233	84.73	3	145	0.7958

Table 8: The secondary structure (coils, turns sheets and α-helixes) predicted by GOR database. Ta indicates *Triticum aestivum*, DHN indicates dehydrin followed by molecular weight of protein and chromosome number

S No.	Protein Name	AA	No. of coils	No. of helix	No. of turns	No. of sheets
1	TaDHN14-8-U	149	30	47	34	38
2	TaDHN15-4-U	151	28	55	30	38
3	TaDHN11-1-7D	112	23	25	22	42
4	TaDHN12-1-7B	125	27	24	28	46
5	TaDHN12-2-7A	124	23	35	24	42
6	TaDHN16-5-6D	167	43	36	26	62
7	TaDHN16-2-6D	160	21	50	31	58
8	TaDHN15-9-6D	155	36	38	25	56
9	TaDHN14-5-6D	144	30	34	18	62
10	TaDHN16-1-6D	162	35	36	23	68

11	TaDHN23-2-6D	231	46	37	30	118
12	TaDHN9-1-6D	93	18	31	12	32
13	TaDHN17-1-6D	179	24	51	22	82
14	TaDHN28-1-6D	262	44	154	40	24
15	TaDHN16-4-6B	166	38	40	29	59
16	TaDHN15-10-6B	158	30	45	24	59
17	TaDHN23-3-6B	231	42	36	38	115
18	TaDHN40-1-6B	405	69	83	53	200
19	TaDHN27-4-6B	259	41	155	40	23
20	TaDHN20-1-6A	`190	29	54	49	58
21	TaDHN14-7-6A	143	23	49	22	49
22	TaDHN15-7-6A	152	41	36	27	48

23	TaDHN16-3-6A	162	30	35	25	72
24	TaDHN22-1-6A	221	45	36	32	108
25	TaDHN101-1-6A	991	216	205	167	403
26	TaDHN9-2-6A	93	17	28	11	37
27	TaDHN19-1-6A	190	30	45	21	94
28	TaDHN28-2-6A	268	47	155	39	27
29	TaDHN15-6-6A	153	28	63	28	34
30	TaDHN15-8-5D	154	45	37	32	40
31	TaDHN15-5-5D	152	41	39	31	41
32	TaDHN13-1-5D	133	20	58	31	24
33	TaDHN14-4-5D	143	35	47	32	49
34	TaDHN15-3-5B	150	37	38	34	41

35	TaDHN15-1-5B	150	42	38	28	42
36	TaDHN14-1-5B	138	19	61	28	30
37	TaDHN14-3-5B	143	32	46	33	32
38	TaDHN15-2-5A	149	36	43	33	37
39	TaDHN14-2-5A	140	18	59	26	37
40	TaDHN14-6-5A	143	29	55	31	28
41	TaDHN41-1-4D	430	65	64	60	241
42	TaDHN43-2-4B	457	59	56	56	286
43	TaDHN43-1-4A	455	60	58	64	273
44	TaDHN27-2-3D	275	81	53	49	92
45	TaDHN22-2-3D	215	60	43	43	69
46	TaDHN27-3-3B	274	83	57	39	95

47	TaDHN22-3-3B	217	54	44	48	71
48	TaDHN27-1-3A	275	78	66	35	96

4.1.12 Synteny analysis

Synteny analysis is conducted to confirm homology as well as authenticate the existence of homologous gene in the flanking regions around the interested gene among different species (Zayed & Badawi, 2019).

Gene duplication plays a crucial role in gene amplification, successive evolution as well as progress of new gene families in wheat. Even tandem duplication occurs when two or more alike gene are in close proximity of 200 kb on the chromosome (Holub, 2001). With the help of McScanX, we compared the rates of dehydrin duplication in *Triticum aestivum* (AABBDD), *Triticum urartu* (AA), *Triticum dicoccoides* (AABB), and *Aegilops tauschii* (DD) (Wang *et al*., 2016). Moreover, due to the lack of the donor's genome sequence for the B sub-genome, *T. dicoccoides (A. tauschii, wild emmer wheat)* was chosen as the source of this sub-genome. On assessment, numerous tandems pairs (in duplicate) of dehydrin genes were found in *T. urartu* (9), *T. aestivum* (29), *T. dicoccoides* (19) and *A. tauschii* (8) (Figure 19) (Table 5.5). Orthologous portions of the genome were found to share collinear clusters of dehydrin genes (Figure 19) after a microcolinearity study was performed on genomes from all of the parent species (Table 5.5). Five clusters of dehydrin genes can be found in *Aegilops tauschii*, and these clusters are collinear with the two progenitors, *Triticum aestivum* and *Triticum Urartu*. In addition, two segmentally duplicated blocks of DHNs of *Triticum aestivum* were also identified. A single cluster of *T. dicoccoides* DHN genes was found to be collinear in *T. urartu*. the findings suggest that both segmental gene duplication and tandem duplication events might have contributed to the diversification of gene family "dehydrin" in wheat. Additionally, our data unveils that wheat dehydrins were acquired from their progenitors as they show the similarity with the number of genes in the chromosomes as that were accorded in the respective sub-genome donor chromosomes. Higher numbers of DHNs genes were shown to be present on chromosomes 5D, 6D, and 6B compared to the sub- genome sources, suggesting abundance of dehydrins due to duplication events.

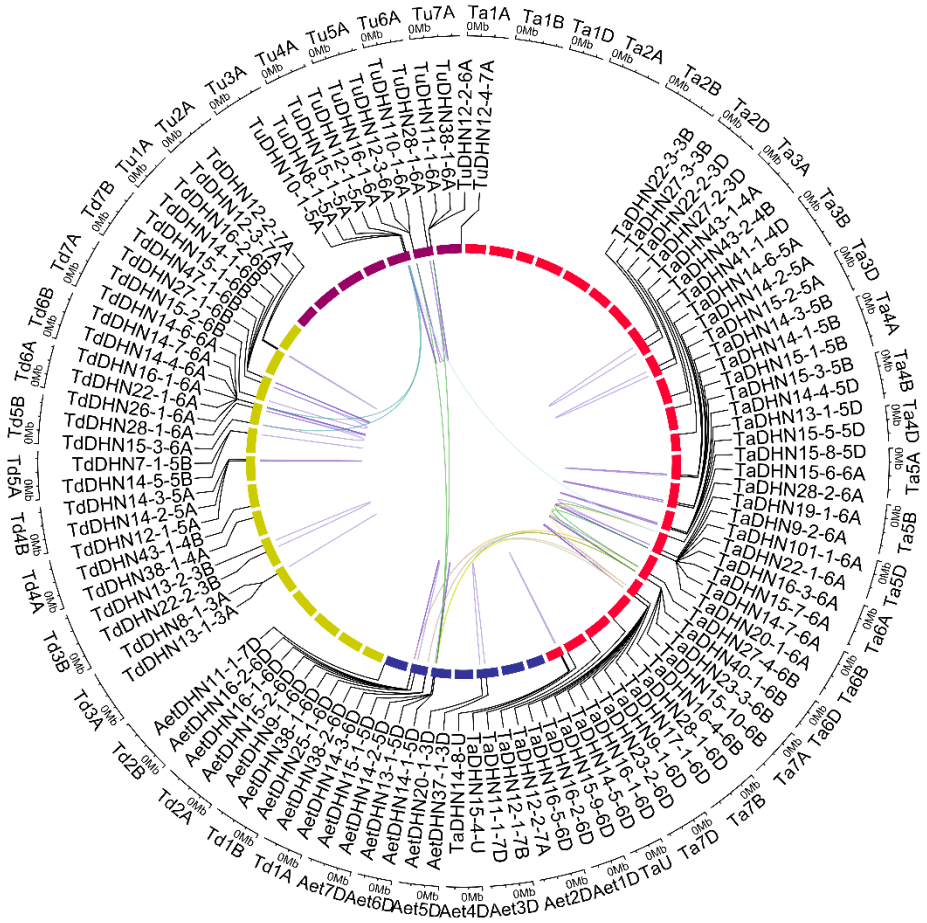


Figure 19: Illustration of collinearity analysis of TaDHN genes mined from *T. aestivum* genome sequence with their progenitor species *(A. tauschii, T. urartu and T. dicoccoides)*

Note for figure 19: the boxes represent in red, blue, purple and yellow represents the T. aestivum (Ta, AABBDD), A. tauschii (AeT, DD), T. urartu (Tu, AA) and T. dicoccoides (Td, AABB) respectively. The green lines represent the collinearity of blocks and show their joining across the corresponding chromosomes in *Triticum urartu* and *Aegilops tauschii.* The purple lines represent the tandemly duplicated genes whereas scale of the circles represents the Megabases.

4.1.13 Identification of cis- regulatory elements

The gene expression is controlled by specific transcription factors after binding to the specific DNA sequences (Luo *et al*., 2013). Thus, the prediction of these DNA binding motifs of transcription factors is a significant approach to analyze the functional aspect of these factors which are called promoter regions. Promoter regions of genomic sequences exhibit cis-regulatory elements, to which transcription factors bind and enhance the gene expression. Regulation of transcription is one of the most important and dynamic processes as it ultimately controls the expression of genes under various conditions (Lenstra *et al*., 2015).

In the present study, a total of 69 cis-regulatory elements have been identified using the PLANTCARE. These *cis*-regulatory elements were grouped into different functional categories, viz. cellular functions, light stress and hormone response. (Figure 5.11; Table 5.5). Among the functionally annotated results 69 regulatory elements, the largest group comprised of light-responsive elements (19) followed by other functions which include flavonoid biosynthetic genes regulation, expression in inducing dehydration etc. (17), stress-responsive (13), hormone regulators (10) and cellular functions (10) (Figure 20). However, about 11 of 69 *cis*-elements belonged to the group with un-annotated functions in the PlantCARE database. The similar cis-regulatory elements acting as transcriptional initiation sites (TATA-Box, ABRE, MBS, MYC, LTRE and Gt1 motif) were found to be present in dehydrin (CdDHN4-P) identified in Bermudagrass (Lv *et al*., 2017).

The group of promoter regions that are motifs comprising of 5'UTR are related to cellular development and helps in high transcription levels. Ry-element, CAT-Box, NON-Box, TATCCATC/C plays important role in meristem and seed specific activation genes. GCN4_motif is responsible for endosperm specific expression, O2-site is responsible for the zein regulation metabolism and circadian control is regulated by circadian elements. GCN4 _motif and Ry-element have been reported for many seed storage protein coding genes (Onodera *et al*., 2001). Also, GATA and GCN4_motif are known to be functional regulatory unit of seed storage protein wit high molecular weight in wheat (Ravel *et al*.,

2014). Ry-element is responsible for the seed and tissue specific expression in plants (Ezcurra *et al*., 2000).

Analysis of cis regulatory elements revealed various stress responsive elements including W-box, WUN- motif, LTR, MBS, DRE, TC-rich and ABRE in the promoter regions in wheat dehydrins. WUN-motif and W-motif are responsible for responding to wound, additionally W-box regulates such genes which are related to fungal infection. It is known that various defence genes in plants contains W-box elements and are target of WRKY proteins (D. Yu *et al*., 2001). MYB -binding site and MBS) responds to the drought specific signals and found in almost all of TaDHNs (Nash *et al*., 1990) . While ABRE is abscisic acid responsive element and DRE is the element which controls the cold and drought conditions(Hattori *et al*., 2002)s. TC-rich repeats help in regulating defence mechanism in plants(Diaz-De-Leon *et al*., 1993). The presence of these stress responsive elements in upstream regions of the TaDHN genes indicates their likely roles in diverse biotic and abiotic stress responses which are not yet characterized.

In addition to stress conditions, cis elements involved in hormonal responses and light responses were also identified in the promoter regions of TaDHNs. G-Box, CAG, GT1-motif, I-box, ABRE4, AE-Box, BOX4, Sp1, as-1 etcetera were identified which are involved in light responses. G-Box motifs are known to be present in promoters of genes that are expressed in response to various stimuli like ABA, light or ethylene. I-boxes are kwon to be light responsive as well as circadian control elements. These have been frequently reported in regulatory regions of sucrose transporters of rice and Arabidopsis, where they play role as a linkage between sugar metabolism and perception (Ibraheem *et al*., 2010). Hormonal responsive elements P-box, TGA, TCA, AuxRR-core, TATC motif, GARE, CCAAT-Box, ABRE3a, CGTCA motif and TGACG motif were identified which controls the gene expression in response to auxins, gibberellins, ABA, salicylic acid and methyl-jasmonate and ethylene (Kim *et al*., 2006; Klessig & Malamy, 1994; Sazegari *et al*., 2015).

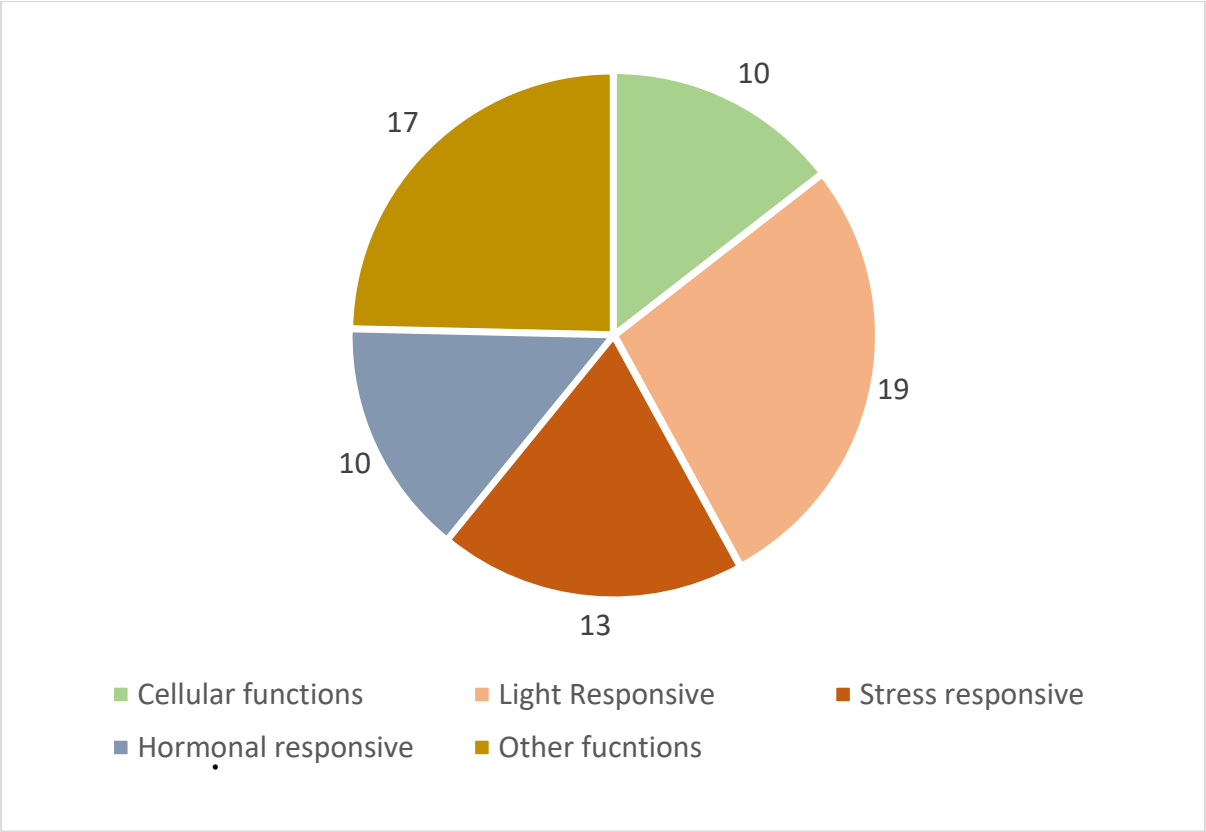


Figure 20: Pie chart showing distribution of cis-regulatory elements based on their biological functions

Table 9: List of cis- regulatory elements using PLANTCARE, within 2000 upstream region of 48 TaDHN genes

Categories based on function of cis-elements	***Cis*-elements**	**Genes**	**Sequence**	**Specific functions of *cis*-elements**
Cellular functions	GCN4_Motif	*TaDHN14-2-5A, TaDHN15-8-5D, TaDHN15-6-6A, TaDHN28-2-6A, TaDHN28-1-6D, TaDHN17-1-6D*	TGAGTCA	Endosperm-specific expression
	RY-element	*TaDHN27-1-3A, TaDHN22-3-3B, TaDHN22-2-3D, TaDHN14-2-5A, TaDHN13-1-5D, TaDHN15-8-5D*	CATGCATG	Tissue and seed-specific regulation
	O2-Site	*TaDHN43-1-4A, TaDHN41-1-4D, TaDHN14-2-5A, TaDHN15-2-5A, TaDHN15-3-5B, TaDHN15-8-5D, TaDHN9-2-6A, TaDHN20-1-6A, TaDHN23-3-6B, TaDHN17-1-6D, TaDHN23-2-6D, TaDHN16-1-6D, TaDHN16-2-6D, TaDHN16-5-6D, TaDHN12-2-7A, TaDHN12-1-7B*	GATGACATGG	cis-acting regulatory element involved in zein metabolism regulation
	Circadian	*TaDHN22-3-3B, TaDHN22-2-3D, TaDHN43-1-4A, TaDHN41-1-4D, TaDHN14-6-5A, TaDHN19-1-6A, TaDHN16-4-6B, TaDHN12-2-7A*	CAAAGATATC	cis-acting regulatory element involved in circadian control

	HD-Zip 3	*TaDHN12-1-7B*	GTAAT(G/C) ATTAC	protein binding site
	Box III	*TaDHN40-1-6B*	atCATTTTCACt	protein binding site
	MSA-like seq	*TaDHN27-1-3A, TaDHN43-1-4A, TaDHN41-1-4D, TaDHN14-6-5A, TaDHN14-3-5B, TaDHN14-5-6D, TaDHN16-5-6D*	TAACCA/ TCAAACGGT	Involve in cell cycle regulation
	CAT-Box	*TaDHN27-1-3A, TaDHN27-3-3B, TaDHN27-2-3D, TaDHN43-2-4B, TaDHN28-2-6A, TaDHN14-6-5A, TaDHN14-2-5A, TaDHN15-2-5A, TaDHN14-3-5B, TaDHN14-1-5B, TaDHN15-1-5B, TaDHN15-3-5B, TaDHN13-1-5D, TaDHN15-8-5D, TaDHN15-6-6A, TaDHN9-2-6A, TaDHN101-1-6A, TaDHN16-3-6A, TaDHN15-7-6A, TaDHN14-7-6A, TaDHN20-1-6A, TaDHN27-4-6B, TaDHN40-1-6B, TaDHN23-3-6B, TaDHN16-4-6B, TaDHN28-1-6D, TaDHN17-1-6D, TaDHN9-1-6D,* *TaDHN14-5-6D, TaDHN15-9-6D, TaDHN16-2-6D, TaDHN12-2-7A, TaDHN12-1-7B, TaDHN11-1-7D*	GCCACT	cis-acting regulatory element related to meristem expression Meristem-specific expression
	CCGTCC motif	*TaDHN22-3-3B, TaDHN27-3-3B, TaDHN22-2-3D, TaDHN43-2-4B, TaDHN41-1-4D, TaDHN14-2-5A, TaDHN15-2-5A, TaDHN15-1-5B, TaDHN14-4-5D, TaDHN15-5-5D, TaDHN15-6-6A, TaDHN28-2-6A, TaDHN19-1-6A, TaDHN101-1-6A, TaDHN22-1-6A, TaDHN16-3-6A, TaDHN15-7-6A, TaDHN20-1-6A, TaDHN27-4-6B, TaDHN23-3-6B, TaDHN23-3-6B, TaDHN15-10-6B, TaDHN16-4-6B, TaDHN28-1-6D,TaDHN17-1-6D, TaDHN9-1-6D, TaDHN23-2-6D, TaDHN15-9-6D,*	CCGTCC	----------

		TaDHN14-5-6D, TaDHN16-2-6D, TaDHN16-5-6D, TaDHN12-2-7A, TaDHN11-1-7D		
	NON-BOX	*TaDHN43-2-4B, TaDHN15-10-6B, TaDHN14-5-6D*	AGATCGACG	Helps in meristem activation
	GATA motif	*TaDHN14-6-5A, TaDHN14-2-5A, TaDHN27-2-3D, TaDHN15-2-5A, TaDHN14-3-5B, TaDHN14-1-5B, TaDHN15-6-6A, TaDHN28-2-6A, TaDHN22-1-6A, TaDHN16-3-6A, TaDHN15-7-6A, TaDHN27-4-6B, TaDHN40-1-6B, TaDHN23-3-6B, TaDHN15-10-6B, TaDHN16-4-6B, TaDHN28-1-6D, TaDHN9-1-6D, TaDHN23-2-6D, TaDHN16-1-6D, TaDHN14-5-6D, TaDHN15-9-6D, TaDHN16-5-6D, TaDHN12-1-7B*	AAGGATAAGG/ GATAGGG	Light responsive element
	G-Box	*TaDHN27-1-3A, TaDHN22-3-3B, TaDHN27-3-3B, TaDHN22-2-3D, TaDHN27-2-3D, TaDHN43-1-4A, TaDHN43-2-4B, TaDHN41-1-4D, TaDHN14-6-5A, TaDHN14-2-5A, TaDHN15-2-5A, TaDHN14-3-5B, TaDHN14-1-5B, TaDHN15-3-5B, TaDHN13-1-5D, TaDHN22-1-6A, TaDHN15-5-5D, TaDHN15-8-5D, TaDHN15-6-6A, TaDHN28-2-6A, TaDHN19-1-6A, TaDHN9-2-6A, TaDHN101-1-6A, TaDHN16-3-6A, TaDHN15-7-6A, TaDHN14-7-6A, TaDHN20-1-6A, TaDHN27-4-6B,* *TaDHN40-1-6B, TaDHN23-3-6B, TaDHN15-10-6B, TaDHN16-4-6B, TaDHN28-1-6D, TaDHN17-1-6D, TaDHN9-1-6D, TaDHN23-2-6D, TaDHN16-1-6D, TaDHN14-5-6D, TaDHN15-9-6D, TaDHN16-2-6D, TaDHN16-5-6D, TaDHN12-2-7A, TaDHN12-1-7B, TaDHN11-1-7D*	CACGTC/TACGTG/ CACGTT	Light responsive element

Light responsive elements	CAG motif	*TaDHN14-1-5B*	GAAAGGCAGAC	Light responsive element
	GT1 motif	*TaDHN27-1-3A, TaDHN27-3-3B, TaDHN41-1-4D, TaDHN14-6-5A, TaDHN15-1-5B, TaDHN13-1-5D, TaDHN15-8-5D, TaDHN101-1-6A, TaDHN15-7-6A, TaDHN14-7-6A, TaDHN40-1-6B, TaDHN23-3-6B, TaDHN9-1-6D, TaDHN23-2-6D*	GGTTAA	Light responsive
	I-Box	*TaDHN27-1-3A, TaDHN27-3-3B, TaDHN43-1-4A, TaDHN43-2-4B, TaDHN14-6-5A, TaDHN15-2-5A, TaDHN14-3-5B, TaDHN15-3-5B, TaDHN15-8-5D, TaDHN15-6-6A, TaDHN14-7-6A, TaDHN20-1-6A, TaDHN40-1-6B, TaDHN15-10-6B, TaDHN17-1-6D, TaDHN16-1-6D*	gGATAAGGTG/ cGATAAGGCG	Light responsive element
	ABRE4	*TaDHN27-1-3A, TaDHN22-3-3B, TaDHN27-3-3B, TaDHN22-2-3D, TaDHN27-2-3D, TaDHN43-1-4A, TaDHN43-2-4B, TaDHN41-1-4D, TaDHN14-6-5A, TaDHN15-2-5A, TaDHN14-1-5B, TaDHN15-1-5B, TaDHN15-3-5B, TaDHN15-5-5D, TaDHN15-8-5D, TaDHN15-6-6A, TaDHN28-2-6A, TaDHN19-1-6A, TaDHN101-1-6A, TaDHN22-1-6A, TaDHN16-3-6A, TaDHN14-7-6A, TaDHN15-7-6A, TaDHN20-1-6A,* *TaDHN27-4-6B, TaDHN40-1-6B, TaDHN15-10-6B, TaDHN16-4-6B, TaDHN17-1-6D, TaDHN9-1-6D, TaDHN23-2-6D, TaDHN14-5-6D, TaDHN15-9-6D, TaDHN16-2-6D, TaDHN16-5-6D*	CACGTA	cis-acting regulatory element involved in light responsiveness
	AE-Box	*TaDHN41-1-4D, TaDHN14-1-5B, TaDHN15-1-5B, TaDHN13-1-5D, TaDHN15-6-6A, TaDHN28-2-6A, TaDHN14-7-6A,*		Light responsive element

		TaDHN20-1-6A, TaDHN14-5-6D, TaDHN16-2-6D, TaDHN16-5-6D, TaDHN12-2-7A	AGAAACTT	
	BOX 4	*TaDHN27-1-3A, TaDHN14-4- 5D, TaDHN14-2-5A, TaDHN15-1-5B, TaDHN15-5-5D, TaDHN15-8-5D, TaDHN9-2-6A, TaDHN101-1-6A, TaDHN23-3-6B, TaDHN15-10-6B, TaDHN23-2-6D, TaDHN12-1-7B*	ATTAAAT	part of a conserved DNA module involved in light responsiveness
	Sp1	*TaDHN27-1-3A, TaDHN22-3-3B, TaDHN27-3-3B, TaDHN22-2-3D, TaDHN27-2-3D, TaDHN43-1-4A, TaDHN43-2-4B, TaDHN41-1-4D, TaDHN15-2-5A, TaDHN14-3-5B, TaDHN14-1-5B, TaDHN13-1-5D, TaDHN15-5-5D, TaDHN28-2-6A, TaDHN22-1-6A, TaDHN16-3-6A, TaDHN15-7-6A, TaDHN20-1-6A, TaDHN27-4-6B, TaDHN40-1-6B, TaDHN23-3-6B, TaDHN15-10-6B, TaDHN28-1-6D, TaDHN17-1-6D, TaDHN9-1-6D, TaDHN23-2-6D, TaDHN16-1-6D, TaDHN15-9-6D, TaDHN16-2-6D, TaDHN16-5-6D*	GGGCGG	light responsive element
	as-1	*TaDHN27-1-3A, TaDHN22-3-3B, TaDHN27-3-3B, TaDHN27-2-3D, TaDHN43-1-4A, TaDHN15-6-6A, TaDHN43-2-4B, TaDHN41-1-4D, TaDHN14-2-5A, TaDHN14-3-5B, TaDHN14-1-5B, TaDHN15-1-5B, TaDHN15-5-5D, TaDHN14-4-5D, TaDHN13-1-5D, TaDHN28-2-6A, TaDHN9-2-6A, TaDHN101-1-6A, TaDHN16-3-6A, TaDHN15-7-6A, TaDHN14-7-6A, TaDHN20-1-6A, TaDHN27-4-6B, TaDHN40-1-6B, TaDHN15-10-6B, TaDHN16-4-6B, TaDHN28-1-6D, TaDHN17-1-6D, TaDHN9-1-6D, TaDHN16-1-6D, TaDHN14-5-6D, TaDHN15-*	TGACG	light responsive element

		9-6D, TaDHN16-2-6D, TaDHN16-5-6D, TaDHN12-2-7A, TaDHN12-1-7B, TaDHN11-1-7D		
	GA-motif	*TaDHN14-6-5A, TaDHN17-1-6D*	ATAGATAA	Light responsive element
	3-AF1 binding site	*TaDHN9-2-6A*	TAAGAGAGGAA	Light responsive element
	TCT motif	*TaDHN27-3-3B, TaDHN43-1-4A, TaDHN14-2-5A, TaDHN15-2-5A, TaDHN14-1-5B, TaDHN13-1-5D, TaDHN15-8-5D, TaDHN9-2-6A, TaDHN101-1-6A, TaDHN15-7-6A, TaDHN14-7-6A, TaDHN40-1-6B, TaDHN15-10-6B, TaDHN17-1-6D, TaDHN9-1-6D*	TCTTAC	Light responsive element
	Chs-CMA2a	*TaDHN15-2-5A, TaDHN14-3-5B, TaDHN14-4-5D, TaDHN16-4-6B, TaDHN17-1-6D, TaDHN9-1-6D, TaDHN16-5-6D*	TCACTTGA/ TTACTTAA	Light responsive element
	LAMP element	*TaDHN41-1-4D*	CTTTATCA	Light responsive element
	GAP-Box	*TaDHN41-1-4D*	CAAATGAA(A/G) A	Light responsive element
	ACE	*TaDHN22-3-3B, TaDHN22-2-3D, TaDHN14-1-5B, TaDHN15-3-5B, TaDHN28-2-6A, TaDHN9-2-6A, TaDHN20-1-6A, TaDHN27-4-6B, TaDHN28-1-6D, TaDHN15-9-6D, TaDHN16-2-6D*	GACACGTATG	Light responsiveness

	A-Box	*TaDHN22-3-3B, TaDHN27-3-3B, TaDHN22-2-3D, TaDHN43-2-4B, TaDHN41-1-4D, TaDHN14-2-5A, TaDHN15-2-5A, TaDHN15-1-5B, TaDHN14-4-5D, TaDHN15-5-5D, TaDHN15-6-6A, TaDHN28-2-6A, TaDHN19-1-6A, TaDHN101-1-6A, TaDHN22-1-6A, TaDHN16-3-6A, TaDHN15-7-6A, TaDHN20-1-6A, TaDHN27-4-6B, TaDHN23-3-6B, TaDHN23-3-6B, TaDHN15-10-6B, TaDHN16-4-6B, TaDHN28-1-6D, TaDHN17-1-6D, TaDHN9-1-6D, TaDHN23-2-6D, TaDHN15-9-6D, TaDHN14-5-6D, TaDHN16-2-6D, TaDHN16-5-6D, TaDHN12-2-7A, TaDHN11-1-7D*	CCGTCC	Light responsiveness
	W-Box	*TaDHN22-3-3B, TaDHN22-2-3D, TaDHN27-2-3D, TaDHN14-6-5A, TaDHN14-2-5A, TaDHN14-3-5B, TaDHN15-3-5B, TaDHN13-1-5D, TaDHN15-6-6A, TaDHN19-1-6A, TaDHN40-1-6B, TaDHN23-3-6B, TaDHN16-1-6D, TaDHN14-5-6D, TaDHN12-2-7A, TaDHN12-1-7B*	TTGACC	Fungal elicitor responsive element, wound-responsive
	ERE	*TaDHN22-2-3D, TaDHN43-1-4A, TaDHN41-1-4D, TaDHN14-6-5A, TaDHN15-2-5A, TaDHN14-1-5B, TaDHN14-4-5D, TaDHN15-6-6A, TaDHN9-2-6A, TaDHN22-1-6A, TaDHN23-3-6B, TaDHN16-4-6B, TaDHN17-1-6D, TaDHN12-2-7A, TaDHN12-1-7B, TaDHN11-1-7D*	ATTTCATA	Ethylene responsive element
	AT rich element	*TaDHN23-3-6B, TaDHN28-1-6D, TaDHN23-2-6D, TaDHN12-1-7B*	ATAGAAATCAA	binding site of AT-rich DNA binding protein (ATBP-1)
	LTR	*TaDHN27-1-3A, TaDHN22-3-3B, TaDHN22-2-3D, TaDHN27-2-3D, TaDHN43-2-4B, TaDHN41-1-4D, TaDHN14-4-5D,*		

Stress responsive		*TaDHN27-2-3D, TaDHN14-2-5A, TaDHN15-8-5D, TaDHN28-2-6A, TaDHN19-1-6A, TaDHN9-2-6A, TaDHN16-3-6A, TaDHN15-7-6A, TaDHN20-1-6A, TaDHN27-4-6B, TaDHN23-3-6B, TaDHN15-10-6B, TaDHN16-4-6B, TaDHN28-1-6D, TaDHN17-1-6D, TaDHN14-5-6D, TaDHN16-5-6D, TaDHN12-2-7A, TaDHN12-1-7B*	CCGAAA	Low temperature response
	MBS	*TaDHN15-2-5A, TaDHN14-3-5B, TaDHN15-1-5B, TaDHN15-3-5B, TaDHN14-4-5D, TaDHN19-1-6A, TaDHN101-1-6A, TaDHN20-1-6A, TaDHN16-4-6B, TaDHN17-1-6D, TaDHN16-2-6D, TaDHN12-2-7A, TaDHN12-1-7B, TaDHN11-1-7D*	CAACTG	MYB binding site involved in drought-inducibility
	STRE	*TaDHN27-1-3A, TaDHN22-3-3B, TaDHN27-3-3B, TaDHN15-1-5B, TaDHN22-2-3D, TaDHN27-2-3D, TaDHN43-1-4A, TaDHN41-1-4D, TaDHN14-6-5A, TaDHN14-2-5A, TaDHN15-2-5A, TaDHN14-1-5B, TaDHN14-3-5B, TaDHN14-4-5D, TaDHN15-3-5B, TaDHN15-5-5D, TaDHN15-8-5D, TaDHN15-6-6A, TaDHN28-2-6A, TaDHN19-1-6A, TaDHN9-2-6A, TaDHN101-1-6A, TaDHN22-1-6A, TaDHN16-3-6A,* *TaDHN15-7-6A, TaDHN14-7-6A, TaDHN20-1-6A, TaDHN27-4-6B, TaDHN40-1-6B, TaDHN23-3-6B, TaDHN15-10-6B, TaDHN16-4-6B, TaDHN28-1-6D, TaDHN17-1-6D, TaDHN9-1-6D, TaDHN23-2-6D, TaDHN16-1-6D, TaDHN14-5-6D, TaDHN15-9-6D, TaDHN16-2-6D, TaDHN16-5-6D, TaDHN11-1-7D, TaDHN12-2-7A, TaDHN12-1-7B*	AGGGG	Stress responsive element
	ABRE	*TaDHN27-1-3A, TaDHN22-3-3B, TaDHN27-3-3B, TaDHN22-2-3D, TaDHN27-2-3D, TaDHN43-1-4A, TaDHN43-2-4B,*		Cold,

		TaDHN41-1-4D, TaDHN11-1-7D, TaDHN14-6-5A, TaDHN14-2-5A, TaDHN15-2-5A, TaDHN15-1-5B, TaDHN14-3-5B, TaDHN14-1-5B, TaDHN15-3-5B, TaDHN14-4-5D, TaDHN13-1-5D, TaDHN15-5-5D, TaDHN15-8-5D, TaDHN15-6-6A, TaDHN28-2-6A, TaDHN19-1-6A, TaDHN9-2-6A, TaDHN101-1-6A, TaDHN16-3-6A, TaDHN22-1-6A, TaDHN15-7-6A, TaDHN14-7-6A, TaDHN20-1-6A, TaDHN27-4-6B, TaDHN40-1-6B, TaDHN23-3-6B, TaDHN15-10-6B, TaDHN16-4-6B, TaDHN28-1-6D, TaDHN17-1-6D, TaDHN23-2-6D, TaDHN16-1-6D, TaDHN14-5-6D, TaDHN15-9-6D, TaDHN16-2-6D, TaDHN16-5-6D, TaDHN12-2-7A, TaDHN12-1-7B	ACGTG/ CGCACGTGTC/ GCAACGTGTC/ TACGTGTC	drought - regulated gene expression
	TC rich repeats	*TaDHN27-2-3D, TaDHN15-5-5D, TaDHN15-6-6A, TaDHN28-2-6A, TaDHN9-2-6A, TaDHN101-1-6A, TaDHN15-10-6B, TaDHN12-2-7A*	GTTTTCTTAC	Defence and stress response
	Myb	*TaDHN27-1-3A, TaDHN22-3-3B, TaDHN27-3-3B, TaDHN22-2-3D, TaDHN27-2-3D, TaDHN43-1-4A, TaDHN43-2-4B, TaDHN41-1-4D, TaDHN14-6-5A, TaDHN15-6-6A, TaDHN14-2-5A, TaDHN15-1-5B,* *TaDHN15-2-5A, TaDHN14-3-5B, TaDHN14-1-5B, TaDHN14-4-5D, TaDHN13-1-5D, TaDHN15-5-5D, TaDHN15-8-5D, TaDHN28-2-6A, TaDHN9-2-6A, TaDHN19-1-6A, TaDHN101-1-6A, TaDHN22-1-6A,* *TaDHN16-3-6A, TaDHN15-7-6A, TaDHN14-7-6A, TaDHN20-1-6A, TaDHN27-4-6B, TaDHN40-1-6B, TaDHN23-3-6B, TaDHN15-10-6B, TaDHN16-4-6B, TaDHN28-1-6D, TaDHN9-1-6D, TaDHN17-1-6D,*	TAACTG/CAACAG/ CAACCA	MYB binding site involved in drought-inducibility

		TaDHN23-2-6D, TaDHN16-1-6D, TaDHN15-9-6D, TaDHN16-2-6D, TaDHN16-5-6D, TaDHN12-2-7A, TaDHN12-1-7B, TaDHN11-1-7D		
	DRE-Core	*TaDHN22-3-3B, TaDHN27-3-3B, TaDHN22-2-3D, TaDHN43-1-4A, TaDHN43-2-4B, TaDHN41-1-4D, TaDHN14-6-5A, TaDHN15-2-5A, TaDHN14-3-5B, TaDHN14-1-5B, TaDHN15-3-5B, TaDHN14-4-5D, TaDHN13-1-5D, TaDHN28-2-6A, TaDHN19-1-6A, TaDHN9-2-6A, TaDHN101-1-6A, TaDHN22-1-6A, TaDHN16-3-6A, TaDHN15-7-6A, TaDHN14-7-6A, TaDHN20-1-6A, TaDHN27-4-6B, TaDHN40-1-6B, TaDHN23-3-6B, TaDHN15-10-6B, TaDHN16-4-6B, TaDHN28-1-6D,* *TaDHN17-1-6D, TaDHN9-1-6D, TaDHN16-1-6D, TaDHN14-5-6D, TaDHN15-9-6D, TaDHN16-2-6D, TaDHN16-5-6D, TaDHN12-2-7A, TaDHN12-1-7B, TaDHN11-1-7D*	GCCGAC	cold- and dehydration-responsive
	WUN-motif	*TaDHN22-2-3D, TaDHN14-4-5D, TaDHN13-1-5D, TaDHN15-8-5D, TaDHN19-1-6A, TaDHN14-7-6A, TaDHN40-1-6B, TaDHN23-2-6D, TaDHN15-9-6D*	AAATTACT	Wound response
	BoxS	*TaDHN22-3-3B, TaDHN43-2-4B, TaDHN14-6-5A, TaDHN15-1-5B, TaDHN14-2-5A, TaDHN15-3-5B, TaDHN13-1-5D, TaDHN15-5-5D, TaDHN15-8-5D, TaDHN9-2-6A, TaDHN101-1-6A, TaDHN20-1-6A, TaDHN27-4-6B, TaDHN23-3-6B, TaDHN17-1-6D, TaDHN9-1-6D, TaDHN14-5-6D, TaDHN16-2-6D, TaDHN16-5-6D, TaDHN12-1-7B*	AGCCACC	Wound response
	GC-motif	*TaDHN27-1-3A, TaDHN27-3-3B, TaDHN22-2-3D, TaDHN43-2-4B, TaDHN14-3-5B, TaDHN15-3-5B, TaDHN14-4-5D, TaDHN28-2-6A, TaDHN15-6-6A, TaDHN22-1-6A, TaDHN16-*	CCCCCG/	enhancer-like element involved

		3-6A, TaDHN15-7-6A, TaDHN14-7-6A, TaDHN20-1-6A, TaDHN27-4-6B, TaDHN15-10-6B, TaDHN16-4-6B, TaDHN28-1-6D, TaDHN17-1-6D, TaDHN16-1-6D, *TaDHN14-5-6D, TaDHN15-9-6D, TaDHN16-2-6D, TaDHN16-5-6D, TaDHN12-2-7A*	AGCGCGCCG	in anoxic specific inducibility
	WRE3	*TaDHN27-1-3A, TaDHN27-3-3B, TaDHN22-2-3D, TaDHN43-1-4A, TaDHN43-2-4B, TaDHN41-1-4D, TaDHN14-6-5A, TaDHN15-2-5A, TaDHN14-1-5B, TaDHN14-3-5B, TaDHN15-3-5B, TaDHN15-5-5D, TaDHN14-4-5D, TaDHN15-8-5D, TaDHN9-2-6A, TaDHN101-1-6A, TaDHN22-1-6A, TaDHN16-3-6A, TaDHN20-1-6A, TaDHN40-1-6B, TaDHN23-3-6B, TaDHN16-4-6B, TaDHN28-1-6D, TaDHN17-1-6D, TaDHN9-1-6D, TaDHN14-5-6D, TaDHN16-5-6D, TaDHN12-2-7A,* *TaDHN12-1-7B, TaDHN11-1-7D*	CCACCT	Wound-response element reported in *Pisum sativum*
	TGACG motif	*TaDHN27-3-3B, TaDHN43-1-4A, TaDHN41-1-4D, TaDHN15-2-5A, TaDHN14-3-5B, TaDHN14-1-5B, TaDHN14-4-5D, TaDHN13-1-5D, TaDHN15-6-6A, TaDHN28-2-6A, TaDHN101-1-6A, TaDHN16-3-6A, TaDHN15-7-6A, TaDHN14-7-6A, TaDHN20-1-6A, TaDHN27-4-6B, TaDHN40-1-6B, TaDHN27-1-3A, TaDHN22-3-3B, TaDHN43-2-4B, TaDHN14-6-5A, TaDHN14-2-5A, TaDHN15-5-5D, TaDHN9-2-6A, TaDHN15-10-6B, TaDHN16-4-6B, TaDHN28-1-6D, TaDHN17-1-6D, TaDHN9-1-6D, TaDHN16-1-6D, TaDHN14-5-6D, TaDHN15-9-6D, TaDHN16-2-6D, TaDHN16-5-6D, TaDHN12-2-7A, TaDHN12-1-7B, TaDHN11-1-7D*	TGACG	Hormonal responsive element

Hormonal response	CGTCA motif	*TaDHN27-1-3A, TaDHN22-3-3B, TaDHN27-3-3B, TaDHN27-2-3D, TaDHN43-1-4A, TaDHN43-2-4B, TaDHN41-1-4D, TaDHN14-6-5A, TaDHN14-2-5A, TaDHN15-2-5A, TaDHN14-3-5B, TaDHN14-1-5B, TaDHN15-1-5B, TaDHN14-4-5D, TaDHN13-1-5D, TaDHN15-5-5D, TaDHN15-6-6A, TaDHN28-2-6A, TaDHN9-2-6A, TaDHN101-1-6A, TaDHN16-3-6A, TaDHN15-7-6A, TaDHN14-7-6A, TaDHN20-1-6A,* *TaDHN27-4-6B, TaDHN40-1-6B, TaDHN15-10-6B, TaDHN28-1-6D, TaDHN17-1-6D, TaDHN9-1-6D, TaDHN16-1-6D, TaDHN14-5-6D, TaDHN15-9-6D, TaDHN16-2-6D, TaDHN16-5-6D, TaDHN12-2-7A, TaDHN12-1-7B, TaDHN11-1-7D*	CGTCA	cis-acting regulatory element involved in the MeJA-responsiveness
	ABRE3a	*TaDHN27-1-3A, TaDHN15-8-5D, TaDHN22-3-3B, TaDHN43-1-4A, TaDHN43-2-4B, TaDHN41-1-4D, TaDHN19-1-6A, TaDHN14-2-5A, TaDHN15-2-5A, TaDHN14-1-5B, TaDHN15-3-5B, TaDHN14-4-5D, TaDHN13-1-5D, TaDHN15-5-5D, TaDHN15-6-6A, TaDHN28-2-6A, TaDHN9-2-6A, TaDHN101-1-6A, TaDHN22-1-6A, TaDHN16-3-6A, TaDHN15-7-6A, TaDHN14-7-6A, TaDHN20-1-6A, TaDHN27-4-6B,* *TaDHN40-1-6B, TaDHN23-3-6B, TaDHN15-10-6B, TaDHN16-4-6B, TaDHN17-1-6D, TaDHN9-1-6D, TaDHN23-2-6D, TaDHN16-1-6D, TaDHN14-5-6D, TaDHN15-9-6D, TaDHN16-2-6D, TaDHN16-5-6D*	TACGTG	ABA regulated element
	P-Box	*TaDHN27-1-3A, TaDHN14-3-5B, TaDHN14-1-5B, TaDHN15-3-5B, TaDHN15-8-5D, TaDHN28-2-6A, TaDHN9-2-6A, TaDHN20-1-6A, TaDHN27-4-6B, TaDHN40-1-6B, TaDHN28-*	CCTTTTG	Gibberellins responsive element

		1-6D, TaDHN23-2-6D, TaDHN14-5-6D, TaDHN15-9-6D, TaDHN12-2-7A, TaDHN11-1-7D		
	CCAAT-Box	*TaDHN27-3-3B, TaDHN43-1-4A, TaDHN43-2-4B, TaDHN41-1-4D, TaDHN14-6-5A, TaDHN14-3-5B, TaDHN15-3-5B, TaDHN15-5-5D, TaDHN15-8-5D, TaDHN19-1-6A, TaDHN9-2-6A, TaDHN15-7-6A, TaDHN14-7-6A, TaDHN27-4-6B, TaDHN40-1-6B, TaDHN28-1-6D, TaDHN17-1-6D, TaDHN16-1-6D, TaDHN14-5-6D, TaDHN15-9-6D, TaDHN16-2-6D, TaDHN16-5-6D, TaDHN12-2-7A, TaDHN12-1-7B, TaDHN11-1-7D*	CAACGG	MYBHv1 binding site
	TCA element	*TaDHN27-1-3A, TaDHN27-2-3D, TaDHN22-3-3B, TaDHN43-1-4A, TaDHN43-2-4B, TaDHN14-6-5A, TaDHN14-1-5B, TaDHN15-1-5B, TaDHN15-3-5B, TaDHN14-4-5D, TaDHN15-5-5D, TaDHN28-2-6A, TaDHN19-1-6A, TaDHN101-1-6A, TaDHN15-7-6A, TaDHN14-7-6A, TaDHN20-1-6A, TaDHN40-1-6B, TaDHN17-1-6D, TaDHN9-1-6D, TaDHN14-5-6D, TaDHN12-2-7A, TaDHN12-1-7B*	TAACTG/CAACAG/ CAACCA	cis-acting element involved in salicylic acid responsiveness
	GARE	*TaDHN43-1-4A, TaDHN15-1-5, TaDHN19-1-6A, TaDHN15-9-6D*	TCTGTTG	Gibberellins responsive element
	TATC motif	*TaDHN22-2-3D, TaDHN43-2-4B, TaDHN15-7-6A*	TATCCCA	Involved in gibberellins - responsiveness
	AuxRR-core	*TaDHN15-7-6A, TaDHN16-4-6B, TaDHN14-5-6D*	GGTCCAT	cis-acting regulatory element

Other functions				involved in auxin responsiveness
	TGA element	*TaDHN22-3-3B, TaDHN22-2-3D, TaDHN43-2-4B, TaDHN41-1-4D, TaDHN14-3-5B, TaDHN15-1-5B, TaDHN14-4-5D, TaDHN13-1-5D, TaDHN15-5-5D, TaDHN15-6-6A, TaDHN15-6-6A, TaDHN28-2-6A, TaDHN101-1-6A, TaDHN22-1-6A, TaDHN20-1-6A, TaDHN27-4-6B, TaDHN40-1-6B, TaDHN23-3-6B, TaDHN15-10-6B, TaDHN16-4-6B, TaDHN28-1-6D, TaDHN17-1-6D, TaDHN9-1-6D, TaDHN23-2-6D, TaDHN14-5-6D, TaDHN15-9-6D, TaDHN16-2-6*	AACGAC	auxin-responsive element
		TaDHN27-1-3A, TaDHN27-2-3D, TaDHN22-3-3B, TaDHN22-2-3D, TaDHN43-2-4B, TaDHN14-6-5A, TaDHN14-2-5A, TaDHN15-2-5A, TaDHN14-3-5B, TaDHN14-1-5B, TaDHN15-1-5B, TaDHN15-3-5B, TaDHN15-5-5D, TaDHN15-6-6A, TaDHN28-2-6A, TaDHN101-1-6A, TaDHN15-7-6A, TaDHN14-7-6A, TaDHN20-1-6A, TaDHN40-1-6B, *TaDHN23-3-6B, TaDHN17-1-6D, TaDHN9-1-6D, TaDHN15-9-6D, TaDHN16-2-6D, TaDHN16-5-6D, TaDHN12-2-7A, TaDHN11-1-7D*	AAACCA	Essential for the anaerobic induction
	AAGAA-motif	*TaDHN27-1-3A, TaDHN22-3-3B, TaDHN14-5-6D, TaDHN43-2-4B, TaDHN41-1-4D, TaDHN15-2-5A, TaDHN15-1-5B, TaDHN14-4-5D, TaDHN13-1-5D, TaDHN15-8-5D, TaDHN15-6-6A, TaDHN28-2-6A,*	gGTAAAGAA/GAAGAA	----------

		TaDHN19-1-6A, TaDHN101-1-6A, TaDHN15-7-6A, TaDHN40-1-6B, TaDHN23-3-6B, TaDHN28-1-6D, TaDHN15-9-6D, TaDHN12-2-7A, TaDHN11-1-7D		
	Pc-CMA2a	*TaDHN14-6-5A*	CAGCCAATCACAG	----------
	TATA-Box	*TaDHN27-1-3A, TaDHN22-3-3B, TaDHN27-3-3B, TaDHN22-2-3D, TaDHN27-2-3D, TaDHN43-1-4A, TaDHN43-2-4B, TaDHN41-1-4D, TaDHN14-4-5D, TaDHN14-6-5A, TaDHN14-2-5A, TaDHN15-2-5A, TaDHN14-3-5B, TaDHN14-1-5B, TaDHN15-1-5B, TaDHN15-3-5B, TaDHN14-4-5D, TaDHN13-1-5D, TaDHN28-2-6A, TaDHN15-5-5D, TaDHN15-8-5D, TaDHN15-6-6A, TaDHN19-1-6A, TaDHN9-2-6A, TaDHN101-1-6A, TaDHN22-1-6A, TaDHN16-3-6A, TaDHN14-7-6A,* *TaDHN20-1-6A, TaDHN27-4-6B, TaDHN40-1-6B, TaDHN23-3-6B, TaDHN15-10-6B, TaDHN16-4-6B, TaDHN28-1-6D, TaDHN17-1-6D, TaDHN9-1-6D, TaDHN16-1-6D, TaDHN14-5-6D, TaDHN15-9-6D, TaDHN16-2-6D, TaDHN16-5-6D, TaDHN12-1-7B, TaDHN11-1-7D*	TACAAAA/ TATA/ ccTATAAAaa	core promoter element around -30 of transcription start
	Motif I	*TaDHN14-2-5A, TaDHN15-2-5A, TaDHN14-1-5B, TaDHN15-1-5B, TaDHN15-3-5B, TaDHN13-1-5D, TaDHN15-5-5D, TaDHN19-1-6A, TaDHN101-1-6A, TaDHN20-1-6A, TaDHN40-1-6B, TaDHN15-10-6B,* *TaDHN17-1-6D, TaDHN9-1-6D, TaDHN16-2-6D*	gGTACGTGGCG	cis-acting regulatory element root specific
	MYC	*TaDHN27-1-3A, TaDHN22-3-3B, TaDHN27-3-3B, TaDHN22-2-3D, TaDHN27-2-3D, TaDHN43-1-4A, TaDHN43-2-4B, TaDHN41-1-4D, TaDHN14-6-5A, TaDHN14-2-5A, TaDHN15-*		

		2-5A, TaDHN14-3-5B, TaDHN14-1-5B, TaDHN15-3-5B, TaDHN14-4-5D, TaDHN13-1-5D, TaDHN15-5-5D, TaDHN15-8-5D, TaDHN15-6-6A, TaDHN28-2-6A, TaDHN19-1-6A, TaDHN9-2-6A, TaDHN101-1-6A, TaDHN22-1-6A, TaDHN15-7-6A, TaDHN14-7-6A, TaDHN20-1-6A, TaDHN27-4-6B, TaDHN40-1-6B, TaDHN23-3-6B, TaDHN15-10-6B, TaDHN16-4-6B, TaDHN28-1-6D, TaDHN9-1-6D, TaDHN23-2-6D, TaDHN16-1-6D, TaDHN14-5-6D, TaDHN15-9-6D, TaDHN16-2-6D, TaDHN16-5-6D, TaDHN12-2-7A, TaDHN12-1-7B, TaDHN11-1-7D	CATTTG/ CATGTG	Involved in dehydration inducible expression
	4cl-CMA1b	*TaDHN27-2-3D*	ATTCCGATAAACT	Light responsive element
	CAAT-Box	*TaDHN27-1-3A, TaDHN22-3-3B, TaDHN27-3-3B, TaDHN22-2-3D, TaDHN28-2-6A, TaDHN15-6-6A, TaDHN27-2-3D, TaDHN43-1-4A, TaDHN43-2-4B, TaDHN41-1-4D, TaDHN14-6-5A, TaDHN15-8-5D, TaDHN14-2-5A, TaDHN15-2-5A, TaDHN14-3-5B, TaDHN15-1-5B, TaDHN14-1-5B, TaDHN15-3-5B, TaDHN14-4-5D, TaDHN13-1-5D, TaDHN15-5-5D, TaDHN19-1-6A, TaDHN9-2-6A, TaDHN101-1-6A, TaDHN22-1-6A, TaDHN16-3-6A, TaDHN15-7-6A, TaDHN14-7-6A,* *TaDHN20-1-6A, TaDHN27-4-6B, TaDHN40-1-6B, TaDHN23-3-6B, TaDHN15-10-6B, TaDHN16-4-6B, TaDHN28-1-6D, TaDHN17-1-6D, TaDHN9-1-6D, TaDHN23-2-6D, TaDHN16-1-6D, TaDHN14-5-6D, TaDHN15-9-6D, TaDHN16-2-6D, TaDHN16-5-6D, TaDHN12-2-7A, TaDHN12-1-7B, TaDHN11-1-7D*	CAAAT/ CAAT/CAAAT/ CCCAATTT/ CCAAT	Present in enhancer and promoter region

	ABREa	*TaDHN27-3-3B, TaDHN22-2-3D*	TACGTG	----------
	TGACG motif	*TaDHN27-3-3B, TaDHN43-1-4A, TaDHN41-1-4D, TaDHN15-2-5A, TaDHN14-3-5B, TaDHN14-1-5B, TaDHN14-4-5D, TaDHN13-1-5D, TaDHN15-6-6A, TaDHN28-2-6A, TaDHN101-1-6A, TaDHN16-3-6A, TaDHN15-7-6A, TaDHN14-7-6A, TaDHN20-1-6A, TaDHN27-4-6B,* *TaDHN40-1-6B, TaDHN27-1-3A, TaDHN22-3-3B, TaDHN43-2-4B, TaDHN14-6-5A, TaDHN14-2-5A, TaDHN15-5-5D, TaDHN9-2-6A, TaDHN15-10-6B, TaDHN16-4-6B, TaDHN28-1-6D, TaDHN17-1-6D, TaDHN9-1-6D, TaDHN16-1-6D, TaDHN14-5-6D, TaDHN15-9-6D, TaDHN16-2-6D, TaDHN16-5-6D, TaDHN12-2-7A, TaDHN12-1-7B, TaDHN11-1-7D*	TGACG	----------
	MYB-Binding site	*TaDHN22-3-3B, TaDHN22-2-3D, TaDHN43-1-4A, TaDHN43-2-4B, TaDHN14-6-5A, TaDHN15-3-5B, TaDHN15-6-6A, TaDHN19-1-6A, TaDHN9-2-6A, TaDHN22-1-6A, TaDHN20-1-6A, TaDHN40-1-6B, TaDHN16-4-6B, TaDHN16-1-6D, TaDHN14-5-6D, TaDHN15-9-6D, TaDHN12-1-7B, TaDHN11-1-7D*	CAACAG	MYB binding site involved in flavonoid biosynthetic genes regulation
	ACTCATCCT	*TaDHN43-2-4B*	ACTCATCCT	----------
	CARE	*TaDHN41-1-4D, TaDHN14-1-5B, TaDHN13-1-5D, TaDHN9-2-6A, TaDHN22-1-6A, TaDHN28-1-6D, TaDHN12-2-7A,*	CAACTCCC	----------

	AT~TATA-Box	*TaDHN27-1-3A, TaDHN22-3-3B, TaDHN22-2-3D, TaDHN41-1-4D, TaDHN14-6-5A, TaDHN14-2-5A, TaDHN15-2-5A, TaDHN14-3-5B, TaDHN14-1-5B, TaDHN15-3-5B, TaDHN15-5-5D, TaDHN15-8-5D,* *TaDHN19-1-6A, TaDHN101-1-6A, TaDHN22-1-6A, TaDHN27-4-6B, TaDHN16-4-6B, TaDHN9-1-6D, TaDHN23-2-6D, TaDHN14-5-6D, TaDHN16-5-6D, TaDHN11-1-7D*	TATATA	----------
	AT~ABRE	*TaDHN22-3-3B, TaDHN22-2-3D, TaDHN20-1-6A, TaDHN16-2-6D*	TACGTGTC	----------
	ATCT-motif	*TaDHN22-3-3B, TaDHN27-3-3B, TaDHN22-2-3D, TaDHN19-1-6A, TaDHN16-1-6D*	AATCTAATCC	----------
	C-Box	*TaDHN22-3-3B, TaDHN16-4-6B, TaDHN15-9-6D*	ACGAGCACCGCC	----------
	3-AF3 binding site	*TaDHN15-3-5B, TaDHN16-4-6B, TaDHN16-5-6D*	CACTATCTAAC	part of a conserved DNA module array (CMA3)

4.2 Expression analysis of wheat dehydrins

4.2.1 Expression analysis of the dehydrin proteins under drought stress conditions using real-time PCR.

High quality of RNA was isolated from PBW 175 seedlings after 5 days of growth. The integrity of total RNA was checked with help of denaturing agarose gel stained with ethidium bromide (EtBr), where isolated RNA sample was run and its intensity was checked by comparing control and stress samples of PBW 175 (Figure 21).

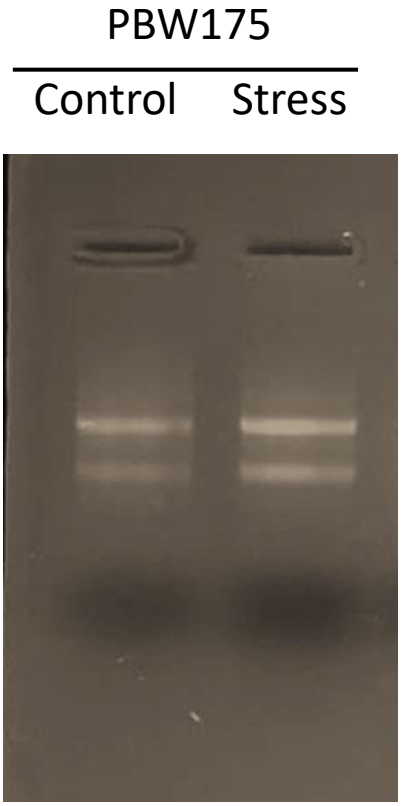


Figure 21: RNA integrity was checked on Agarose gel stained with *ethidium bromide (EtBr)*

To evaluate possible induction of dehydrin expression in response to drought conditions, gene expression was measured as the relative change in expression at 72h of drought treatment in 5 days old seedlings of drought tolerant PBW175 for 48 dehydrin genes classified into 17 homologous gene groups (Table 3). After 3 days of drought stress treatment, the transcript level of dehydrins was observed to increase

significantly (six-fold) in stressed seedlings as compared to control in eight groups which were divided on the basis on homologous dehydrin genes. Amongst the upregulated genes, the highest expression was found in group 16 (TaDHN16-5-6D, TaDHN16-1-6D and TaDHN16-4-6B) highlighted with red in graphical representation, followed by group 12 (TaDHN22-1-6A, TaDHN23-2-6D and TaDHN23-3-6B). The least upregulated expression was observed in group 17 (TaDHN17-1-6D) (Figure 21). These findings are in consonance with the previous published findings for class 1 cytoplasmic glutathione reductase (GR) proteins in wheat which were significantly upregulated at different time intervals (1 hr and 6-hour) of drought stress. In addition to this, the increased expression of the osmotic and salt responsive dehydrin genes (Gh_A05G1554 (GhDHN_03) and Gh_D05G1729 (GhDHN_04) in cotton irrespective of the spatial and temporal constraints also bear attestation to the fact that these are highly implicated in the stress tolerance (Kirungu *et al*., 2020). The upregulation of dehydrin genes demonstrates that the genes could be playing a significant role in enhancing drought stress tolerance in wheat. On the other hand, the relative expression level of nine homologous dehydrin gene groups was found to be non-significant and were totally unresponsive to the stress conditions for the remaining nine groups. Such similar finding has been reported recently for Class 2 cytoplasmic TaGR wheat genes (Madhu *et al*., 2022).

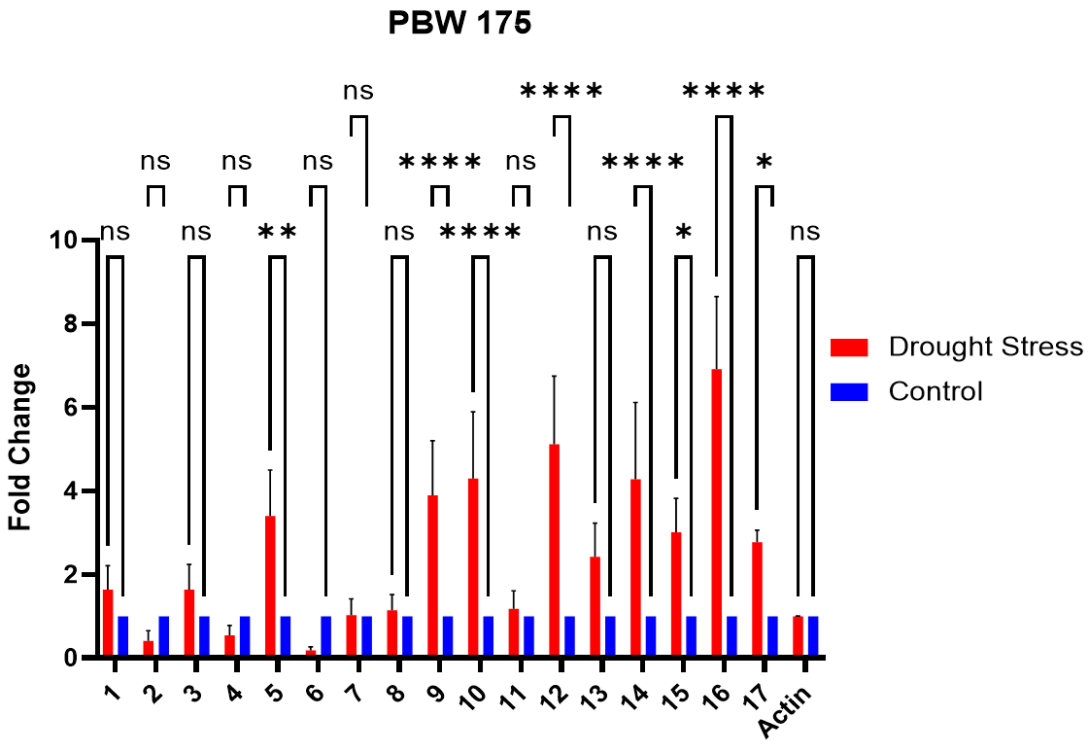


Figure 22: Expression of dehydrin genes in drought-stressed wheat seedlings from drought tolerant cultivar (PBW175). The vertical bar shows the fold change. Bars with red colour are folded change values of stressed samples, while blue shows the control values. *sign indicates the significant difference between control and samples on basis of 2-way ANOVA, alpha ≤ 0.05.

4.3 SDS-PAGE and Western blotting

The SDS-PAGE profile of five days old seedlings of PBW 175 subjected to three consecutive days of drought treatment followed by 24-hour post stress harvest is shown in Figure 23. Each lane was loaded with 50µg of protein samples and it was observed that separated proteins ranged from low molecular weight to high molecular weight. Notably, in the stressed and the post stress harvested samples, a medium molecular weight protein at approximately 50 kDa (marked by an arrow) (Figure 23) was observed to be highly expressed during all the three days of stress treatment followed by the post stress treatment of 24 hour. The separated proteins were then identified by western blotting using dehydrin specific antibody. Such similar pattern of

separated proteins was observed in previous studies of common bean, where differential pattern of drought-responsive proteins was observed. Out of seven, five proteins were found to be upregulated during water limiting condition while two were downregulated, suggesting the strong interaction and varied activation of such stress responsive genes at developmental stages (N. Gupta *et al*., 2019). Another study conducted by (Kasim *et al*., 2019) revealed that 14 days old seedlings of carrot were treated with drought stress by holding the watering for 14 and 22 days respectively. SDS-PAGE analysis of both the stages resulted in newly synthesised protein bands of similar patterns in stressed plants while in control plants bands were not seen.

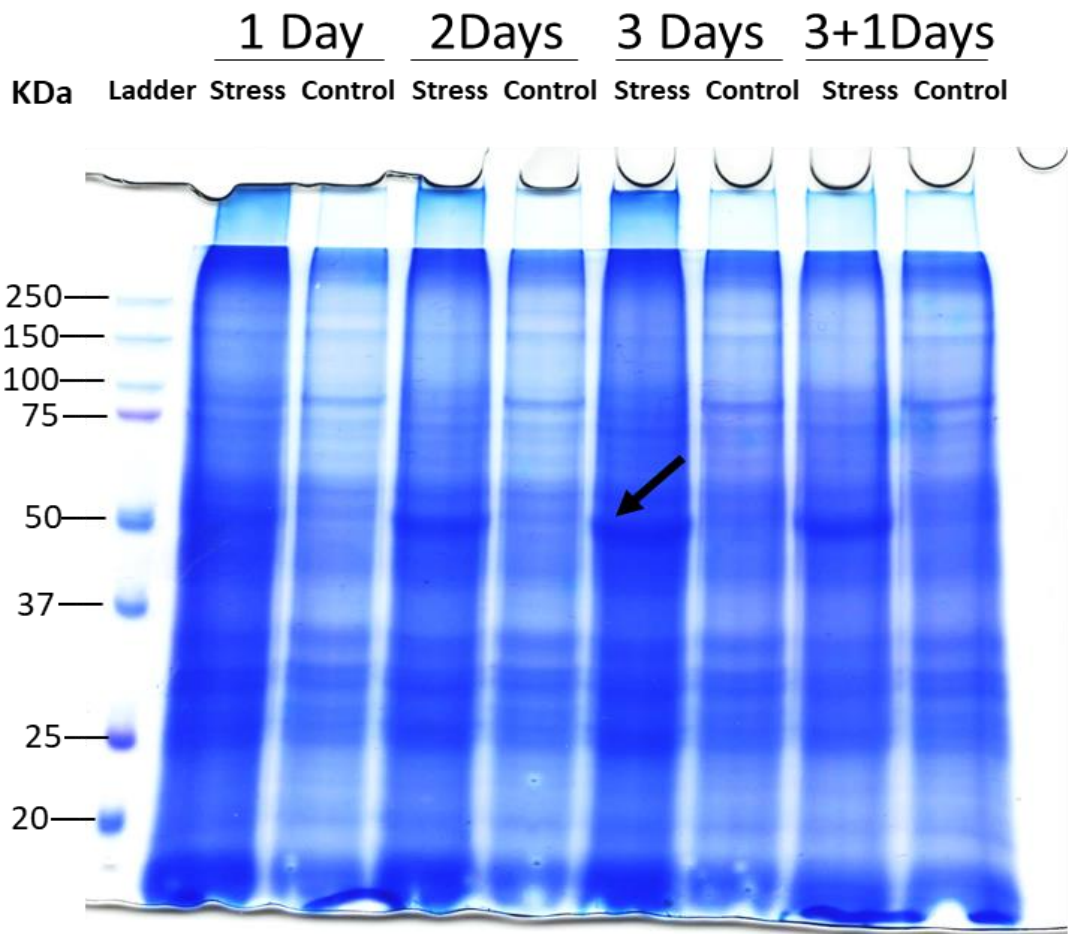


Figure 23: SDS-PAGE profiles in PBW 175 (drought tolerant variety) of *Triticum aestivum*. In the stressed and the post stress harvested samples, a medium molecular weight protein at approximately 50 kDa (marked by an arrow)

Western blot analysis revealed different protein bands of approximately molecular weights 55kDa, 65kDa and 75kDa, which were found to be differentially regulated on different days of stress treatment followed by post-stress harvest (Figure 24). During 2nd day of stress treatment, protein bands with molecular weight of 55kDa, 65kDa and 75kDa were considerably induced under the stress conditions. Interestingly, there was a remarkable increase in the expression of protein band of approximately 65kDa, during 3rd day of stress treatment. Upon re-watering the 3 days old stressed seedlings, just like 2nd day of stress treatment, again there was an enhanced expression of proteins bands with molecular weights 55kDa, 65kDa and 75kDa.

Similar results were observed in *Araucaria angustifolia* embryos, where dehydrins were immune analyzed under heat stress and resulted in the induced accumulation of three protein bands of 20 kDa, 26 kDa and 29 kDa respectively (Farias-Soares *et al*., 2013). In Birch (*Betula pubescens* Ehrh.) also, western blot analysis revealed the presence of dehydrin bands of 24 kDa and 30 kDa against cold stress (Rinne *et al*., 1999; Tiwari & Chakrabarty, 2021). In yet another study carried out using the wild and Tibetan hulless barley, it was demonstrated that high molecular weight dehydrins were induced more in the hulless barley under the effect of Polyethylene Glycol (PEG) and salt stress treatment of 24 hours (Du *et al*., 2011). All these studies therefore bear testimony to the fact of improved stress tolerance owing to the increased dehydrin expression.

Upon re-watering the 3-day old stressed seedlings, up-regulation of dehydrin expression was observed for stressed seedlings and this was again significantly higher in comparison to the control seedlings. These findings therefore signify that dehydrins might be engaged in the repair of cellular damage caused during the stress conditions, which are similar with the findings of (Rakhra *et al*., 2017) in which they found the expression of the WZYb dehydrin-like protein was found to be higher in the wheat cultivar PBW343 during the stress recovery process (i.e., rehydration after drought

stress), suggesting a potential association between WZYb and the repair of cellular damage. This observation indicates that WZYb may play a role in protecting cellular structures during stress and in facilitating recovery after stress.

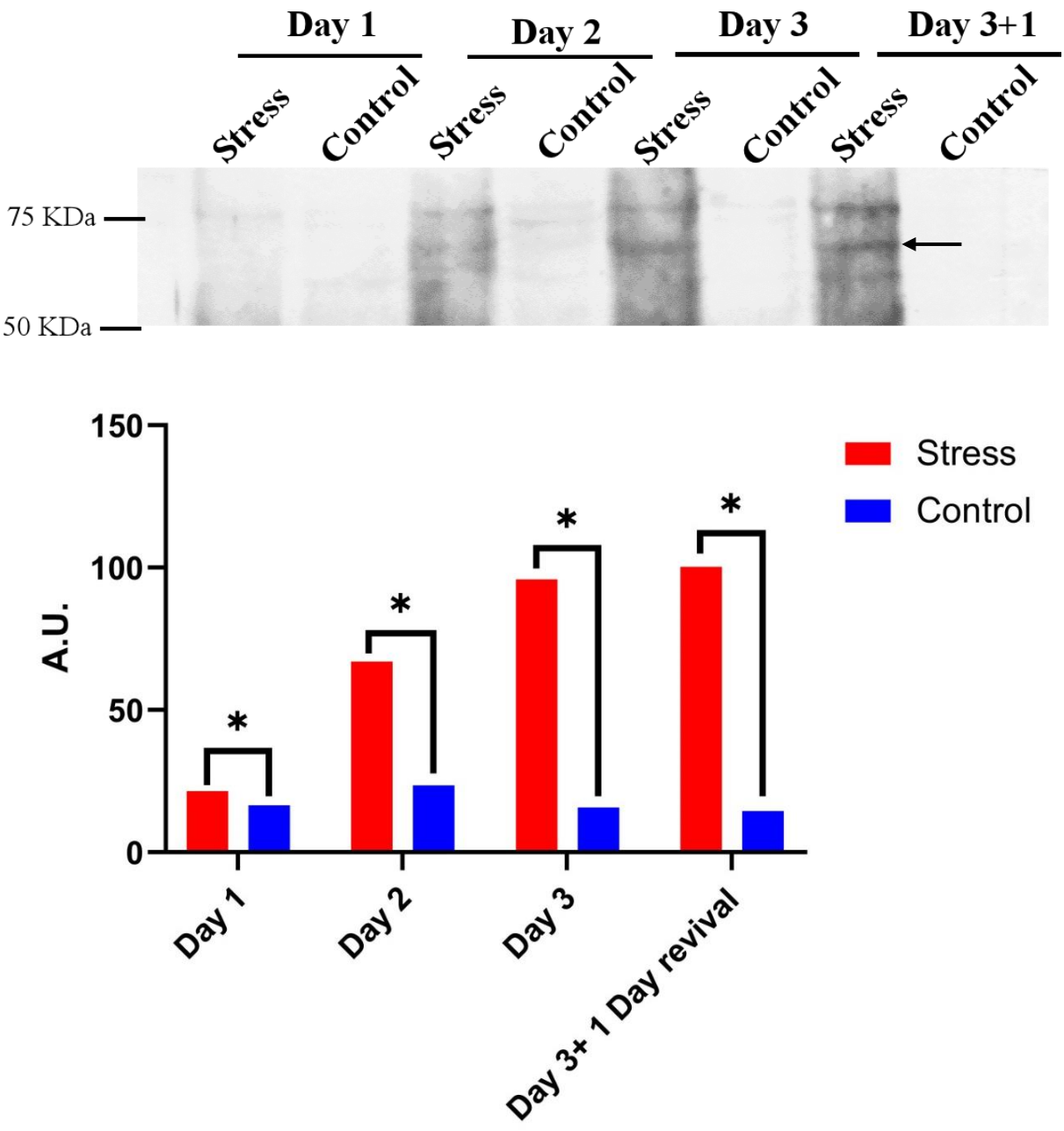


Figure 24: Western blot analysis comparing expression control and stress seedlings, showing proteins bands (above), graph representing statistical analysis in basis of paired T-test. Red strands showing the stress samples expression while blue shows the control samples.

4.4 Digital expression analysis

The investigation of dehydrin gene expression in wheat was carried out by analysing data from WheatExp, a publicly accessible database that provides gene expression profiles for polyploid wheat based on homologs (Pearce *et al*., 2015). To assess gene expression, RNA sequencing (RNAseq) was performed on various plant tissues, including spike, root, leaf, grain, and stem at 10-, 20-, and 30-days post anthesis (DPA), as well as senescing leaf at 12 and 22 DPA, under heat and drought stress conditions. For the stress treatments, 7-day-old seedlings were exposed to heat (40°C), drought, or a combination of both (H. Liu *et al*., 2015). The tblastn program was used to query the protein sequences of wheat dehydrins against the wheat expression database in this study. The expression values for each transcript that showed the best match were gathered and compiled into a text file. Out of 48 TaDHN genes, expression of 20 was supported by expression data, whereas information regarding other 28 TaDHN genes was not available (Figure 23).

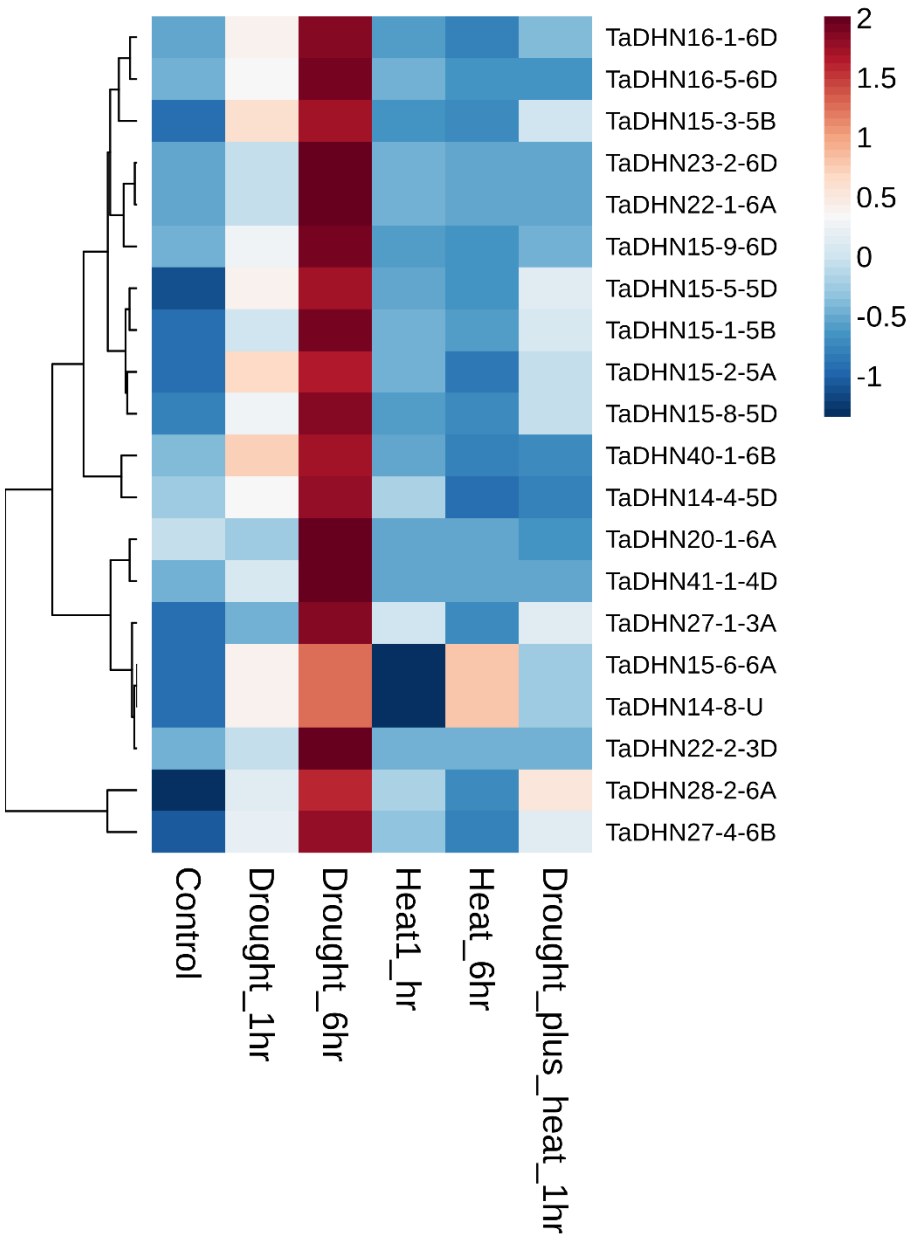


Figure 25: Heatmap for expression of 20 identified TaDHNs under different stress (drought and heat) conditions (heat, drought, heat plus drought). The scale ranges from 2 to -1, which represents the upregulated expression above 0 and down regulated expression below 0

The highest expression was observed under drought conditions of 6 hours, where all 20 dehydrin genes were found to be upregulated. However, under mild drought conditions (1 hour), only seven out of 20 dehydrins (TaDHN16-1-6D,

TaDHN15-3-5B, TaDHN15-5-5D, TaDHN15-2-5A, TaDHN40-1-6B, TaDHN15-6-6A and TaDHN14-8-U) showed a slight increase in the expression (Figure 23). Notably, during mild heat stress (1 hr.), none of the dehydrins showed any upregulated expression. As the duration of heat stress increased to 6 hrs., we observed that there was a mild increase (1 to 1.5) in the expression of two dehydrins (TaDHN15-6-6A and TaDHN14-8-U).

Interestingly, when the heatmap expression analysis of dehydrins were studied under the combined effect of mild drought and heat stress of 1hr), most of dehydrins were downregulated except TaDHN28-2-6A which was observed to be slightly upregulated (Figure 23).

Validation of digital heatmap expression analysis with the qRT-PCR

The findings of the digital heatmap expression analysis were compared with the qRT-PCR of the 17 homologous dehydrin gene groups (Figure5.13) under drought stress. The homolog genes (TaDHN16-1-6D and TaDHN16-5-6D) belonging to group 17 (Figure5.11) showed the highest upregulated expression in both experimental as well as digital analysis. Significant expression was observed in TaDHN23-2-6D, TaDHN22-1-6A (group 12) and TaDHN15-9-6D (group 15) in qRT-PCR and heat map. Dehydrins belonging to group 7 (Figure 5.11) (TaDHN15-3-6B, TaDHN15-5-5D, TaDHN15-1-5B, TaDHN15-2-5A and TaDHN15-8-5D) expressed almost same as that of control in experimental analysis but showed a good expression in digital analysis, which may be due to difference in stress conditions or a wheat variety.

Chapter-5

Summary

In recent years, there has been a significant increase in crop losses caused by drought stress, which can be attributed to global warming and changes in rainfall patterns (A. Gupta *et al*., 2020). Drought-tolerant plants utilize a variety of metabolites and low-molecular-weight proteins as part of their mechanism to counteract the harmful impacts of drought stress (Riyazuddin *et al*., 2022).

During periods of stress, such as drought, dehydrins (DHNs) proteins are a class of proteins that tend to accumulate within plants (Y. Liu *et al*., 2017). With their highly hydrophilic nature, these proteins play a crucial role in protecting plant cells during periods of water deficiency (Tommasini *et al*., 2008). Recent research has provided evidence that dehydrin proteins contribute to the development of drought tolerance in plants through various mechanisms, including but not limited to: increasing water retention capacity, enhancing chlorophyll content, preserving the photosynthetic machinery, facilitating the accumulation of compatible solutes, and triggering ROS detoxification (Halder *et al*., 2017; Tunnacliffe & Wise, 2007). Studies involving overexpression have suggested that targeting dehydrin proteins may be a viable approach for mitigating the detrimental impacts of drought stress and producing drought-tolerant crops in order to address food security concerns in the future (Riyazuddin *et al*., 2021)

Dehydrins are ubiquitous proteins and found in various organisms like plants, fungi, algae and cyanobacteria (Ingram & Bartels, 1996). Dehydrins plays an important role in growth and development , transcription regulation, photosynthesis, protein folding and stress conditions (M. Hara, 2010a). However, studies regarding these proteins in wheat are scanty. The genome sequence of hexaploid wheat (*Triticum aestivum*) has been sequenced in past few years by the International Wheat Genome Sequencing Consortium. Therefore, to characterize and role of dehydrin proteins in wheat, genome-wide identification and analysis of these genes was carried out using IWGSC RefSeq assembly 2.0.

Salient Findings

48 dehydrins genes were identified from *T. aestivum* by in silico analysis TaDHNs. Domain analysis revealed mmultifunctionality of proteins by the presence of multiple dehydrin domains for 12 proteins, while 36 of proteins are having single dehydrin domain. Motif analysis resulted in the identification of 15 different motifs, out of which 6 motifs are associated with dehydrin domain.

Further, *in-silico* subcellular location of dehydrin genes revealed that most of the dehydrins are localized in cytoplasm (35), while others are present in nucleus (5) and some of them are secreted (8). On basis of the molecular weights, proteins range from 9.65kDa to 101.60 kDa. Isoelectric point (pI) ranges from 5.19-10.7.

Phylogenetic analysis revealed about the TaDHNs having clustering pattern that explains about the presence of extra functional domains as well as their subcellular localization. Gene structure analysis revealed the exons and introns organisation of different dehydrin wheat genes. Four genes lack intron, while 1 intron is present in remaining 44 genes. TaDHNs shows homolog-dependent variability in number of introns. Considerable diversity was observed in the wheat dehydrin 5'UTR and 3' UTR regions. Majorly, genes (42) were determined to have both the regions, whereas 6 genes were determined to lack the same.

The distribution of TaDHNs on chromosomes was analysed through chromosome mapping, and the results showed that nearly all TaDHNs were found on 15 chromosomes, with two additional dehydrin genes on unidentified U chromosomes that had uneven gene density. It was observed that the D chromosomes contained the highest number of dehydrin genes.

Synteny analysis determined the tandem repeats and collinear genes of *Triticum aestivum* with its progenitors *Aegilops tauschii*, *Triticum dicoccoides* and *Triticum urartu*. This gene duplication data plays an important role in gene amplification and their families and their subsequent evolution in wheat. Also, the dehydrin proteins were found to be highly disordered, through disorder nature prediction tool. The secondary structure prediction of identified dehydrin proteins indicated the presence of random coils as well as alpha helices.

The functional partners for dehydrin proteins were identified for protein-protein interaction analysis, which revealed that most of the functional partners belong to

dehydrin family. Gene ontology analysis revealed the functional roles of genes and their biological processes in which they are involved under various abiotic stress. The enrichment analysis shows the involvement of dehydrin genes in various processes which were classified into 14 diverse pathways. Most of the dehydrin genes were found to be involved in abiotic stimulus, response to oxygen containing compound and response to acid chemicals.

Promoter analysis revealed the identification of cis- regulatory elements. Present study revealed total of 69 cis-regulatory elements, which are further divided into abiotic stress responsive, hormone specific, biotic stress responsive and guard cell specific elements.

Expression analysis of TaDHN genes under drought stress was carried out through qRT-PCR and western blotting. The qRT-PCR results revealed the upregulation of dehydrin genes in stressed samples as compare to control, which demonstrate that the genes could be playing a significant role in enhancing drought stress tolerance in wheat. Western blotting resulted in dehydrin protein band of approximately 64kDa.

To conclude, this study is the first to investigate and describe the WZYb gene belonging to the dehydrin family in wheat. Thus, the novel finding about the characteristics of this stress-responsive gene family will aid in improvement of crop via conventional plant breeding approach.

Chapter-6

Conclusion

The present study focuses on the identification, characterization and expression analysis of 48 wheat dehydrin genes under abiotic and biotic stress conditions. Dehydrins are known to play a significant role in stress response and maintain homeostasis under stress conditions. The study provides a detailed in-silico analysis of dehydrins including their genome-wide analysis, phylogenetic analysis, gene structure analysis, and functional analysis.

- The identified dehydrins were found to range in molecular weight from 9.65 kDa to 101.60 kDa, and were named as TaDHN, where Ta represents *Triticum aestivum* and DHN indicates dehydrin followed by molecular weight and chromosome number.
- The study revealed that 35 TaDHNs were single domain proteins, while the rest 13 proteins were multiple domains, indicating constant diversity and multifunctionality of dehydrin genes.
- The subcellular localization analysis revealed that dehydrins were present in cytoplasm, nucleus, and secretory pathway.
- The study also analyzed the gene structure of dehydrins and found that beside ORF, introns were also present in both 5' and 3' UTRs in almost all proteins.
- The protein-protein interaction analysis identified functional partners belonging to dehydrin family such as DHN3, COR410, and CS120.
- The synteny analysis showed that collinear genes and tandem repeats formed a significant part of the wheat genome. Functional versality was observed on the basis of gene ontology analysis.
- The digital expression analysis provided a temporal- and stress-dependent pattern, which could provide further insights into the possible functional divergence in dehydrin gene family.
- Experimental analysis using qRT-PCR and western blotting revealed a significant upregulation of dehydrin expression under stress conditions.

Overall, the present study provides a comprehensive analysis of dehydrins in wheat and their role in stress response. The findings could be useful in improving crop resilience and developing stress-tolerant wheat varieties.

Future aspects

- The newly identified multifunctional TaDHNs, which were characterized in this study, could enhance our comprehension of plant response and acclimation to multifactorial and abiotic stresses.

- This study would lay the foundation for future work wherein these genes could serve as a valuable resource (genetic marker) for plant breeding experiments on developing plants with improved abiotic stress tolerance.

- Overall, this study provides a framework for investigating the diverse functions of dehydrin genes in other plant species under various abiotic stress conditions.

Weblinks

Cj-Chen Tbtools software - https://github.com/CJ-Chen/TBtools

Clustal omega - http://www.ebi.ac.uk/ Tools/msa/clustalo/

Compute_pi - http://web.expasy.org/compute_pi/

EMBOSS extractseq - https://www.bioinformatics.nl/cgi-bin/emboss/extractseq

Ensembl plants' database - https://plants.ensembl.org/index.html

ESPript3.0 - http://espript.ibcp.fr/ESPript/ESPript/

GOR database - http://cib.cf.ocha.ac.jp/bitool/GOR/

GSDS2.0 server - http://gsds.cbi.pku.edu.cn/

Heatmapper web database - http://www.heatmapper.ca/ expression/?qt1m4dc=1

Interactive tree of life - https://itol.embl.de/

LOCTREE3 - https://rostlab.org/services/loctree3/

MatGat v 2.02 - http://www.angelfire.com/nj2/arabidopsis/MatGAT.html

PFAM - http://pfam.xfam.org

PlantCARE database - http://bioinformatics.psb.ugent.be/webtools/plantcare/html/

PONDR - http://www.pondr.com/

Prosite - https://prosite.expasy.org/

ShinyGO web server - http://bioinformatics.sdstate.edu/go74/

SignalP 4.1 - http://www.cbs.dtu.dk/services/SignalP/

TargetP2.0 server - https://services.healthtech.dtu.dk/service.php?TargetP-2.0

Uniprot - https://www.uniprot.org/uniprot/?query=wzyb&sort=score

WheatExp - https://wheat.pw.usda.gov/WheatExp/

International Wheat Genome Sequencing Consortium (IWGSC) - https://www.wheatgenome.org/

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Appendices

1. Protein sequences of TaDHN

>TaDHN14-8-U

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>TaDHN12-1-7B

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LPGQH

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>TaDHN14-4-5D

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>TaDHN15-3-5B

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>TaDHN15-1-5B

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>TaDHN14-1-5B

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>TaDHN14-3-5B

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>TaDHN15-2-5A

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SSSSEDDGMGGRKKKGMKEKIKEKLPGGHKDNQQHMATGTGTGGAYGPGTGTGAAYGQQG

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>TaDHN14-2-5A

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>TaDHN14-6-5A

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>TaDHN41-1-4D

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>TaDHN43-2-4B

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GGTGTGITGTHGTGHTAGYDATGTHGTGHTAGYGTTGTGTTGVGIAGTHGAVGTHPHGGV

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>TaDHN43-1-4A

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>TaDHN22-2-3D

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>TaDHN27-3-3B

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>TaDHN22-3-3B

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>TaDHN27-1-3A

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GTTATAGTYGQPAPTGMTGTGAHGTMAAGEKKGMKDKIMEKLPGGHKNEQHAMPTAGAYG

QPGMTGTGVHGNTAPGGGYGGQPGHAGMTGAGTHGSATTGGAYGHQGHPGVTGTGAHGTT

ATGGAYSQQGHAGVTGTGEKKGIMDKIKEKLPGQH

2. Reagents used for qRT-PCR

   RNA isolation-Qiagen RNAeasy mini Kit (plant)

   cDNA isolation-iScript^{TM} cDNa synthesis kit

   RT PCR Reaction

   2X Sybr green – 5µl

   Forward primer - 0.5µl

   Reverse primer - 0.5µl

   cDNA (Template) – 0.5µl

   MQ water – up to 10µl

3. Western Blotting analysis

   QB Buffer

   Buffer composition:

   i. 100 mM Tris-Cl (pH-6.8)
   ii. 4% SDS (w/v)
   iii. 200 mM DTT

   HBA Buffer

   Buffer composition

   i. 100mM Tris-Cl pH-7.4
   ii. 10% sucrose
   iii. 5 mM EDTA (pH-8.0)
   iv. 0.19% EGTA
   v. 0.28% β-mercaptoethanol (freshly added)

1 mM Phenyl methyl sulfonyl fluoride (freshly added

RIPA (Radioimmunoprecipitation Assay) Buffer

Buffer composition

i. 25 mM Tris-Cl (pH-7.5)
ii. 150 mM NaCl
iii. 1% NP40
iv. 1% Sodium deoxycholate
v. 0.1% SDS
vi. 1 mM Sodium orthovanadate
vii. 1X Protease inhibitor cocktail

List pf Publications

- Intrinsic disordered nature and prediction of the secondary structure in wheat dehydrins in Research Journal of Biotechnology

List of conferences

- 5th international conference on advances in agriculture technology and allied sciences (ICAATAS 2022) on June 4-5, 2022


SARSD/ICAATAS/2022/133

5th INTERNATIONAL CONFERENCE ON
ADVANCES IN AGRICULTURE TECHNOLOGY AND ALLIED SCIENCES
(ICAATAS 2022) on JUNE- 4-5, 2022

CERTIFICATE

This is to certify that Prof./Dr./Mr./Mrs./Ms. Ridhi Joshi
Actively participated/presented a paper (Oral/Poster) entitled Identification & Characterization of Dehydrins in Wheat

As Delegate/Scholar/Student in the 5th INTERNATIONAL CONFERENCE ON ADVANCES IN AGRICULTURE TECHNOLOGY AND ALLIED SCIENCES (ICAATAS 2022) on JUNE- 4-5, 2022, held at MS Swaminathan School of Agriculture, Centurion University of Technology and Management, Paralakhemundi, Gajapati, Odisha – 761211, India

Mr. Akhil Gupta, President SARSD
Dr. Mehjabeen, Oraganizing Convener
Dr. Ashirbachan Mahapatra, Oraganizing Secretary
Dr. S.P. Nanda, Conference Director
Dr. Ankush Raut, Chief Oraganizing Secretary

- Recent trends in Smart and Sustainable Agriculture for Food Security" (SSAFS-2022) held from 21-22 January 2022

LOVELY FACULTY OF TECHNOLOGY AND SCIENCES

[Under the Aegis of Lovely Professional University, Jalandhar-Delhi G.T. Road, Phagwara (Punjab)]

Certificate No.241842

Certificate of Participation

This is to certify that **Dr./Mr./Ms. Ridhi Joshi** of **Lovely Professional University, Jalandhar** has participated in Poster presentation on the topic entitled **Dehydrins: Stress responsive proteins in wheat** in the International Conference on **"Recent trends in Smart and Sustainable Agriculture for Food Security" (SSAFS-2022)** held from 21-22 January 2022, organized by School of Agriculture, Lovely Professional University, Punjab.

Date of Issue :31-03-2022
Place : Phagwara (Punjab), India

Prepared by
(Administrative Officer-Records)

Convener
Dr. Ramesh Kumar

Organizing Secretary
Dr. Sarvjeet Kukreja

- Sustainability: Life on Earth 2021 (ICS-LOE 2021) held on 17-18 December 2021

LOVELY FACULTY OF TECHNOLOGY AND SCIENCES

[Under the Aegis of Lovely Professional University, Jalandhar-Delhi G.T. Road, Phagwara (Punjab)]

Certificate No.241842

Certificate of Participation

This is to certify that **Dr./Mr./Ms. Ridhi Joshi** of **Lovely Professional University, Jalandhar** has participated in Poster presentation on the topic entitled **Dehydrins: Stress responsive proteins in wheat** in the International Conference on **"Recent trends in Smart and Sustainable Agriculture for Food Security" (SSAFS-2022)** held from 21-22 January 2022, organized by School of Agriculture, Lovely Professional University, Punjab.

Date of Issue :31-03-2022
Place : Phagwara (Punjab), India

Prepared by
(Administrative Officer-Records)

Convener
Dr. Ramesh Kumar

Organizing Secretary
Dr. Sarvjeet Kukreja

- 4th International conference Global efforts on Agriculture, forestry, environment and food security (GAFEF-2022) at Institute of Forestry,

Tribhuvan University, Pokhara Campus, Pokhara, Nepal on September 17-19 2022

4th International Conference

Global Efforts on Agriculture, Forestry, Environment and Food Security

(Theme: Climate Change and Its Impact)

(GAFEF-2022)

CERTIFICATE

This is to certify that Prof./Dr./Mr./Ms. RIDNI JOSHI
of LOVELY PROFESSIONAL UNIVERSITY, PHAGWARA, PUNJAB, INDIA
Actively participated/presented a paper (Oral/Poster) entitled IDENTIFICATION & CHARACTERIZA-
-TION OF STRESS RESPONSIVE PROTEINS: DEHYDRINS IN WHEAT
As Delegate/Scholar in the "4th International Conference on "Global Efforts on Agriculture, Forestry, Environment and Food Security (Theme: Climate Change and Its Impact) (GAFEF-2022)" at Institute of Forestry, Tribhuvan University, Pokhara Campus, Pokhara, Nepal on September 17-19, 2022.

Prof. C. P. Singh
Conference Chairperson
President, AETDS
Former Professor
GBPUAT, Pantnagar, India

Dr. Arjun Prasad Bastola
Conference Director
Institute of Forestry
TU, Pokhra, Nepal

Dr. Santosh Marahatta
Organizing Secretary
AFU, Chitwan, Nepal

Dr. Wajid Hasan
Chief-Organizing Secretary
Secretary, AETDS
KVK Jehanabad,
BAU Sabour, Bihar, India

AETDS/PC/GAFEF/20