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ORIGINAL ARTICLE

Identification of universal stress proteins in wheat and functional characterization during abiotic stress

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Abstract

Key message **TaUSPs are localized in Endoplasmic reticulum and form homo and hetero dimers within themselves. They play significant role in multiple abiotic stress responses in yeast heterologous system and in plants.**

Abstract Universal Stress Proteins are stress responsive proteins present in a variety of life forms ranging from bacteria to multicellular plants and animals. In this study we have identified 85 *TaUSP* genes in the wheat genome and have characterised their abiotic stress responsive members in yeast under different stress conditions. Localization and Y2H studies suggest that wheat, USP proteins are localized in the ER complex, and extensively crosstalk amongst themselves through forming hetero and homodimers. Expression analysis of these *TaUSP* genes suggests their role in adaptation to multiple abiotic stresses. TaUSP_5D-1 was found to have some DNA binding activity in yeast. Certain abiotic stress responsive *TaUSP* genes are found to impart tolerance to temperature stress, oxidative stress, ER stress (DTT treatment) and $LiCl_2$ stress in the yeast heterologous system. *TaUSP_5D-1* overexpression in *A. thaliana* imparts drought tolerance via better lateral root network in transgenic lines. The *TaUSP* represents an important repertoire of genes for engineering abiotic stress responsiveness in crop plants.

Keywords Abiotic stresses · Drought stress · Endoplasmic reticulum · Universal stress proteins (USP) · Unfolded protein response (UPR)

Abbreviations

AANH	Adenine nucleotide alpha hydrolases
CDS	Coding sequence
DAB	3,3′: Diaminobenzidine
DTT	Dithiothreitol
ER	Endoplasmic reticulum
EV	Empty vector
H_2O_2	Hydrogen peroxide
ROS	Reactive oxygen species
TaUSP	*Triticum aestivum* Universal stress protein

Introduction

Plants being sessile organisms are highly influenced by different stresses exerted by the changing environment. Plant growth, development, survival and yield depends upon the mechanism with which they adapt to tolerate such unpropitious environmental conditions. In response to such conditions plants have evolved advanced and complex defence mechanisms. These responses may include the production of reactive oxygen species (ROS), change in redox potential or cellular level of Ca^{2+}, disruption of ion homeostasis, and adjustment of membrane fluidity (Choudhury et al. 2017; Gilroy et al. 2016). Abiotic stresses are multigenic in nature and multiple stress responsive genes act in a synchronized manner to attain tolerance to abiotic stresses (Tuteja 2007). One such family of genes that provides tolerance to a plethora of abiotic stresses is the Universal Stress Proteins (USPs). These genes are known to be present in a profusion of organisms, ranging from bacteria to higher life forms like plants and animals (Vollmer et al. 2018).

The Universal Stress Protein (USP) was first discovered in *Escherichia coli* as a 13.5 kDa cytosolic protein which was induced under a broad range of nutrient starvation

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stresses (Nystrdm and Neidhardt 1992). Proteins of this class play a crucial role in cell survival during H_2O_2 stress, osmotic stress, heat shock, exposure to DNA-damaging agents and UV light treatment in *Escherichia coli* (Nystrom and Neidhardt 1994; Nachin et al. 2005). USP genes have been broadly classified into two categories based on the structural homology of the proteins encoded by these genes with MJ0577 protein of *Methanocaldococcus jannaschii* and USPA protein of *Haemophilus influenza*. USPs having structural homology with USPA protein of *Haemophilus influenza* lack the ATP-binding residues (Sousa and Mckay 2001). The USPs which have a structural homology with MJ0577 protein contain an ATP-binding motif G-2X-G-9X-G-(S/T) at their C-terminal region and a five β-strands—four α-helical α/β-core structure (Aravind et al. 2002; Siegele 2005). After the discovery of *USP* in *E. coli*, many proteins containing at least one USP domain, with a conserved set of 140–160 amino acid residues and other diverse functional motifs have been found from variety of other organisms including bacteria, archaea, plants, and metazoans (Forêt et al. 2011; Vollmer et al. 2018).

In plants, the USP family thrives abundantly and a single plant genome may harbour typically 20–50 members, that may go up to 142 as in *Brassica napus*. (Li et al. 2010; Chi et al. 2019). The *Arabidopsis thaliana* genome consists of 44 *USP* encoding genes (Kerk et al. 2003), which confer tolerance under different abiotic stress conditions. Hypoxia-Responsive Universal stress protein, HRU1 (*At3g03270*) is involved in ROS homeostasis under anoxia (Gonzali et al. 2015). Plants over-expressing *At3g53990* gene show tolerance to heat shock and oxidative stress via redox-dependent chaperone activity (Jung et al. 2015). At3g53990, a cold stress responsive USP possesses strong nucleic acid melting activity and functions as a RNA chaperone to provide tolerance to cold stress (Melencion et al. 2017). Other AtUSPs are identified to be playing various roles in multiple abiotic stresses (Bhuria et al. 2019) but their detailed biochemical and function characterization is still limited in plants. Other than *Arabidopsis thaliana*, USPs have been studied in *Solanum lycopersicum,* SlRd2 a USP domain containing protein regulates Calcineurin B-like interacting protein kinase (SlCipk6*)*-mediated ROS generation. *Solanum pennellii*, SpUSP confers drought tolerance via regulation of stomatal closure, in seedling and adult stages in tomato.: (Loukehaich et al. 2012; Gutiérrez-Beltrán et al. 2017). Many of the UspA proteins have also been characterized in rice (Sauter et al. 2002), cotton (Maqbool et al. 2009) legumes (Sinha et al. 2016), and *Salicornia* (Udawat et al. 2016).

At the cellular level, USPs are essentially involved in protein scaffolding, preventing the denaturation of globular macromolecules, and aid in cellular protein transport (Vollmer et al. 2018). However, the precise molecular function and regulation of USPs under diverse abiotic stresses are still unknown in many plants. No literature till date has reported any characterization of USPs in *Triticum aestivum.* In this study we attempt to identify *TaUSP* genes within the genome of *Triticum aestivum*, and characterization of abiotic stress responsive *TaUSP* genes under different abiotic stress in yeast and *A.thaliana* heterologous systems.

Material and methods

Identification and phylogenetic analysis of USP genes

USP gene family members were identified by HMM search using wheat transcriptome data. We found 106 USP domain containing proteins encoded by 85 genes in the wheat genome. The putative USP genes were confirmed by using NCBI-CDD search (Marchler-Bauer et al. 2011). They were named as *TaUSP_1A-1* to *TaUSP_7D-2*, according to their annotated gene IDs. For example, gene ID TraesCS1A02G106600.1 has been named as *TaUSP_1A-1*, in which 1A is chromosome number and 1 signifies the first transcript on it. Further, phylogenetic analysis was done, based on full-length protein sequences with single transcript per gene. Multiple sequence alignment was carried out using CLUSTALW program, and the phylogenetic tree was constructed using the MEGA7 software with Neighbor-Joining method.

Structural and domain analysis of abiotic stress responsive *TaUSP* gene members

The HMM profiles for USP (PF00582) were retrieved from Pfam database[74] https://pfam.xfam.org. Each protein sequence was checked on wheat expression database (http://www.wheat-expression.com). Six *TaUSP* genes were selected based on their expression levels under different abiotic stresses. Search was performed for these six genes against Ensembl Genomes 49 (http://plants.ensembl.org/index.html). Their chromosomal location, structural organisation (introns, exons, and transcript length) and isoelectric points of the proteins encoded by the selected *TaUSP* gene family members were analysed. The conserved motifs in the selected *TaUSP* gene family members were identified using a motif-based sequence analysis tool, MEME suite 5.3.0 (http://meme-suite.org). The sequences were searched for a total of 5 motifs along with the default parameters.

Plant growth conditions and stress treatment

Bread wheat (*T. aestivum*) variety PBW343 was used in this study. Seeds were surface-sterilized with 4% sodium hypochlorite for 20 min, followed by five washes with

autoclaved RO water. Seeds were then grown on a cotton bed in a growth chamber (Conviron, Canada) maintained at 22 ± 1 °C with a 16-h photoperiod. After 10 days the seedlings were subjected to different abiotic stresses such as cold (4 °C for 24 h), salt (200 mM NaCl for 24 h), heat (42 °C for 2 h) and drought (200 mM mannitol for 24 h)(Amoah et al. 2019; Chauhan et al. 2011; Khurana et al. 2013; Hamdi et al. 2020). After the stress treatment, seedlings of control and treated plants were frozen in liquid nitrogen and stored at − 80 °C until RNA isolation. The seeds of the transgenic *A. thaliana* were germinated on half strength MS media and allowed to grow for 7 days in a growth chamber maintained at 22 °C with a photosynthetic flux density of 300 µ mol m^{-2} s^{-1}, 60% humidity, and a photoperiod duration of 16/10 h light/dark phase. For drought stress, the plants were transferred to a 200 mM mannitol, supplemented MS media plates after 7 days. Phenotype was observed after 7 days. For expression level of *TaUSP_5D-1* in reproductive tissues, *T. aestivum* plants were grown in open fields during growth seasons and tissue samples were collected for RNA isolation.

RNA isolation and expression analysis

RNA isolation was done by using RNeasy plant mini kit (Qiagen, Germany) as per the instructions. On-column DNaseI treatment was given to the RNA samples to remove any genomic DNA contamination. Thereafter 2 µg RNA was used for the synthesis of cDNA using the High Capacity cDNA Archive kit (Applied Biosystems, United States). For RT-PCR analysis the cDNA was mixed with 200 nM of each primer and SYBR Green PCR Master Mix (Applied Biosystems). ABI Prism 7000 sequence detection system and software (PE Applied Biosystems) was used for the analysis as per the manufacturers' instructions. Relative fold-change was calculated using expression of *TaGAPDH*, a housekeeping gene as the reference point. Three biological replicates and three technical replicates were used for plotting the graphs (Meena et al. 2022).

Cloning of abiotic stress responsive *TaUSP* gene family members

For cloning purpose full length CDSs of all the six abiotic stress responsive *TaUSP* gene family members (500–550 bp) were amplified with their respective gene specific primers. Control cDNA was isolated from 10-day-old seedlings of *T. aestivum* (cv. PBW343) and used as the template for gene amplification. The amplified products were cloned into the Gateway entry vector (pENTR™/D-TOPO) and then into the destination vectors pGBKT7 and pGADT7 under T7 promoter for yeast-2-hybrid assay in *S. cerevisiae* strain AH109. All the six genes were also cloned into destination vector pSITE-3CA under CaMV35S promoter for subcellular localization. The cloning was done following the Gateway™ cloning strategy (Directional TOPO cloning kit and LR clonase enzyme mix II kit, Invitrogen Inc., United States). Abiotic stress responsive *TaUSP* genes were further cloned into p426GPD, between EcoRI and HindIII (for *TaUSP_1A-3, TaUSP_3B-1* and *TaUSP_6D-5*) and between BamHI and HindIII (for *TaUSP_1B-1, TaUSP_1D-1* and *TaUSP_5D-1*) under GPD promoter for stress assays. Stress assays were done in *S. cerevisiae* strain BY4741. For generating overexpression lines full length CDS of *TaUSP_5D-1* was cloned into gateway destination vector pMDC32, under CaMV*35S* promoter.

Subcellular localization of abiotic stress responsive *TaUSP* genes

For subcellular localization pSITE-3CA-*TaUSP* constructs with the CDS fused in frame with the N-terminal of YFP were used. Each construct was coated on gold particles and bombarded at a pressure of 1100 psi on the onion epidermal peels, plated on 1/2MS media (Lee et al. 2008; Nebenführ 2014). The PDS-1000 bombardment system (Bio-Rad, Canada) was used for the purpose. After bombardment the onion peels were incubated for 12 h at 27 °C in dark. After the incubation period, fluorescence was observed in confocal microscope (Leica, Germany). As the fluorescence seemed to appear in some organelles, expression of each gene was further analysed with the help of ER and Golgi organelle markers.

Yeast-two-hybrid interaction of proteins encoded by abiotic stress responsive *TaUSP* genes

Proteins encoded by each of the six *TaUSP* gene family members were checked for the interaction with every other selected member of the family. Each gene was cloned into gateway destination vectors pGBKT7(DBD) and pGADT7(AD). To check for the interaction between two proteins, constructs were used for co-transformation into *S. cerevisiae* strain AH109, harboring the HIS3 reporter gene. Yeast supplement dropout media, lacking leucine and tryptophan (-LW) was used to select the transformants. Further, the interactions were checked with dilutions on selective media, lacking histidine, leucine and tryptophan (-HLW), other medium lacking adenine, histidine, leucine and tryptophan (-AHLW) and one medium lacking histidine, leucine and tryptophan (-HLW) supplemented with of 1 mM 3AT (3-Amino-1,2,4-triazole) (Meena et al. 2020).

In-silico promoter analysis for abiotic stress responsive *TaUSP* genes

Genomic sequences of all the six members were fetched from the EnsemblePlant database (http://plants.ensembl.org/index.html). A 1.5 kb region, upstream to the transcription start site was taken. The upstream sequence for each of the six members was then searched for various abiotic stress responsive elements using the Plant Care and PLACE databases (Suppl. Table 1) (Lescot et al. 2002).

Autoactivation assay of *TaUSP*_5D-1 and BiFC assay for *TaUSP*_5D-1 interactions

USP genes are known to have DNA binding activity (Melencion et al. 2017). So the DNA binding activity of the selected *TaUSP* genes was checked via yeast-2-hybrid. *TaUSP* genes (CDS) were cloned into pDEST-pGBKT7. Yeast cells (strain AH109) were co-transformed with these constructs and with EV pDEST-pGADT7. The DNA binding activity was analysed by drop-out assays on yeast supplement dropout media, lacking histidine, leucine and tryptophan (-HLW) and -HLW with X-α-gal. The activity was also checked by transforming yeast cells with pGBKT7- *TaUSP_5D-1* construct and then doing a drop out assay on SD/-HW medium (Meena et al. 2022). Because of DNA binding activity of *TaUSP_5D-1*, its interactions with protein encoded by other *TaUSP* genes were further confirmed via BiFC assay. For BiFC assay *TaUSP_5D-1* was cloned in pSITE-nEYFP-N1 and genes encoding interacting partners were cloned in pSITE-cEYFP-N1. Both the constructs were coated on gold particles and bombarded with a pressure of 1100 psi on the onion epidermal peels, plated on 1/2MS medium (Lee et al. 2008). All other steps were same as in the localization procedure.

Stress tolerance assays in yeast

For stress tolerance assays, yeast strain BY4741 harboring p426GPD EV and p426GPD-*TaUSP* constructs were grown in liquid synthetic defined medium lacking uracil containing 2% Glc (w/v) for 24 h at 30 °C. Subsequently, they were diluted to the different concentrations (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) with uniform initial OD_{600} of 0.4 and 10 µL of each dilution was spotted onto solid YPD medium, supplemented with the different stress agents. Yeast dropouts were allowed to grow for 3 days at 30 °C on SD/-Ura medium supplemented with DTT, $LiCL_2$ H_2O_2 (Gutiérrez-Beltrán et al. 2017). For temperature stress, yeast cultures overexpressing *TaUSP* genes were grown overnight in SD/-Ura medium and $O.D_{600}$ was adjusted to 0.4. These cultures were then grown for 1 h at 42 °C and 46 °C for heat stress. For cold stress cultures were grown at 0 °C and -20 °C for 1 h (Wang et al. 2017; Jiang et al. 2009; Qin et al. 2015).

Overexpression of TaUSP 5D-1 in *Arabidopsis thaliana*

The floral dip method was used to transform *Arabidopsis* plants with the GV3101 strain of *Agrobacterium tumefaciens* carrying the pMDC32- *TaUSP 5D-1* (Zhang et al. 2006). After successive generations of selection on MS-agar medium, supplemented with 15 mg/l hygromycin, positive transgenic plants were identified. PCR using gene-specific primers was used to confirm the plants. Members of the T3 generation with 100% hygromycin resistance were considered homozygous. The level of ectopic expression in homozygous plants was checked by RT-PCR analysis.

Histochemical ROS detection

Nitro blue tetrazolium (NBT) and 3,3′-Diaminobenzidine (DAB) staining was done to measure the amount of reactive oxygen species (ROS), produced in response to DS in transgenic Arabidopsis and WT plants (Agarwal and Khurana 2018). For this, 3-week-old Arabidopsis WT and overexpression transgenic seedlings were exposed to DS (200 mM mannitol for 3 days), and then the plants were overnight stained by incubating them in NBT and DAB stains separately. The following day, the seedlings were washed with water and then dipped in a bleaching solution to remove the chlorophyll (ethanol, acetic acid, and glycerol in a ratio of 3:1:1). Following the wash, plants were observed under a bright field light microscope (Leica) and images were acquired for the comparison of ROS in transgenics and the WT *Arabidopsis* plants.

Results

Identification, phylogenetic and structural analysis of USP genes in *Triticum aestivum*

A total of 108 USP domain containing proteins encoded by 85 genes were found to be present in the wheat genome, using HMM search and *Triticum aestivum* ensemble genome_52. The size of TaUSP gene ranged from 423 bp (*TaUSP_2D-6*) to 1989 bp (*TaUSP_6D-5*) and the molecular weights of proteins varied from 15.23 kDa (TaUSP_2D-6) to 71.93 kDa (TaUSP_4A-1). The predicted theoretical isoelectric points, ranged from 4.67 (TaUSP_2A-5) to 11.83 (TaUSP_3B-2). Structural and molecular parameters for selected TaUSP encoding proteins are summarised in Table 1. Phylogenetic analysis revealed that *TaUSP* genes can be grouped into four groups based on their sequence similarity (Fig. 1). Gene structure analysis found that most of these USPs had four exons except *TaUSP_1B-1.1*, with three exons (Fig. 2a). These

Table 1 Description of abiotic stress responsive TaUSPs in *Triticum aestivum*

Gene Name	Gene ID	Nucleotide (bp)	Exon	Amino acids	Mw (KDa)	Iso-electric Point	Chromo-somal location	Abiotic Stress (Cold, Heat, Salt, Drought stress) with highest transcript level
TaUSP_1A-3	TraesCS1A02G280700.1	731	4	166	18.4	6.7	1A	Cold stress
TaUSP_1B-1	TraesCS1B02G124100.1	752	3	197	22.0	8.9	1B	Drought stress
TaUSP_1D-1	TraesCS1D02G108300.1	1134	4	166	17.7	6.4	1D	Drought stress and Cold stress
TaUSP_3B-1	TraesCS3B02G107200.1	839	4	160	17.2	6.9	3B	Drought stress
TaUSP_5D-1	TraesCS5D02G104200.1	1050	4	169	18.0	7.3	5D	Drought stress
TaUSP_6D-5	TraesCS6D02G303600.1	686	5	165	17.8	9.1	6D	Heat stress

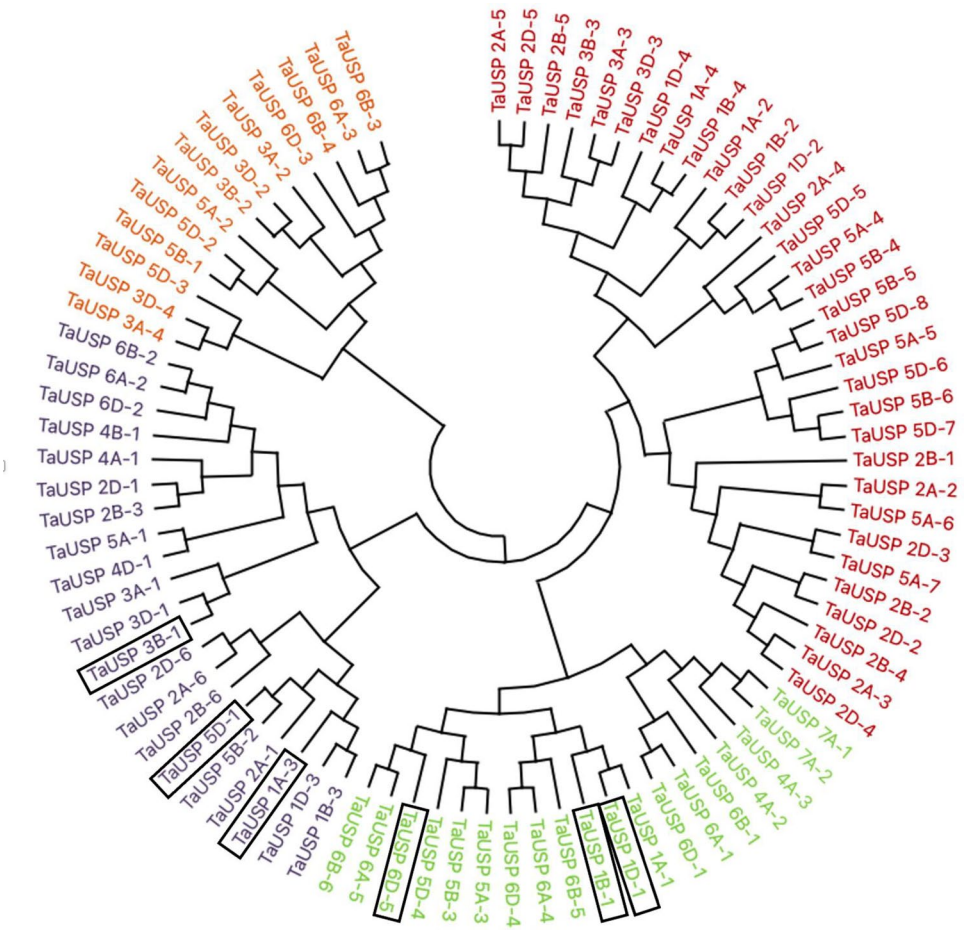

Fig. 1 Phylogenetic analysis of USP gene family in *Triticum aestivum*. Sequence alignment for total TaUSP protein sequences was done by CLUSTALW program and phylogenetic tree was constructed based on Neighbor-Joining method using MEGA7. Selected Abiotic stress responsive *TaUSP*s are highlighted in boxes

genes are found to be diversified in their functions with the presence of different domains, like Ubox, protein kinase catalytic domain, N-terminal serine threonine kinase, adenine nucleotide alpha hydrolases (Suppl. File 4), along with the USP domain. Proteins encoded by selected abiotic stress responsive *TaUSPs* were analysed for such domains and two prominent domains, USP domain and AANH_like superfamily domain were found in these proteins (Fig. 2b). Sequence analysis of the encoded proteins revealed that except for TaUSP_1B-1, all the abiotic stress response *TaUSP* genes code for proteins which contain a ATP binding motif similar to MJ0577 *Methanocaldococcus jannaschii* USP protein (Fig. 3a) (Chi et al. 2019). Phylogenetic analysis of abiotic stress responsive *TaUSP* genes revealed that they are more closely related to gene encoding MJ0577 protein than *Haemophilus influenza*_ USPA homolog (Fig. 3b).

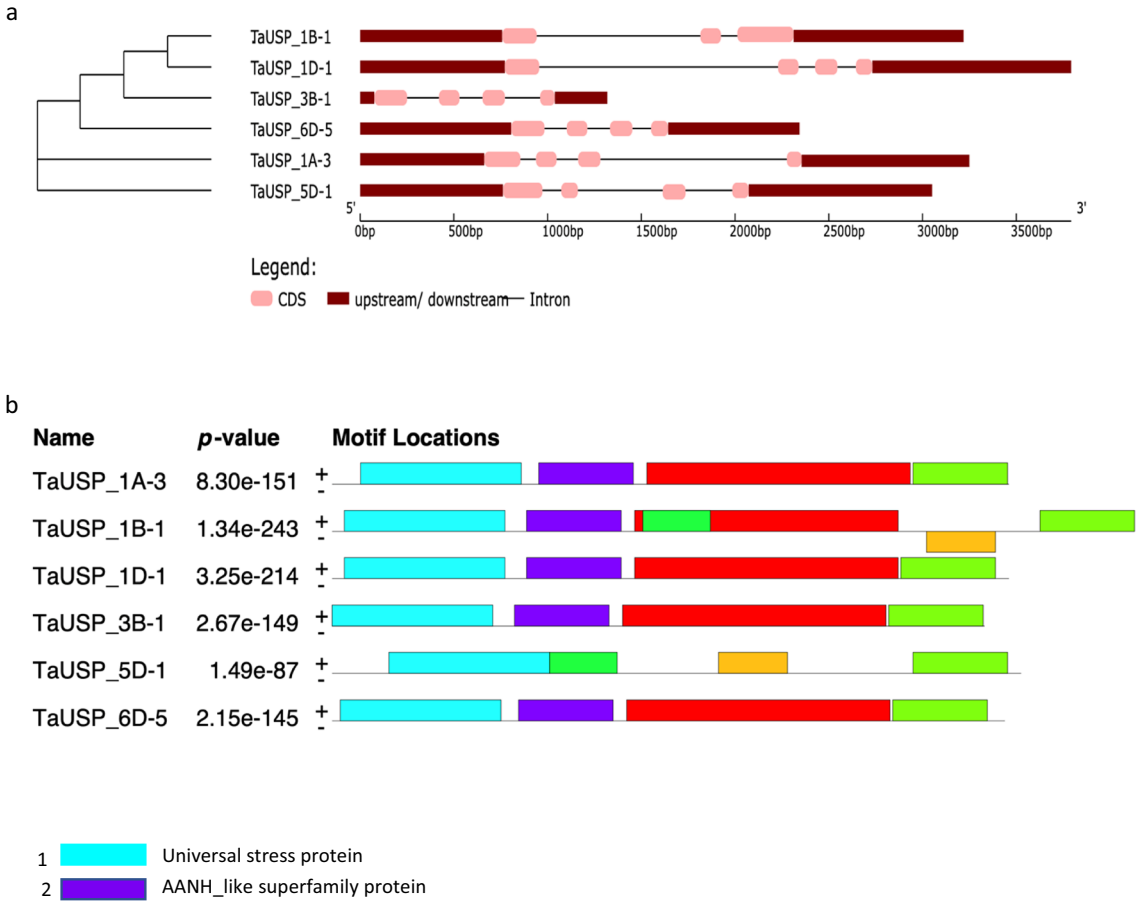

Fig. 2 Structural and domain analysis of selected *TaUSP* members. **a** Gene structure analysis of selected *TaUSP* members. Exons are depicted using solid boxes and introns are shown using lines. **b** Protein domain structure was constructed using MEME Suite software

Expression profile of abiotic stress responsive *TaUSP* genes under different abiotic stresses

Six genes, based on the transcript levels under abiotic stress on online wheat expression database were selected for further analysis. Their roles in different abiotic stresses were validated via quantitative real-time PCR (qPCR). Ten-day-old *Triticum aestivum* var. PBW343 seedlings were subjected to the above mentioned abiotic stresses. These members expressed differentially under different abiotic stresses. *TaUSP_1A-3* and *TaUSP_1D-1* were upregulated in cold stress and downregulated in heat stress. *TaUSP_3B-1* was upregulated in salt and drought stress condition, while *TaUSP_6D-5* was upregulated solely under heat stress and downregulated in all other stress treatments (Fig. 4). Transcript levels of *TaUSP_5D-1* were also checked in reproductive tissues of *Triticum aestivum* var. PBW343 (Suppl. Figure 8).

Subcellular localization of TaUSP proteins

Subcellular localization of TaUSP proteins was done to narrow down the possible physiological functions of abiotic stress responsive *TaUSP* genes. All the gene constructs encoding TaUSP proteins fused to YFP in frame were bombarded on onion epidermal peels. YFP signal for the TaUSP-YFP proteins was observed in cytoplasmic structures resembling ER and Golgi bodies (Fig. 5). To confirm this result further, co-localization with ER and Golgi body specific organelle markers was done. Proteins encoded by abiotic stress responsive *TaUSP* genes were predominantly localized in ER with some small traces in the Golgi bodies. These results were relatable to the cellular functions of *USP* being associated with protein scaffolding, holding and preventing the denaturation of globular macromolecules, and cellular protein transport (Vollmer et al. 2018).

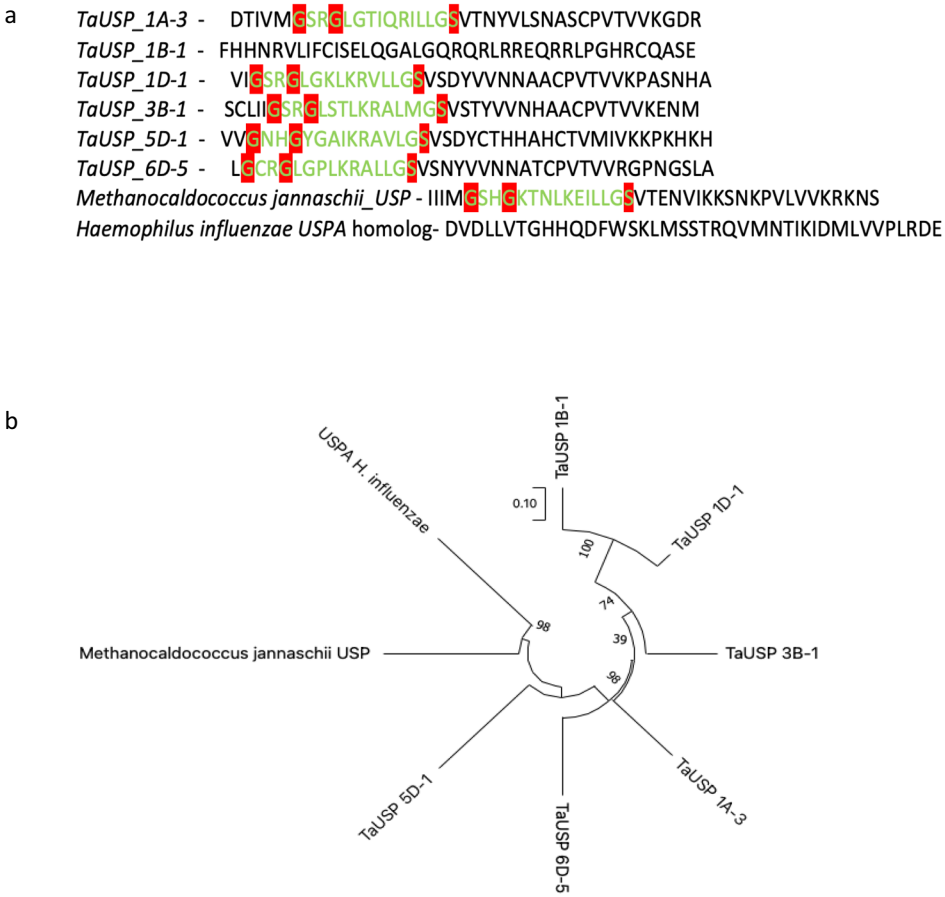

Fig. 3 Protein sequence comparison analysis of selected TaUSPs with MJ0577 protein and *H. influenzae* USP. **a** Nucleotide sequence analysis for ATP binding motif in *TaUSPs*, *MJ0577 USP* and *H. influenzae USP*. **b** Phylogenetic analysis based on protein sequence homology depicting evolutionary relation between MJ0577, TaUSPs and *H. influenzae USP*

Yeast-two-hybrid and BiFC assays for *TaUSP*_5D-1

USP proteins are known to form homo-dimers and hetero-dimers amongst themselves (Nachin et al. 2008). Therefore, proteins encoded by abiotic stress responsive *TaUSP* genes were checked for homo- and hetero-dimerization in *S. cerevisiae* strain AH109. In total 7 interactions were found, 3 being homo and 4 heterologous interactions (Fig. 6). The strength of interactions was checked by dropout assays up to a dilution of 10^{-4} on dropout yeast media, -LW, -HLW, -AHLW and 3AT (1 mM). Proteins encoded by *TaUSP_1A-3*, *TaUSP_1D-1* and *TaUSP_5D-1* were found to form homodimers. TaUSP_6D-5 and TaUSP_5D-1 had the maximum number of interactions amongst the candidate proteins. TaUSP_5D-1 and TaUSP_6D-5 were found to be forming heterodimers with three other abiotic stress responsive TaUSP proteins, respectively. Certain USP proteins are known to bind DNA (Melencion et al. 2017), so construct having all abiotic stress responsive pDEST-pGKT7:*TaUSP* genes were used with empty vector pDEST-pGADT7. Only TaUSP_5D-1 was found to have the property of autoactivation when co-transformed with empty vector pDEST-pGAD (Fig. 7a). To confirm the growth of yeast cells transformed with pGBKT7:*TaUSP_5D-1*, was checked on SD/-HW media. Further, all the interactions for TaUSP_5D-1 were confirmed with BiFC assay in onion epidermal peels and were found to be positive (Fig. 7b).

Stress assays in *TaUSP* overexpressing yeast

To functionally validate the abiotic stress responsive *TaUSP* genes in mediating different stress responses, yeast model system was used. Overexpression of abiotic stress responsive *TaUSP* in BY4741 (*Mat*α *met15*Δ*0 his3*Δ*1 ura3*Δ*1 leu2*Δ*0*) imparted tolerance to the yeast for a variety of stresses. The comparison was done with the yeast transformed with empty vector p426GPD. All abiotic stress responsive *TaUSP*: p426GPD constructs were transformed into *S. cerevisiae* strain BY4741 and treated with different stress conditions. *TaUSP_1A-3* overexpressing yeast was found to be more tolerant to cold stress (0 °C), even at freezing stress of (− 20 °C), as compared to yeast transformed with empty vector. *TaUSP_5D-1* and *TaUSP_6D-5* transformed yeast cells were found to be better in growth in comparison to empty vector transformed yeast cells even at a sub-lethal temperature of 46 °C (Fig. 8a). Because abiotic

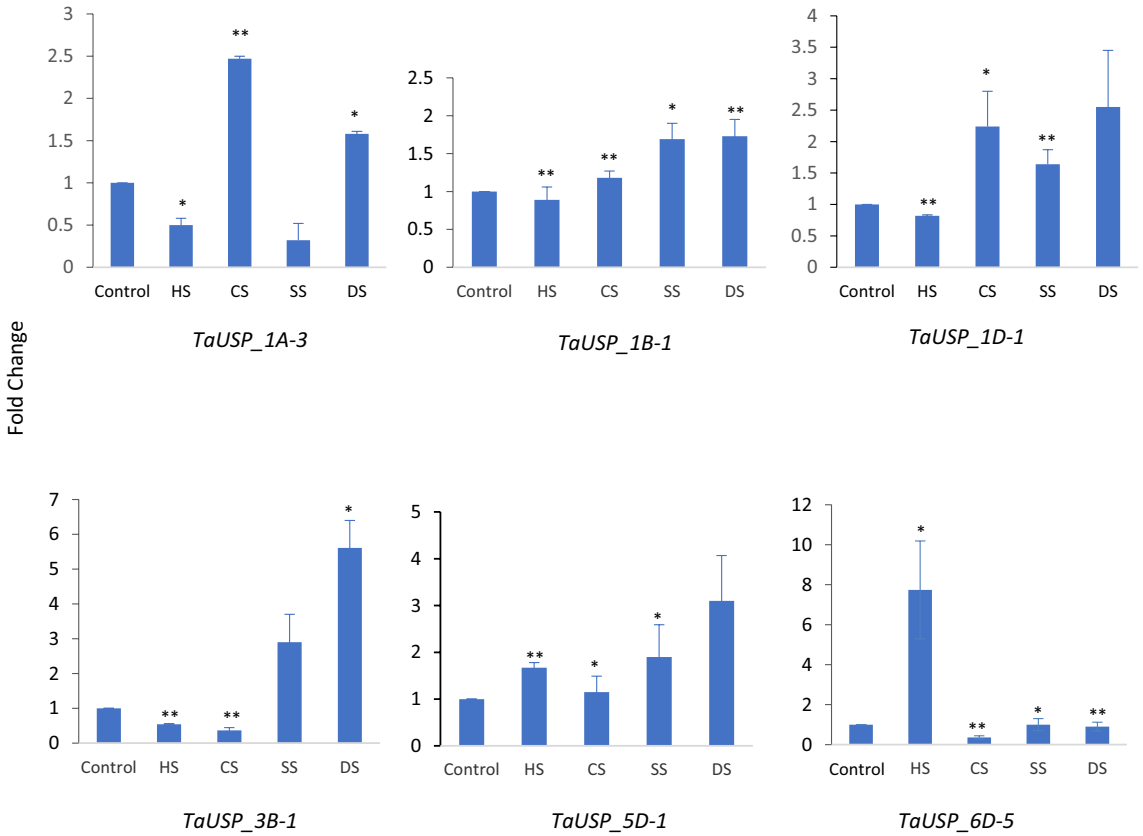

Fig. 4 Relative expression of different abiotic stress responsive *TaUSPs* in 10-day old *Triticum aestivum* seedlings under heat stress (HS), cold stress (CS), salt stress (SS) and drought stress (DS). Error bars are plotted using the standard error of three biological replicates. Three technical replicates were run for each biological replicate. The asterisk indicates the fold-changes calculated as significant (Students' *T* test; p value ≤ 0.05)

stress responsive *TaUSP* genes were majorly localized in ER, so DTT stress was given to the yeast to check for their probable role in UPR, and it was found that *TaUSP_3B-1* and *TaUSP_6D-5* imparted tolerance to DTT stress in yeast (Fig. 8c). *TaUSP_3B-1* was found to be providing tolerance to yeast for H_2O_2 and $LiCl_2$ stress (Fig. 8d and b). *TaUSP_6D-5* and *TaUSP_1D-1* overexpressing yeast cells were growing better in oxidative stress (H_2O_2) than the empty vector transformed yeast cells (Fig. 8b).

Overexpression of *TaUSP_5D-1* enhanced drought tolerance in *A. thaliana*

To elucidate the functional role of *TaUSP* gene in plants, overexpression transgenic lines of *Arabidopsis* were generated for *TaUSP_5D-1*. These lines were confirmed using gene-specific semi quantitative PCR and real-time PCR in transgenic line and WT (Suppl. Figure 2). Overexpression lines showed drought tolerance and better lateral root growth under drought stress (200 mM mannitol) at seedling stage. Since abiotic stress accelerates the ROS accumulation in plants, leading to oxidative damage, ROS levels were checked via DAB and NBT staining in WT and transgenic lines. Transgenic lines showed lesser ROS levels as compared to the WT plants. Further transcription profiling for drought stress responsive genes was done in WT and transgenic lines (Agarwal and Khurana 2020). *At.RD22, At.DREB2A* and *At.RD29B* were found to be upregulated in transgenic lines as compared to the WT plants under control conditions (Fig. 9).

Discussion

The identification of total *TaUSP* genes and characterization of abiotic stress responsive *TaUSP* genes is an important step for understanding their downstream signalling and to identify the pathways they regulate. No assigned function of these proteins in wheat was priorly available. The large number of *TaUSP* genes made us to prioritize the characterization of abiotic stress responsive *TaUSP* genes.

In *E. coli,* the UspA protein accumulates in response to a wide range of stresses in organisms, providing survival advantage under adverse growth conditions (Freestone

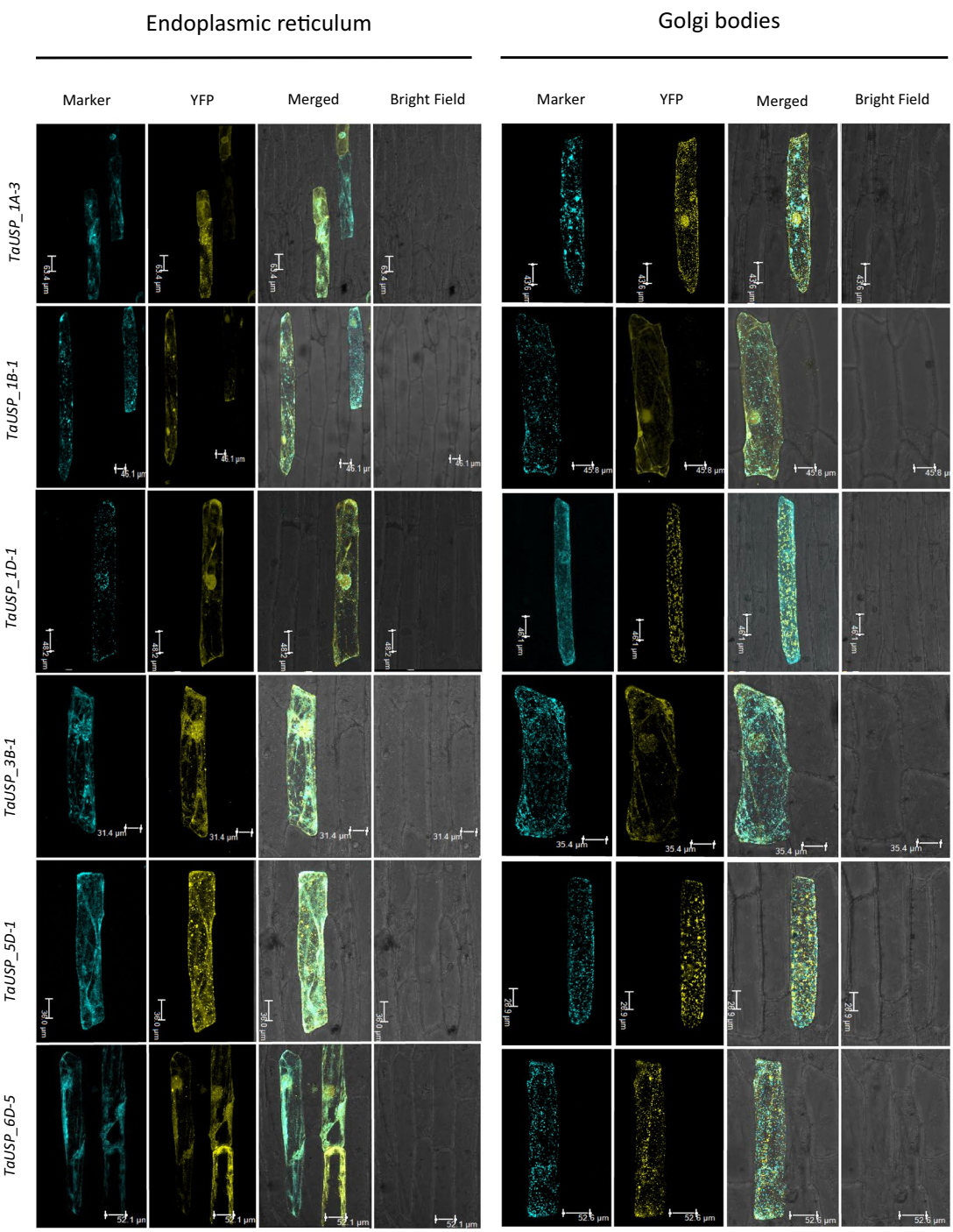

Fig. 5 Subcellular localisation of abiotic stress responsive TaUSP proteins along with different organelle markers. CDS of TaUSPs were cloned in frame with YFP protein and the expression was and observed in onion epidermal cells under confocal microscope. ER and Golgi body markers were used to locate the protein in onion epidermis cells. YFP yellow fluorescent protein

et al. 1997; Jung et al. 2015). Members of the UspA family have been found to regulate plant response to various abiotic stress conditions (Loukehaich et al. 2012: Gonzali et al. 2015: Jung et al. 2015: Gutiérrez-Beltrán et al. 2017: Melencion et al. 2017 and Chi et al. 2019). In wheat *TaUSP* genes appear to actively impart tolerance to the abiotic stress treatments. The expression pattern of these genes was interesting to study because of varied response of

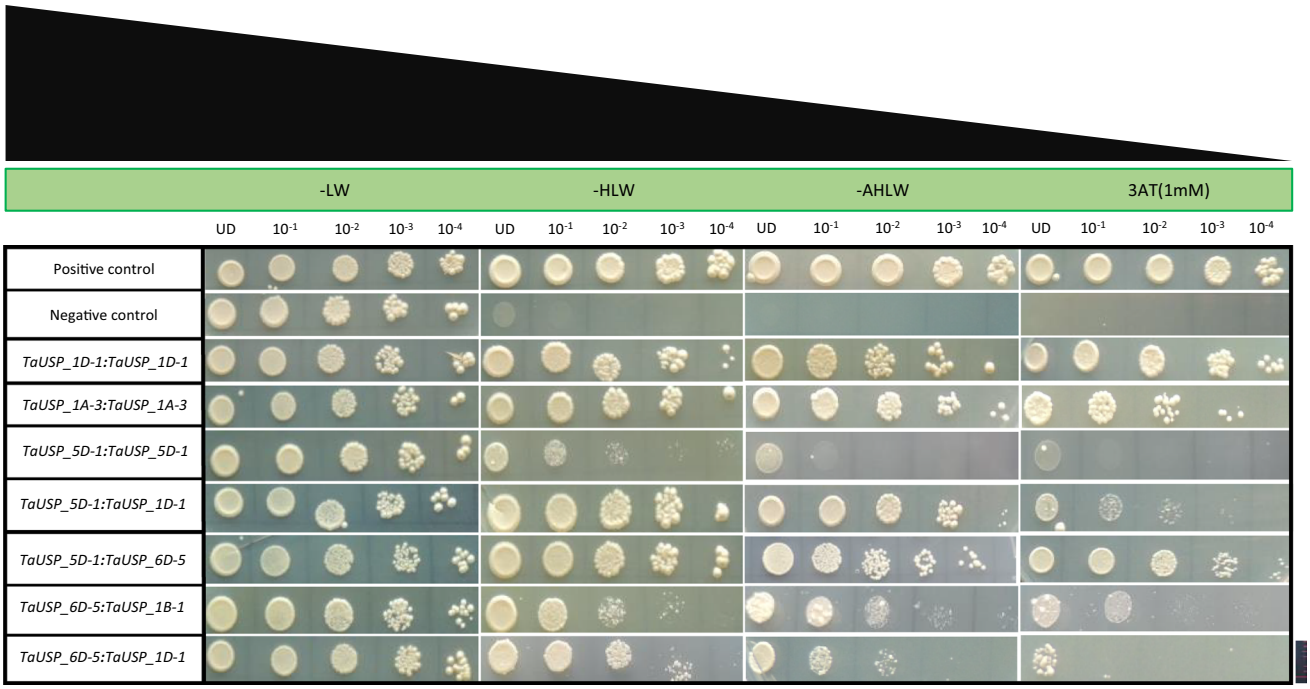

Fig. 6 Yeast-2-hybrid assay for homodimerization and heterodimerization study of TaUSPs. Yeast cells were co-transformed with pGBKT and pGAD *TaUSP* constructs and the growth was analyzed on SD/-Leu/-Trp (-LW), SD/-Leu/-Trp/-His(-HLW), SD/-Leu/-Trp/-His/-Ade (-AHLW) and SD/-Leu/-Trp/-His(-HLW) + 1 mM 3-aminotriazole (3-AT) media. Interaction between pGAD-T-Antigen and pGBKT-p53 was used as positive control, and interaction between pGAD-T-Antigen and pGBKT-Lam was used as a negative control

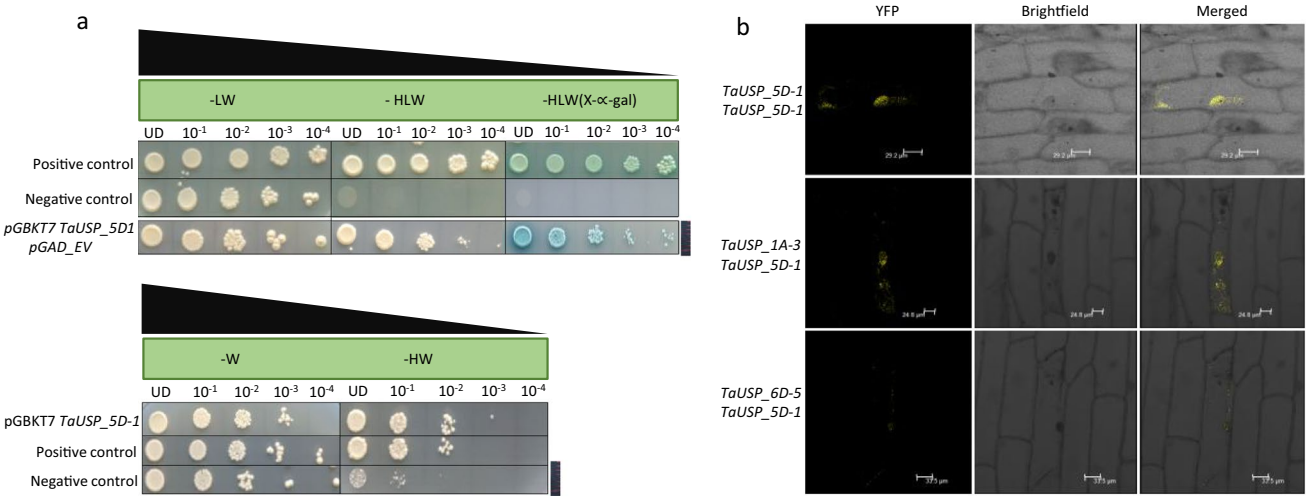

Fig. 7 DNA binding activity check in yeast. a. Growth of yeast cells harbouring pDEST-pGBKT7*::TaUSP_5D-1* and pDEST-pGADT7 EV was analysed on SD/-Leu/-Trp, SD/-His/-Leu/-Trp and SD/-His/-Leu/-Trp supplemented with X-α-GAL. pGADT7-T/pGBKT-53 was taken as positive interaction control and pGADT7-T/pGBKT-Lam was taken as negative interaction control. Growth of yeast cells transformed with fusion constructs pDEST- GBKT7:: *TaUSP_5D-1,* harbouring histidine reporter gene was analysed on SD/-Trp (-W) medium and on SD/-Trp/-His (-HW) medium. Yeast cells transformed with the empty vector pGBKT7 (EV) alone were used as a negative control and pDEST -pGBKT::TaHsfA6b were taken as positive control respectively. b. BiFC assay in onion epidermal cells to confirm *TaUSP_5D-1* interactions

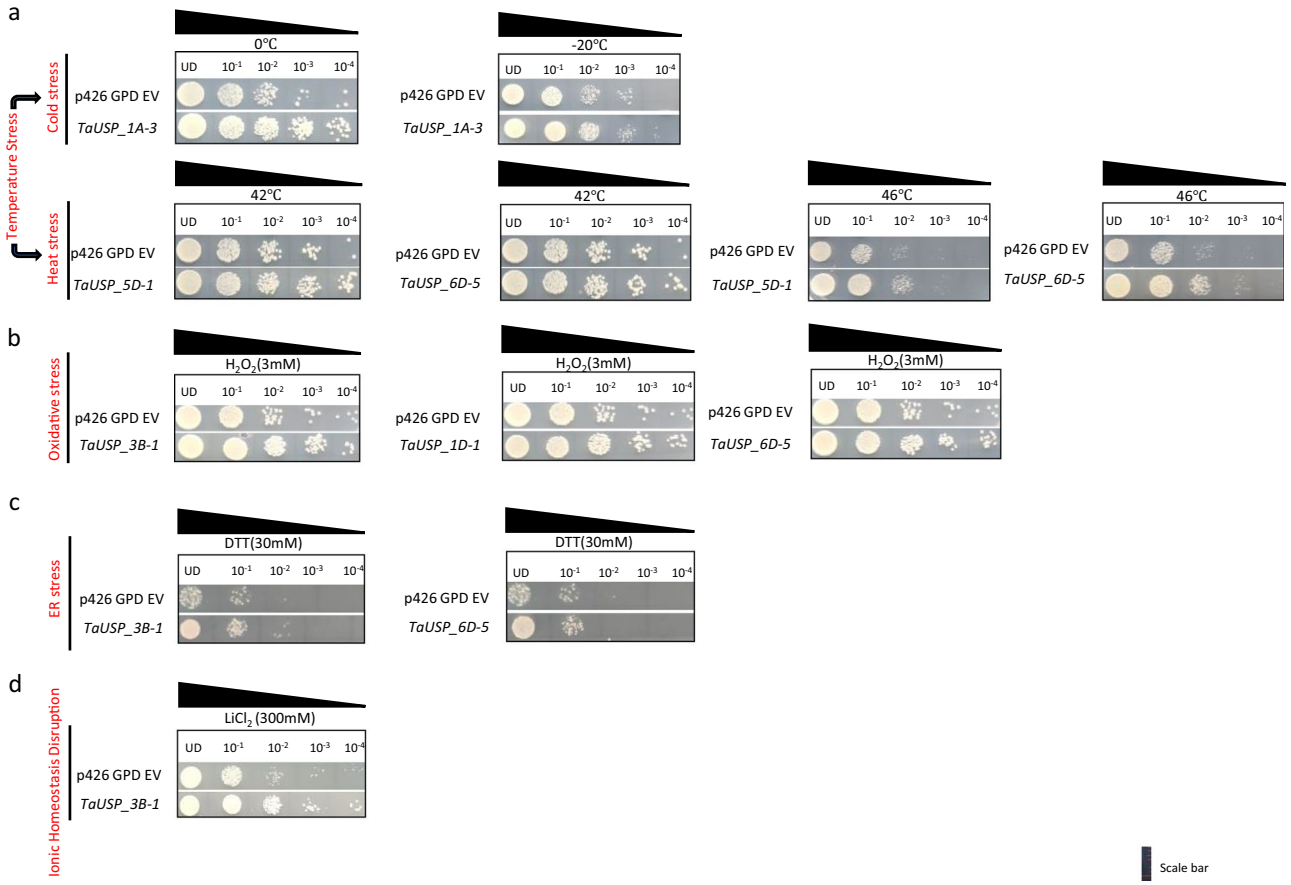

Fig. 8 Overexpression of *TaUSP* genes impart tolerance to *S.cerevisiae* under different stress conditions. The strain BY4741 was transformed with p426GPD (EV), p426GPD:*TaUSP_1A-3*, p426GPD:*TaUSP_5D-1*, p426GPD:*TaUSP_6D-5*, p426GPD:*TaUSP3B-1*. All mentioned *TaUSP* gene constructs and EV were grown in liquid culture for, **a** heat stress (42 °C and 46 °C for 1 h) and cold stress (0 °C and − 20 °C for 1 h), serially diluted samples were spotted on plates of SD/-Ura media. Yeast dropouts were allowed to grow for 3 days at 30 °C on SD/-Ura media for **b** oxidative stress (3 mM H_2O_2), **c** ER stress (30 mM DTT) and **d** Ionic homeostatis disruption (300 mM $LiCl_2$)

different *TaUSP*s in the same stress (Fig. 4). *TaUSP_1A-3* and *TaUSP_1D-1* were upregulated in cold stress but downregulated in heat stress condition. However, *TaUSP_6D-5* showed the exact opposite pattern of transcript levels in the same afore mentioned stresses. Same was the case with *TaUSP_3B-1* and *TaUSP_6D-5* in drought and salt stress. These observations point towards the fact that different abiotic stress responsive *TaUSP* genes may respond to same stress through a different downstream pathway.

Although it has been reported that UspA proteins contain a dimerization domain in their sequence, little is known about its biological relevance. *E.coli* Usp proteins have been shown to form homodimers and/or heterodimers in vivo, resulting in adaptation to different stresses (Nachin et al. 2008; Heermann et al. 2009). Abiotic stress responsive *TaUSP* genes were also found to be forming hetero/ homo dimers (Fig. 6), suggesting that these genes crosstalk among themselves to respond to various stress conditions. Homodimerization is an essential need for these genes, in certain plants, to function for better adaptability towards external stresses (Gutiérrez-Beltrán et al. 2017). However the Y2H assay data for wheat USPs showed that not all TaUSPs essentially formed homodimers, some of them rather only formed heterodimers but these heterodimer formations were preferentially within the same clade of genes (Fig. 1 and Fig. 6).

All abiotic stress responsive *TaUSP* genes, except *TaUSP_1B-1*, were found to be closely related to gene encoding MJ0577, and code for proteins with ATP binding motif at their C-terminal as is present in MJ0577 (Fig. 3). The ATP binding functionality in a few members of the UspA family has brought about the hypothesis that nucleotide binding USP proteins ought to feature as molecular switches, through sensing ATP levels for the duration of stress to detect cell metabolic status (O'Toole and Williams 2003; Persson et al. 2007; Drumm et al. 2009). Autoadenylation is found in bacterial USPs in stationary phase (Weber and Jung 2006) and it has been proven to be a key aspect

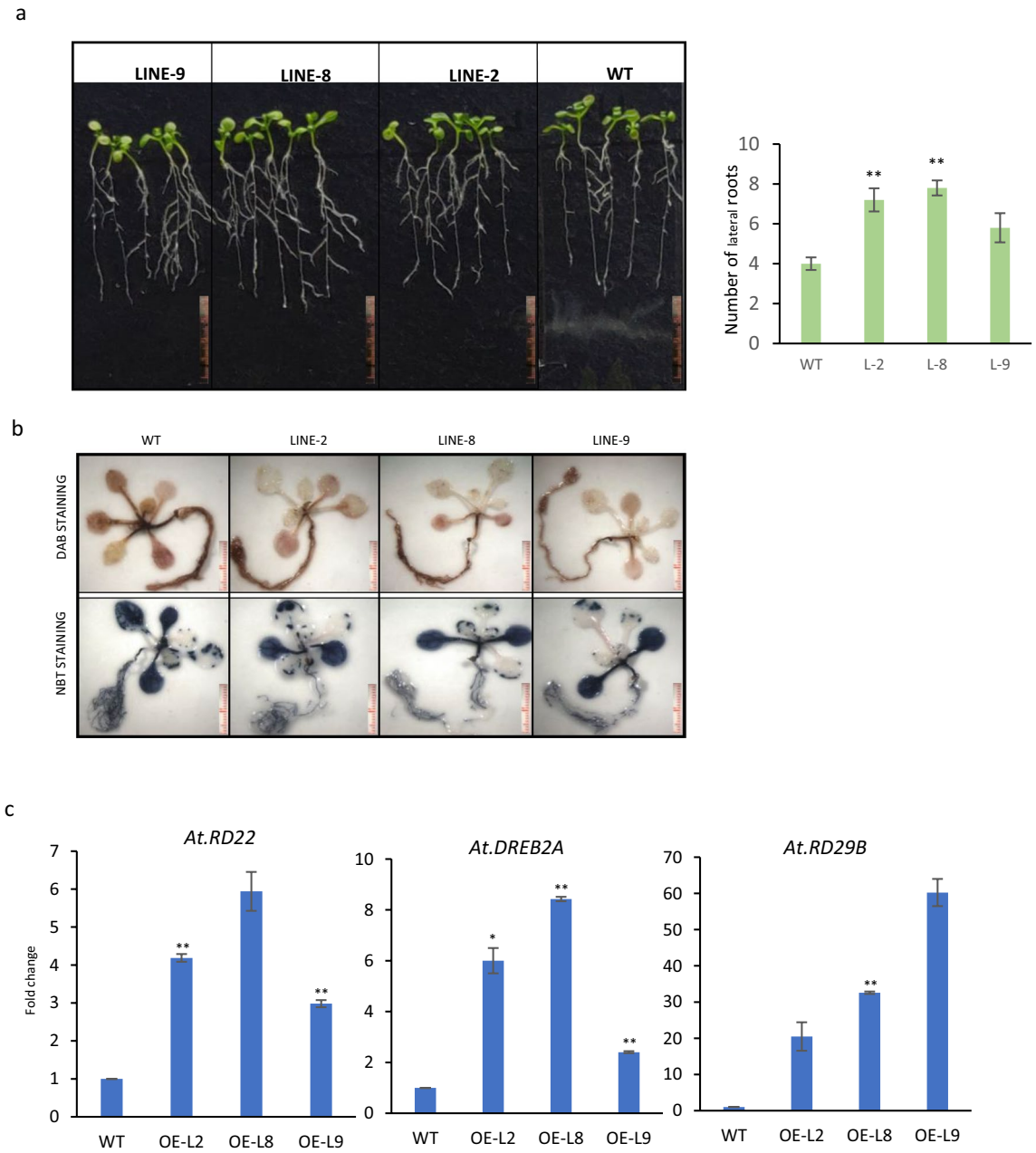

Fig. 9 Phenotypical and physiological analysis of *TaUSP_5D-1* overexpression *Arabidopsis* transgenics. **a** Transgenic plants overexpressing *TaUSP_5D-1* under drought conditions. Plants were grown under control conditions for 7 days, then transferred to mannitol (200 mM)-supplemented MS media. Phenotype with increased number of lateral roots in transgenics was observed after seven-days. **b** Two-week-old seedling of WT and transgenics were given drought stress for three days on 200 mM mannitol-supplemented MS medium and stained with DAB and NBT to analyse the H_2O_2 and superoxide anion accumulation, respectively, after the stress treatment **c** Expression analysis of drought stress marker genes. Transcript analysis of drought stress marker genes in WT and transgenics stress conditions. Transcript levels were normalized to WT, and *AtActin* was used as a housekeeping gene. Values represent data from three biological replicates and three technical replicates. Error bars indicate values ± SD. Asterisks on top of the error bars represent the significance levels (Student's *t*-test; *p*-value ≤ 0.05)

for microbial survival in O_2 depletion during the course of growth arrest and virulence. In fact, it has been demonstrated that the capacity of UspA protein Rv2623 from *M. tuberculosis* to regulate its growth and latency in the host, depends on its ATP-binding activity (Drumm et al. 2009). Presence of the ATP binding motifs in abiotic stress responsive *TaUSPs* might be the stress sensing mechanism used by these USP proteins which is majorly conserved in

euryarchaeota and plant kingdom. *TaUSP_5D-1* also shows some DNA binding activity which implies its role in DNA damage repair under stress conditions (Fig. 7a), however, this needs to be further studied for a better understanding.

TaUSPs were majorly localized in endoplasmic reticulum and some residual amount in golgi bodies in the cell. ER is known to be the major site for synthesis and protein folding in cells (Gidalevitz et al. 2013). During different abiotic stress conditions, demand for protein folding exceeds the production capacity, which leads to accumulation of unfolded or incorrectly folded proteins resulting in ER stress (Fernández-Bautista et al. 2017).Various stresses like heat, drought and salt lead to ER stress. Localization of TaUSPs in ER (Fig. 5), led to speculation of their involvement in UPR stress. To check for the same, DTT (ER stressor) treatment was given to *TaUSP* OE yeast. Transformation of yeast with *TaUSP_3B-1* and *TaUSP_6D-5* imparted better growth under DTT stress, pointing towards the possibility of their roles in UPR under drought and heat stress, respectively, in plants. Transformation with *TaUSP_3B-1* also imparted better growth to the yeast under $LiCl_2$, depicting its role in maintaining ion homeostasis in cells under salt stress (Gutiérrez-Beltrán et al. 2017). In the yeast system *TaUSP_6D-5* and *TaUSP_5D-1* imparted tolerance to heat stress even at a sub-lethal temperature of 46 °C, and *TaUSP_1A-3* imparted tolerance even at a temperature of 0 °C and − 20 °C (Fig. 8). To elucidate the function of *USP* genes in planta, gain of function approach in *A.thaliana* was used. Overexpression lines for *TaUSP_5D-1* were generated and analyzed for their phenotype and tolerance in drought stress conditions. It was found that plants overexpressing the selected *TaUSP* better performed under drought condition and had a dense rooting system due to a greater number of lateral roots as compared to the WT plants. Results reveal that *TaUSP_5D-1* provides drought tolerance to plants via improving lateral root system of the plant. At molecular level transgenic lines even showed less ROS accumulation under stress condition as compared to the WT (Fig. 9).

In conclusion, wheat abiotic stress responsive *USP* genes are good candidates for imparting abiotic stress tolerance to crops through genetic engineering. *TaUSP_6D-5* and *TaUSP_3B-1* connect heat stress and drought stress to UPR, respectively in the yeast system. The *TaUSP_6D-5* also stands as a good candidate gene for heat stress tolerance and should be further studied for the downstream singling pathway for its functioning. Similarly, *TaUSP_3B-1* providing tolerance to $LiCl_2$ in yeast and its upregulation in drought and salt stress suggests the possibility of its function in maintaining cellular homeostasis for imparting drought and salt stress in plants. Further overexpression of *TaUSP_5D-1* in plant imparts drought stress tolerance via alteration in root morphology. The other abiotic stress responsive *TaUSP* genes can also be used to engineer crops to impart tolerance to cold stress (*TaUSP_1A-3* and *TaUSP_1D-1*), drought and salt stress.

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Author contributions statement AS performed the experiments and written the manuscript. AS and CS analysed the data and discussed the outcomes. PK and AKS conceived the idea and provided logistic support. All authors read and approved the final version of the manuscript.

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Data availability Enquiries about data availability should be directed to the authors.

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

Agarwal P, Khurana P (2018) Characterization of a novel zinc finger transcription factor (TaZnF) from wheat conferring heat stress tolerance in *Arabidopsis*. Cell Stress Chaperones 23:253–267. https://doi.org/10.1007/s12192-017-0838-1

Agarwal P, Khurana P (2020) TaZnF, a C3HC4 type RING zinc finger protein from *Triticum aestivum* is involved in dehydration and salinity stress. J Plant Biochem Biotechnol 29:395–406. https://doi.org/10.1007/s13562-019-00546-8

Amoah JN, Ko CS, Yoon JS, Weon SY (2019) Effect of drought acclimation on oxidative stress and transcript expression in wheat (*Triticum aestivum* L.). J Plant Interact 14:492–505. https://doi.org/10.1080/17429145.2019.1662098

Aravind L, Anantharaman V, Koonin Ev (2002) Monophyly of Class I aminoacyl tRNA synthetase, USPA, ETFP, photolyase, and PP-ATPase nucleotide-binding domains: Implications for protein evolution in the RNA world. Proteins: structure. Funct Genet 48:1–14. https://doi.org/10.1002/prot.10064

Bhuria M, Goel P, Kumar S, Singh AK (2019) Genome-wide identification and expression profiling of genes encoding universal stress proteins (USP) identify multi-stress responsive USP genes in *Arabidopsis thaliana*. Plant Physiol Rep 24:434–445. https://doi.org/10.1007/s40502-019-00468-6

Chauhan H, Khurana N, Tyagi AK et al (2011) Identification and characterization of high temperature stress responsive genes in bread wheat (*Triticum aestivum* L.) and their regulation at various stages of development. Plant Mol Biol 75:35–51. https://doi.org/10.1007/s11103-010-9702-8

Chi YH, Koo SS, Oh HT et al (2019) The physiological functions of universal stress proteins and their molecular mechanism to protect plants from environmental stresses. Front Plant Sci. https://doi.org/10.3389/fpls.2019.00750

Choudhury FK, Rivero RM, Blumwald E, Mittler R (2017) Reactive oxygen species, abiotic stress and stress combination. Plant J 90:856–867. https://doi.org/10.1111/tpj.13299

Drumm JE, Mi K, Bilder P et al (2009) Mycobacterium tuberculosis universal stress protein Rv2623 regulates bacillary growth by

ATP-binding: requirement for establishing chronic persistent infection. PLoS Pathog. https://doi.org/10.1371/journal.ppat.1000460

Fernández-Bautista N, Fernández-Calvino L, Muñoz A, Castellano MM (2017) HOP3, a member of the HOP family in *Arabidopsis*, interacts with BiP and plays a major role in the ER stress response. Plant Cell Environ 40:1341–1355. https://doi.org/10.1111/pce.12927

Forêt S, Seneca F, de Jong D et al (2011) Phylogenomics reveals an anomalous distribution of USP genes in metazoans. Mol Biol Evol 28:153–161. https://doi.org/10.1093/molbev/msq183

Freestone P, Nyström T, Trinei, M, Norris V (1997) The universal stress protein, UspA, of *Escherichia coli* is phosphorylated in response to stasis. J Mol Biol 274(3):318–324. https://doi.org/10.1006/jmbi.1997.1397

Gidalevitz T, Stevens F, Argon Y (2013) Orchestration of secretory protein folding by ER chaperones. Biochim Biophys Acta Mol Cell Res 1833:2410–2424

Gilroy S, Białasek M, Suzuki N et al (2016) ROS, calcium, and electric signals: key mediators of rapid systemic signaling in plants. Plant Physiol 171:1606–1615. https://doi.org/10.1104/pp.16.00434

Gonzali S, Loreti E, Cardarelli F et al (2015) Universal stress protein HRU1 mediates ROS homeostasis under anoxia. Nat Plants. https://doi.org/10.1038/nplants.2015.151

Gutiérrez-Beltrán E, Personat JM, de la Torre F, del Pozo O (2017) A universal stress protein involved in oxidative stress1 is a phosphorylation target for protein kinase CIPK6. Plant Physiol 173:836–852. https://doi.org/10.1104/pp.16.00949

Hamdi K, Brini F, Kharrat N et al (2020) Abscisic acid, stress, and ripening (Tt ASR1) gene as a functional marker for salt tolerance in durum wheat. Biomed Res Int. https://doi.org/10.1155/2020/7876357

Heermann R, Weber A, Mayer B et al (2009) The universal stress protein UspC scaffolds the KdpD/KdpE signaling cascade of *Escherichia coli* under salt stress. J Mol Biol 386:134–148. https://doi.org/10.1016/j.jmb.2008.12.007

Jiang C, Xu J, Zhang H et al (2009) A cytosolic class i small heat shock protein, RcHSP17.8, of *Rosa chinensis* confers resistance to a variety of stresses to *Escherichia coli*, yeast and *Arabidopsis thaliana*. Plant Cell Environ 32:1046–1059. https://doi.org/10.1111/j.1365-3040.2009.01987.x

Jung YJ, Melencion SMB, Lee ES et al (2015) Universal stress protein exhibits a redox-dependent chaperone function in arabidopsis and enhances plant tolerance to heat shock and oxidative stress. Front Plant Sci. https://doi.org/10.3389/fpls.2015.01141

Kerk D, Bulgrien J, Smith DW, Gribskov M (2003) *Arabidopsis* proteins containing similarity to the universal stress protein domain of bacteria. Plant Physiol 131:1209–1219. https://doi.org/10.1104/pp.102.016006

Khurana N, Chauhan H, Khurana P (2013) Wheat chloroplast targeted sHSP26 promoter confers heat and abiotic stress inducible expression in transgenic *Arabidopsis* plants. PLoS One. https://doi.org/10.1371/journal.pone.0054418

Lee LY, Fang MJ, Kuang LY, Gelvin SB (2008) Vectors for multicolor bimolecular fluorescence complementation to investigate protein-protein interactions in living plant cells. Plant Methods. https://doi.org/10.1186/1746-4811-4-24

Lescot M, Déhais P, Thijs G et al (2002) PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. Nucleic Acids Res. https://doi.org/10.1093/nar/30.1.325

Li WT, Wei YM, Wang JR et al (2010) Identification, localization, and characterization of putative USP genes in barley. Theor Appl Genet 121:907–917. https://doi.org/10.1007/s00122-010-1359-9

Loukehaich R, Wang T, Ouyang B et al (2012) SpUSP, an annexin-interacting universal stress protein, enhances drought tolerance in tomato. J Exp Bot 63:5593–5606. https://doi.org/10.1093/jxb/ers220

Maqbool A, Zahur M, Husnain T, Riazuddin S (2009) GUSP1 and GUSP2, two drought-responsive genes in *Gossypium arboreum* have homology to universal stress proteins. Plant Mol Biol Rep 27:109–114. https://doi.org/10.1007/s11105-008-0049-0

Marchler-Bauer A, Lu S, Anderson JB et al (2011) CDD: a conserved domain database for the functional annotation of proteins. Nucleic Acids Res. https://doi.org/10.1093/nar/gkq1189

Meena S, Deb S, Samtani H, Khurana P (2020) Dissecting the molecular function of *Triticum aestivum* STI family members under heat stress. Front Genet. https://doi.org/10.3389/fgene.2020.00873

Meena S, Samtani H, Khurana P (2022) Elucidating the functional role of heat stress transcription factor A6b (TaHsfA6b) in linking heat stress response and the unfolded protein response in wheat. Plant Mol Biol 108:621–634. https://doi.org/10.1007/s11103-022-01252-1

Melencion SMB, Chi YH, Pham TT et al (2017) RNA chaperone function of a universal stress protein in arabidopsis confers enhanced cold stress tolerance in plants. Int J Mol Sci. https://doi.org/10.3390/ijms18122546

Nachin L, Nannmark U, Nyström T (2005) Differential roles of the universal stress proteins of *Escherichia coli* in oxidative stress resistance, adhesion, and motility. J Bacteriol 187:6265–6272. https://doi.org/10.1128/JB.187.18.6265-6272.2005

Nachin L, Brive L, Persson KC et al (2008) Heterodimer formation within universal stress protein classes revealed by an in silico and experimental approach. J Mol Biol 380:340–350. https://doi.org/10.1016/j.jmb.2008.04.074

Nebenführ A (2014) Identifying subcellular protein localization with fluorescent protein fusions after transient expression in onion epidermal cells. Methods Mol Biol 1080:77–85. https://doi.org/10.1007/978-1-62703-643-6_6

Nystrdm T, Neidhardt FC (1992) Cloning, mapping and nucleotide sequencing of a gene encoding a universai stress protein in *Escherichia coli*. Mol Microbiol. https://doi.org/10.1111/j.1365-2958.1992.tb01774.x

Nystrom T, Neidhardt FC (1994) Expression and role of the universal stress protein, UspA, of *Escherichia coli* during growth arrest. Mol Microbiol. https://doi.org/10.1111/j.1365-2958.1994.tb00334.x

O'Toole R, Williams HD (2003) Universal stress proteins and Mycobacterium tuberculosis. Res Microbiol 154:387–392

Persson Ö, Valadi Å, Nyström T, Farewell A (2007) Metabolic control of the *Escherichia coli* universal stress protein response through fructose-6-phosphate. Mol Microbiol 65:968–978. https://doi.org/10.1111/j.1365-2958.2007.05838.x

Qin D, Wang F, Geng X et al (2015) Overexpression of heat stress-responsive TaMBF1c, a wheat (*Triticum aestivum* L.) multiprotein bridging factor, confers heat tolerance in both yeast and rice. Plant Mol Biol 87:31–45. https://doi.org/10.1007/s11103-014-0259-9

Sauter M, Rzewuski G, Marwedel T, Lorbiecke R (2002) The novel ethylene-regulated gene OsUsp1 from rice encodes a member of a plant protein family related to prokaryotic universal stress proteins. J Exp Bot 53:2325–2331. https://doi.org/10.1093/jxb/erf096

Siegele DA (2005) Universal stress proteins in Escherichia coli. J Bacteriol 187:6253–6254

Sinha P, Pazhamala LT, Singh VK et al (2016) Identification and validation of selected universal stress protein domain containing drought-responsive genes in pigeonpea (*Cajanus cajan* L.). Front Plant Sci. https://doi.org/10.3389/fpls.2015.01065

Sousa MC, Mckay DB (2001) Structure of the universal stress protein of *Haemophilus influenzae* mitomycin C and irradiation with

ultraviolet light [3]. When expressed at elevated levels during cell growth arrest, the UspA protein becomes phosphorylated. Structure. https://doi.org/10.1016/S0969-2126(01)00680-3

Tuteja N (2007) Abscisic acid and abiotic stress signaling. Plant Signal Behav 2:135–138

Udawat P, Jha RK, Sinha D et al (2016) Overexpression of a cytosolic abiotic stress responsive universal stress protein (SbUSP) mitigates salt and osmotic stress in transgenic tobacco plants. Front Plant Sci. https://doi.org/10.3389/fpls.2016.00518

Vollmer AC, Bark SJ (2018) Twenty-five years of investigating the universal stress protein: function, structure, and applications. Advances in applied microbiology. Academic Press Inc., Cambridge, pp 1–36

Wang M, Zou Z, Li Q et al (2017) Heterologous expression of three *Camellia sinensis* small heat shock protein genes confers temperature stress tolerance in yeast and *Arabidopsis thaliana*. Plant Cell Rep 36:1125–1135. https://doi.org/10.1007/s00299-017-2143-y

Weber A, Jung K (2006) Biochemical properties of UspG, a universal stress protein of *Escherichia coli*. Biochemistry 45:1620–1628. https://doi.org/10.1021/bi051301u

Zhang X, Henriques R, Lin SS et al (2006) Agrobacterium-mediated transformation of *Arabidopsis thaliana* using the floral dip method. Nat Protoc 1:641–646. https://doi.org/10.1038/nprot.2006.97