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Understanding the impact of heat stress on crop plants

The thesis of the PhD dissertation

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Title: Understanding the impact of heat stress on crop plants

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1. INTRODCUTION

Barley (*Hordeum vulgare* L.), the fourth most produced cereal globally (160 million tonnes annually), serves as a key crop for animal feed, malting, brewing, and human nutrition. Its self-pollinating genetics position it as a model for temperate cereals. Climate change, however, is associated with increasing heat stress, which disrupts cellular homeostasis through membrane damage, protein denaturation, and reactive oxygen species accumulation. Plants counter this via heat shock factors (HSFs) that bind heat shock response elements (HSEs) to target genes, triggering cascades of secondary HSFs, heat shock proteins and antioxidants that restore proteostasis.

In barley, HS during flowering and grain filling severely curtails yield by repressing developmental genes and inducing stress-responsive genes. Beyond regulation at the transcription initiation level, the regulation of RNA Polymerase II (RNAPII) elongation proves essential for adaptation, particularly for HSP transcripts. RNAPII pauses, or arrests at barriers like nucleosomes, DNA secondary structures, or mis-incorporated nucleotides, leading to backtracking: the nascent RNA 3' end extrudes through the funnel pore, promoting TFIIS recruitment. TFIIS stimulates cleavage of the extruded RNA, thereby realigning the 3' end for resumption of the transcriptional process.

Prior studies, including ours, established TFIIS's necessity for heat survival in Arabidopsis. TFIIS is also transcriptionally induced by heat stress across plants like rapeseed and barley, suggesting adaptive roles. Using CRISPR/Cas9 mutagenesis, we disrupted the barley *TFIIS* gene and demonstrated its requirement for heat tolerance, elucidating the molecular basis of elongation control under stress.

In a concurrent side project, we have studied barley grain development under diverse climatic conditions in a field trial. We have shown that GW2 (which encodes a RING-type E3 ubiquitin ligase involved in ubiquitin-mediated protein degradation and regulates barley grain size and protein content) is heat-stress regulated; However, current evidence does not support a role for GW2 as a core component of the canonical heat shock response pathway.

2. OBJECTIVES

Barley (*Hordeum vulgare* L.) is an important crop plant; therefore, understanding the molecular mechanisms underlying its adaptation to climatic stress is of utmost importance.

The specific objectives of the study were the following:

I. Understanding the requirements of TFIIS in barley under ambient temperature conditions.
II. Describe the actions of TFIIS during the heat stress adaptation process in barley.
III. To elucidate the role and mechanism of the GW2.1 gene in the regulation of barley grain development.

3. MATERIALS AND METHODS

3.1 Plant materials, growth circumstances, and treatments for heat stress sensitivity

Barley plants (*Hordeum vulgare* L. cv. 'Golden Promise', wild type, wt) were grown in controlled growth chambers (MLR-350; Sanyo, Tokyo, Japan) at 20 °C under long-day conditions (16 hours light/8 hours dark), as described previously. In heat stress (HS) studies, both wild-type and *tfIIs-cr* mutant seedlings were originally germinated in Jiffy peat pellets (compressed peat-based growth substrates enclosed in biodegradable mesh, which provide a uniform and well-aerated medium for early seedling development under controlled conditions) for one week (20 °C, long-day). The plants in jiffy were then transferred in pairs, with each pot holding one wild-type and one mutant and grown for one additional week. At the 14th day of age, the plants underwent a continuous moderate heat stress regimen (thermotolerance to moderately high temperature regime, TMHT), characterised by incubation at 40 °C. Leaf tissues were obtained from both non-treated and heat-treated plants immediately after the treatment for molecular analysis. Sampling points included non-treated (NT), 1 hour at 40 °C (1 h), 2 hours at 40 °C (2 h), and 1 day at 40 °C (1 d).

Sampling was performed immediately after treatment. Tissue collection was standardised as follows: (i) the second fully expanded leaf was selected from each plant; (ii) the mid-section of the leaf blade was excised to ensure developmental consistency; (iii) for each biological replicate, leaf material was pooled from at least 3 individual plants of the same genotype and treatment group to reduce plant-to-plant variability. A minimum of three independent biological replicates were used for RNA and protein analyses. For molecular analyses, the harvested leaf segments were immediately frozen in liquid nitrogen and stored at −80 °C until further processed.

3.2 Creation of CRISPR mutants

The barley *TFIIS* gene locus and its genomic sequence were previously documented in the Ensembl Plants database (accession no. *HORVU.MOREX.r3.5HG0524690*; originally *HORVU5Hr1G111700*). Potential CRISPR/Cas9 target sites were generated using the

CRISPOR program. Guide RNAs (sgRNAs) were meticulously chosen to reduce off-target effects, yielding two mutant constructs: sgRNA1, which targets the first exon to damage the whole protein-coding region, and sgRNA2, which introduces mutations upstream of the catalytically active TFIIS-type Zn-finger domain.

Immature barley embryos were transformed using *Agrobacterium tumefaciens* strain AGL1 to produce mutant plants. Transgenic plants were produced from the infected calli. Genomic DNA was extracted from each regenerated plant using extraction and dilution buffers (Sigma-Aldrich, E7526 and D5688). The target areas were amplified using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, F537S) from both wild-type and transgenic strains. Mutations at the target loci were identified by T7 endonuclease I digestion (NEB M0302). Homozygous mutant lines were chosen and cultivated in greenhouse conditions.

3.3 Germination assessment

Fully developed barley seeds from the wild-type and *tfIIs-cr1*, *tfIIs-cr2*, and *tfIIs-cr3* mutant lines were first immersed in a gibberellic acid solution (200 mg L^{-1}; G0907, www.duchefa-biochemie.com) and then incubated in darkness for 24 hours. After incubation, the seeds were sterilised using bleach. In each duplicate, 30 seeds were positioned on filter paper inside 9 cm Petri dishes containing 6 mL of sterile distilled water. The dishes were then placed in a temperature-controlled growth chamber (Sanyo MLR-350) maintained at 20 °C under long-day conditions (16 hours of light and 8 hours of darkness) with cold white lighting. Germination was observed daily, and seeds exhibiting visible radicle emergence were documented as germinated.

3.4 Embryo rescue

Mature barley seeds from both the wild-type and *tfIIs-cr1* mutant lines were immersed in water and incubated in darkness for 24 hours. Following incubation, embryos were meticulously extracted from the seeds using forceps and positioned scutellum side up on a callus induction medium. The medium comprised 4.3 g L^{-1} Murashige and Skoog plant salt base (Duchefa, www.duchefa.com), 30 g L^{-1} Maltose, 1.0 g L^{-1} Casein hydrolysate, 350

mg L^{-1} Myo-inositol, 690 mg L^{-1} Proline, 1.0 mg L^{-1} Thiamine HCl, 2.5 mg L^{-1} Dicamba (Sigma-Aldrich, www.sigma-aldrich.com), and 3.5 g L^{-1} Phytagel. Upon the observation of root and shoot growth, the regenerated seedlings were relocated to Jiffy and cultivated under regulated light cabinet settings for further research or heat stress treatment.

3.5 DNA extraction and Sanger sequencing

Total DNA was isolated from 60 mg of barley leaf tissue in 600 ul of extraction buffer µl of extraction buffer (0.1 M glycine–NaOH, pH 9.0, 100 mM NaCl, 10 mM EDTA, 2% SDS) and extracted with equal volumes of phenol–chloroform (pH 8.0) and precipitated with ethanol and resuspended in sterile water. The PCR products were sequenced using the Sanger chain-termination method. Sanger sequencing reactions were prepared using the Sequencing manufacturer's protocol (Starseq, www.starseq.com) and the sample was sent for sequencing. Sequence chromatograms were evaluated using MacVector software.

3.6 RNA extraction and qRT-PCR

Total RNA was isolated from 30–60 mg of barley leaf tissue in 600 µl of extraction buffer (0.1 M glycine–NaOH, pH 9.0, 100 mM NaCl, 10 mM EDTA, 2% SDS) and extracted with equal volumes of phenol–chloroform (pH 4.3) and chloroform, precipitated with ethanol and resuspended in sterile water. For qRT-PCR analysis, 2.5 µg of extracted RNA was subjected to DNase I treatment according to the manufacturer's protocol (NEB, M0303, www.neb.com) to eliminate any contaminating genomic DNA. The RNA was then precipitated using ethanol and reconstituted in sterile water. First-strand cDNA synthesis was conducted using 1 µg of DNase-treated RNA and random primers in accordance with the manufacturer's guidelines (NEB, E6300, www.neb.com). Quantitative PCR reactions were conducted using qPCR Master Mix (NEB, M3003, www.neb.com) on a LightCycler 96 real-time PCR system (Roche). Each experiment was performed with a minimum of three biological replicates, and the results are shown with standard error bars. The statistical significance of differences between samples was evaluated using an unpaired two-tailed Student's t-test.

3.7 Western blotting analysis

The middle section of the second leaves of barley plants was homogenised in a 2× SDS loading buffer solution (100 mM Tris HCl, pH 6.8; 20% glycerol; 2% SDS; 2 mM DTT; 0.05% BPB), subjected to boiling for 5 minutes, and then centrifuged at 14,000× g for 10 minutes at 4°C. The supernatants were subjected to fractionation using 10% SDS gels, followed by the execution of immunoblots. The principal antibodies used were HSP70 (AS08 371), HSP90 (AS08 346), HSP101 (AS07 253), and sHSP-CI (AS07 254), sourced from Agrisera. HRP-conjugated anti-rabbit IgG (A6154: Sigma-Aldrich) was used as the secondary antibody. The detection reagent used was the Clarity ECL solution (Bio-Rad; www.bio-rad.com), and the pictures were quantified using Image Lab 5.1 (Bio-Rad). Rubisco large subunit (RbcL) stain-free signals were used as a loading control.

3.8 Purification of protein aggregates

Protein extracts were prepared by homogenising 0.1 g of fresh plant leaf material in 2.4 ml of isolation buffer [25 mM HEPES, pH 7.5, 200 mM NaCl, 0.5 mM Na2EDTA, 0.1% (v/v) Triton X-100, 5 mM ε-amino-N-caproic acid, 1 mM benzamidine] utilising a mortar and pestle followed by a Cole-Parmer PTFE glass tissue grinder. Two millilitres of total extract were subjected to centrifugation at 16,000×g for 15 minutes at 4°C to separate the soluble and insoluble fractions. The soluble fraction was denatured by the addition of 0.5 volume of 2 × SDS-PAGE buffer and subjected to heating for 5 minutes at 95°C. The insoluble pellet was washed six times by resuspension in the isolation buffer containing 0.1 g of quartz sand (Sigma-Aldrich) and vortexing. The insoluble pellet was resuspended in 400 ml of 2 × SDS-PAGE sample buffer and cleared via centrifugation at 1500×g for 1 minute (insoluble fraction). Samples were separated using SDS-PAGE and then stained with Coomassie Blue, followed by a silver staining technique. The whole lanes of insoluble fractions were measured using Image Lab 5.1 (Bio-Rad), and ratios were calculated based on the Rubisco large subunit (RbcL) and/or stain-free signals.

3.9 Mass spectrometry and data analysis

The protein isolation and mass spectrometry analysis of wild-type and TFIIS-mutant protein samples were conducted concurrently throughout all experimental methods to

provide directly comparable findings. Gel aided sample preparation (GASP) was conducted as previously described and evaluated in a single run on the mass spectrometer. The digestion mixtures were acidified and subsequently transferred to a single-use trapping mini-column (Evotip; 1/8 of the samples) before being analysed using a data-dependent LC–MS/MS method with an Evosep One (LC: 15 SPD; MS1: R:120,000) online coupled with a linear ion trap-Orbitrap (Orbitrap-Fusion Lumos, Thermo Fisher Scientific) mass spectrometer operating in positive ion mode.

Data collection was conducted in a data-dependent manner, with multiply charged ions picked throughout the cycle period from each MS survey scan for ion-trap HCD fragmentation; MS spectra were obtained in the Orbitrap (R=60,000), while MSMS was performed in the ion-trap. Raw data were analysed utilising Proteome Discoverer (v 3.0) and queried against the Uniprot *Hordeum vulgare* database (as of 2024.06.20, comprising 40,549 proteins) employing the Bionyc search engine (v5.15.1) for ms1-based quantification and the Protein Prospector search engine (v5.15.1) for TFIIS detection with the specified parameters: Enzyme: trypsin with a maximum of two missed cleavage sites; mass accuracies: 5 ppm for precursor ions and 10 ppm for fragment ions (both monoisotopic); fixed modification: carbamido-methylation of cysteine residues; variable modifications: acetylation of protein N-termini; methionine oxidation; cyclisation of N-terminal glutamine residues. Acceptance criteria: minimum scores of 22 and 15; maximum E values of 0.01 and 0.05 for protein and peptide identifications, respectively.

3.10 Bioinformatics tools and analyses

To examine the sequence conservation of TFIIS homologues, sequences were discovered and retrieved from the Ensembl Plants and UniProt databases. Protein alignments were conducted using Jalview software www.Jalview.org. The forecast and depiction of the 3D structure of TFIIS domain II were obtained from and produced by AlphaFold DB.

3.11 Gene ontology study

Gene Ontology (GO) enrichment analysis was performed to functionally categorise the proteins identified by LC–MS/MS in soluble and insoluble fractions. Lists of significantly

detected proteins were first filtered to remove contaminants and low-confidence identifications. Barley protein identifiers were converted to their corresponding gene IDs using the Ensembl Plants database. GO enrichment analysis was conducted using the agriGO v2.0 toolkit. Overrepresentation analysis was performed separately for Biological Process (BP), Molecular Function (MF), and Cellular Component (CC) categories. The background reference set consisted of the annotated barley (*Hordeum vulgare* L.) genome. Statistical significance of GO term enrichment was calculated using Fisher's exact test, followed by false discovery rate (FDR) correction according to the Benjamini–Hochberg method. GO terms with FDR-adjusted $P < 0.05$ were considered significantly enriched. Enriched GO categories were visualised using REVIGO to remove redundant terms and summarise functionally related categories. The analysis allowed identification of biological pathways and stress-related processes overrepresented under heat stress conditions.

4. RESULTS

4.1 Conservation of TFIIS in monocots

In order to analyse the evolutionary conservation and possible functional importance of the TFIIS protein in cereals, a thorough sequence conservation analysis was carried out. A search for protein sequences for TFIIS homologs was made for the major monocot crops, including barley, maize, wheat, rice and *Brachypodium* and selected gymnosperm, early dicotyledonous, and eudicot species for the purpose of interspecific comparison

The α-helices of domain I and II, and the β-sheets of domain II were predicted in all TFIIS homologs. Apart from some scattered amino acid differences in some lineages, we observed a monocot-specific amino acid stretch inserted in domain II between α6- and α7 helices; these additional α-helices were named α6-b and α6-c, to preserve the nomenclature of the other. Based on the three-dimensional structure prediction, the α6-b and α6-c helices, consisting mainly of charged amino acids, are located on the surface of the protein. Importantly, the catalytically essential cysteine residues forming the zinc-finger domain and the catalytically active DE di-amino acid are ubiquitously present, suggesting that the homolog genes likely encode functional TFIIS proteins.

These results collectively demonstrate that TFIIS is evolutionarily highly conserved across land plants, with minor lineage-specific divergence.

4.2 Establishment of independent TFIIS mutant barley lines

To investigate the functional role of the TFIIS protein in developmental processes and stress responses in monocot crops, we generated independent mutant lines of barley with disrupted TFIIS function using CRISPR-based mutagenesis. For targeted gene editing, two different single-guide RNA (sgRNA) sequences were designed. The first, sgRNA1, was aimed at the first exon of the TFIIS gene, while the second, sgRNA2, targeted a region located just upstream of the catalytically essential zinc finger domain.

The sgRNA1-induced mutations led to frameshifts early in the gene, which likely completely abolished TFIIS protein production, thus generating a knock-out (KO) mutant.

The sgRNA1-derived line, harbouring a +T insertion, was named *tfIIs-cr1*. Seeds produced from this lines were germination incompetent; therefore, this line could be propagated only through embryo rescue method. Besides these, we have tested several additional sgRNA1-mutated plants; no other homozygous viable mutant could be identified.

In contrast, the sgRNA2-induced mutations resulted in frameshifts near the 3′ end of the gene, upstream to the zinc-finger catalytic motif domain these lines are predicted to produce truncated TFIIS proteins that retain the N-terminal and central domains but lack the entire zinc finger region of domain III critical for the protein's catalytic activity. These mutants, therefore, may produce non-functional TFIIS variants. Importantly, both sgRNA2-derived mutant lines were fertile and produced viable seeds: these were named *tfIIs-cr2* and *tfIIs-cr3*.

To confirm TFIIS protein absence, we analysed the tfIIs-cr lines through mass spectroscopy, in comparison with wild-type samples. The mass spectroscopy analysis confirmed that CRISPR mutagenesis was efficient; our barley plants likely are unable to produce functional TFIIS.

4.3 Barley requires TFIIS for proper reproductive development

We examined the growth of *tfIIs-cr* mutants under standard conditions. The *tfIIs-cr1* plants exhibited mildly reduced growth relative to the wild type. The development of *tfIIs-cr2* and *tfIIs-cr3* plants closely resembled that of wild-type plants in all facets of vegetative growth, including leaf morphology and overall plant stature.

All *tfIIs-cr* mutants exhibited impaired reproductive development. The *tfIIs-cr1* seeds exhibited minor elongation and failed to germinate, despite being pre-treated in darkness with gibberellic acid to enhance root and shoot development. The germination-incompetent phenotype was consistently seen across several plants over successive cultivation periods.

The reproductive development in the *tfIIs-cr2* and *tfIIs-cr3* plants was likewise impacted, but to a lesser degree: the seeds from both lines exhibited a significantly delayed germination rate. In addition to the diminished germination capacity, the *tfIIs-cr1, -cr2,* and *-cr3* lines exhibited thousand grain weights (TGW) decreased to 75%, 68%, and 77%,

respectively, in comparison to the wild type. The combined findings indicate that TFIIS is essential under ambient circumstances for the reproductive fitness of barley plants in terms of both quality (impaired seed germination) and quantity (decreased TGW).

4.4 Barley *tfIIs-cr* mutants are sensitive to heat stress

To test if TFIIS activity is needed for HS tolerance in barley, we subjected wild-type control and the *tfIIs-cr* mutant lines to heat stress; plants were pre-grown at 20 °C for 30 days in soil. Each pot contained a control (wild-type) plant and a mutant plant planted side-by-side. The plants were then heat-treated at 40°C for a 1-to-3-day time series. All the *tfIIs-cr* lines were heat-sensitive, exhibiting modest symptoms after 1 day and a strong retardation or lethality after 2 days of treatment.

4.5 The TFIIS is an essential component of an effective HSR

To uncover the molecular basis of TFIIS actions in barley, we examined the molecular changes of marker HS-transcripts. The heat shock protein (HSP) transcript levels were analysed over a time series in order to gain insight into the early and late (persistent) HSR. Accumulation of *HSP70-4, HSP90* and *HSP101* mRNA transcripts during the early phase of heat stress (1-2 hrs at 40°C) was significantly lower in the *tfIIs-cr* mutants in comparison with wild-type plants. Under persistent heat stress (1 day at 40°C), wt plants showed reduced HSP expression, whereas transcript levels remained elevated in the *tfIIs-cr* mutants.

We propose that rapid transcriptional activation of HSPs is essential during the initial stage of heat stress, as observed at TMHT/1-2h. In the absence of TFIIS, elongation efficiency is compromised, resulting in a delayed accumulation of HSPs. This deficit in the early heat period likely leads to a higher concentration of denatured/unfolded proteins. The unresolved proteins later may trigger a positive transcriptional feedback loop (HSFs' activity is upheld due to a shortage of buffering HSPs), ultimately driving the sustained, elevated expression of HSPs observed during the later stages of heat stress (TMHT/1d).

To examine whether transcriptional dynamics of HSPs could be observed at the protein level, we analysed NT and TMHT-treated barley protein changes in wild-type and *tfIIs-cr* mutants. Wild-type plants exhibited high accumulation of HSP70, HSP90, and HSP101 proteins after early heat stress, whereas *tfIIs-cr* mutants accumulated markedly reduced levels of these chaperones. In later stages of heat stress, the differences between wild-type and mutants were diminished, indicating that *tfIIs-cr* mutants can offset the initial shortfall in HSP accumulation, likely by prolonging the length of mRNA transcription and/or protein translation. Collectively, our findings indicate that TFIIS is essential for the prompt activation and appropriate regulation of the heat stress response in barley.

4.6 Impaired HSP synthesis in the absence of TFIIS induces proteotoxicity

To evaluate the downstream effects of early insufficient accumulation of HSP chaperones, we examined the levels of protein aggregates. We separated the soluble and insoluble protein fractions of the protein extracts and calculated the insoluble/soluble protein ratios in untreated and 1-day heat-treated wild-type and *tfIIs-cr2* plants. A markedly elevated accumulation of insoluble proteins was seen in the *tfIIs-cr2* plants under heat stress, indicating a proteotoxic state. sHSP-CI holdases were present in both the soluble and insoluble fractions, but only in *tfII-cr2*. The accumulation of sHSP-CI holdases in the insoluble fraction indicates that the proteostatic ability to maintain unfolded proteins in a soluble state was likely surpassed. Presence of sHSP-CI in *tfIIs-cr2* line is indicative of an elevated proteotoxicity.

To elucidate the effect of TFIIS deficiency on plant proteotoxicity at the whole proteome level, we conducted mass spectrometry on soluble and insoluble protein fractions from wild-type and *tfIIs-cr2* plants. Similar quantities of proteins were identified in both lines and treatments (soluble + insoluble proteins) Upon analysing the qualitative and quantitative disparities between wt and *tfIIs-cr2*, we observed an elevated number of proteins having higher abundances in the insoluble fraction of the *tfIIs-cr2* line. Gene ontology (GO) analysis of these proteins showed enrichment in RNA/protein metabolism pathways, primarily localised to cytoplasm, organelles, and stress granules.

4.7 TFIIS is induced by heat and autoregulates its own locus

To decipher TFIIS regulation during HSR, we analysed its mRNA changing dynamics during our time series treatment: a significant accumulation of TFIIS mRNA was observed in all timepoints, including 1h, 2h and 1d TMHT treatments, with a peak at 2h. Since barley TFIIS locus contains two canonical HSE cis elements, we postulate that transcriptional regulation of the locus, at least partially, occurs at the transcriptional initiation level, through the action of HSFs.

We have also analysed changes of TFIIS transcripts in *tfIIs-cr* mutants. In the *tfIIs-cr1,* the *TFIIS* mRNA remains at a basal level throughout all treatments notably, in this line, the basal level (at NT situation) is already lower compared to wild-type plants. In the *tfIIs-cr1* line, the single nucleotide insertion (+T) occurs at the beginning of the gene/transcript, leading to a transcript with PTC that generates an extremely long 3'UTR, estimated to be 1794 bp. As the long 3'UTR (above ~500 bp) is a strong inducer of NMD, likely the *tfIIs-cr1* transcript is very efficiently targeted for decay.

In the *tfIIs-cr2* and *tfIIs-cr3* lines, the TFIIS mRNA level at ambient conditions is similar to wild type, but upon exposure to early heat, it cannot be induced, implying that its accumulation requires the activity of the TFIIS protein itself, either directly or indirectly. During the latter time point (at 1d), accumulation of the TFIIS transcript is not suppressed (or attenuated). The late accumulation of TFIIS mRNA could be caused by a positive feedback regulation, in an attempt to replenish the missing activity of TFIIS needed for general transcriptional elongation.

These observations are in unison with earlier observations made in *Arabidopsis*, and further confirm that the TFIIS locus is regulated at both transcriptional initiation and elongation levels for quantitative production of the TFIIS protein needed for an efficient heat stress response. In summary, these data confirm an elevated proteotoxic status of *tfIIs-cr2* compared to wt plants in response to heat exposure.

Based on these findings, we propose a working model of TFIIS function in barley (Fig. 1).

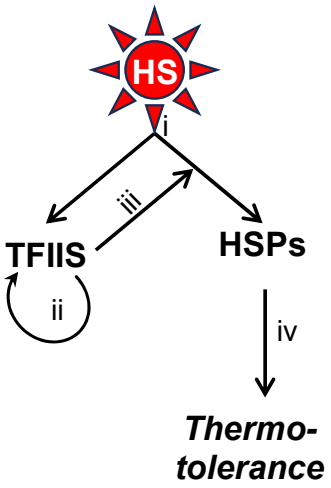

Figure 1: Working model for TFIIS action during heat stress response in barley: (i) Elevated temperature induces expression of HSPs and TFIIS; (ii) TFIIS elongation factor positively feed-back regulates itself and (iii) further enhances expression of HSP transcription; (iv) the timely accumulation of HS-transcripts and proteins ensures efficient thermotolerance in barley plants.

Firstly, elevated temperature activates heat shock factors (HSFs) that orchestrate transcription of heat shock-responsive genes, including those encoding HSPs and TFIIS. Secondly, elevated TFIIS protein levels enhance transcription elongation of these heat shock-responsive genes. Finally, these heat activated molecular players maintain proteostasis essential for efficient thermotolerance.

4.8 Expression of GW2.1, a grain quality regulator, is HS-dependent

Barley is highly sensitive to elevated temperatures, especially around flowering and grain filling, when short episodes of heat can strongly reduce spike fertility and final yield. GW2.1 is a ubiquitin ligase having roles in barley grain maturation during the filling period. GW2.1 may be HS-regulated and may have a role in integrating exogenous temperature signals to coordinate barley grain protein contents and maturation. Within a collaborative study, we investigated how heat stress influences GW2.1 expression and can potentially affect barley productivity. During this analysis, we studied the wild-type plant alongside the CRISPR-edited *gw2.1* barley mutant. Four different *gw2.1* mutants were created using two different sgRNA guides, and homozygous T2 generation was used for the study.

For this examination, wild-type and *gw2.1* mutant plant samples were collected at ambient (non-treated, NT), at 1 hour HS for early HSR and at 1d for late (persistent) HSR response. First, we analysed how *GW2.1* mRNA accumulation is altered in *CRISPR* mutants. Mutant lines expressed significantly lower levels of *GW2.1* mRNA, approximately 50% compared to WT. Likely frameshift mutations in the GW2.1 ORF in mutant lines generated extended 3′ UTR regions, which triggered destabilisation of transcripts during translation through NMD.

To scrutinise the HS responsiveness of the GW2.1 gene, we examined samples and observed a mild but significant decline in GW2.1 transcript levels after 1h of heat stress and a strong downregulation upon persistent stress (1d). To mimic mild heat stress that barley typically experiences under field conditions at late vegetative and reproductive stages, we also treated 30-day-old (late vegetative stage) and 75-day-old plants (being at the early spike developmental stage) to 30 °C for one day: a modest, non-significant reduction of *GW2.1* expression in leaves, but a significant downregulation in developing green spikes compared to control plants maintained at 18 °C. Collectively, these results demonstrate that *GW2.1* expression is highly sensitive to heat stress.

To evaluate whether *GW2.1* activity is involved in the HS tolerance pathway, we analysed selected HSP production as a molecular readout of HS response. We quantified the accumulation of HSP70 and HSP90 proteins in both WT and mutant plants. Both proteins accumulated to similar levels following early (1hour) or late (1day) heat treatment in both wild-type and *gw2.1* mutant lines.

Finally, we conducted an HS-sensitivity assay by comparing wild-type and *gw2.1* mutant survivals: no significant differences in thermotolerance between WT and mutant seedlings could be found. These observations indicate that although GW2 transcription is HS-responsive, GW2.1 does not directly influence the canonical HSR pathway or barley's HS tolerance.

5. DISCUSSION

Cereal crops have been cultivated worldwide since their grains serve as the basis for animal feed and human nutrition. Deciphering the molecular mechanisms that coordinate development and stress response pathways in crops is therefore of great importance. Although transcriptional regulation is vital for delivering genetic information to operate cellular machinery, knowledge of this process in monocot species is very limited. This gap in our knowledge of monocot systems is becoming increasingly problematic in light of global warming and climate change, especially for agricultural crop breeding programs on barley, wheat, maize, and rice.

Elongation factors play essential roles in transcriptional regulation, as well documented in dicots and in non-plant organisms such as yeast or mammals. However, the research on transcriptional elongation control in monocots is scarce, with only a few studies done. It was shown that SPT4 and SPT5 elongation factor paralogs are needed for proper development and reproduction in *Oryza sativa* and *Arabidopsis thaliana*. The hypomorph *Osspt5-1* mutant rice has a semi-dwarf stature and a shortened lifecycle, while strong mutant *Osspt4* or *Osspt5-1* homozygous rice progenies could not be obtained, while heterozygous plants were impaired in their grain development. These observations evidence the vital roles of transcriptional elongation control in rice.

The transcription elongation co-factor TFIIS emerges as an important candidate which mediates stress resilience in *Arabidopsis*. TFIIS is known to assist RNAPII reactivation after transcriptional arrest and is critical for rapid transcriptional reprogramming during heat stress TFIIS's role in monocots' transcriptional regulation remained largely unexplored so far. To understand the transcriptional elongation regulation in monocot crop species, we studied the barley TFIIS. Independent CRISPR lines were obtained using two different sgRNA guides, one sgRNA targeting the gene locus within the first exon, aiming to create knock-out mutants, and the other sgRNA, which targeted the locus just upstream of the catalytically active site, therefore creating a non-functional TFIIS mutation. From these groups of transformants, we have selected independent lines to exclude phenotypic alterations caused by positional effects, insertional mutations (the Crispr-Cas9 construct

insertion itself may create mutations), and off-target editing events. Finally, 3 independent lines were further pursued in the study.

We detected a mild reduction in the vegetative growth of *tfIIs-cr1*, but no developmental alterations in the *tfIIs-cr2* and *-cr3* lines. In *Arabidopsis,* the *tfIIs* mutant plants display essentially normal phenotype. The absence of alterations of vegetative development in *tfIIs-cr* lines suggests that (i) TFIIS is less needed during the vegetative growth phase, or (ii) TFIIS absence may be masked by alternative pathways that are functioning efficiently under ambient conditions.

The absence of TFIIS, however, caused reproductive fitness costs, in terms of both qualitative and quantitative traits of the barley grains: (i) homozygous mutants originating from sgRNA1 mutagenesis were either sterile (therefore could not be further studied) or (ii) germination incompetent (the *tfIIs-cr1*), (iii) mutants originating from sgRNA2 mutagenesis had a significantly decreased germination rate (the *tfIIs-cr2* and *tfIIs-cr3*), and (iv) all *tfIIs-cr* mutants had a significantly reduced TGW value of about 70-75% versus wild-type plants. These findings are in accordance with findings in rice. Whether the seed/grain developmental fault in *tfIIs-cr* lines is due to a general loss of plant fitness or due to the impairment of TFIIS-mediated direct regulation is unknown. Furthermore, germination was impacted at different levels, with *tfIIs-cr1* lines unable to, while *tfIIs-cr2* and *-cr3* were able to germinate, although at a reduced rate compared to wild type. It is possible that the non-functional truncated version of TFIIS (in *tfIIs-cr2* and *-cr3* lines) retains some activities needed during grain development (e.g., protein interaction, allosteric impact on RNAPII upon binding, or other unknown functions) that are not disrupted in *tfIIs-cr2* and *-cr3* (even though the TFIIS protein level is likely very low!), which could fulfil roles during grain maturation.

Transcriptional reprogramming is essential for temperature adaptation in *A. thaliana*. A central player in this process is the TFIIS elongation. Here, we show that TFIIS activity becomes vital under HS in barley. Exposure to high temperatures (40 °C) discloses a striking disparity between wild-type and *tfIIs-cr* mutant plants. Wild-type plants' higher survival rate is supported molecularly by the quick induction of HS-transcripts and production of defensive HSPs, enabling them to maintain protein homeostasis. In contrast,

the *tfIIs* mutants showed delayed and impaired induction of HS-responsive genes, which ultimately leads to proteotoxicity. Mass spectrometry and GO term analysis (Table 1) showing increased accumulation of insoluble and stress-associated proteins in the *tfIIs* mutants support this working model. We also show that TFIIS is induced by HS and is likely autoregulated. Furthermore, the molecular events of HSR and the HS-tolerant phenotype of both mutant groups (the knock-out *tfIIs-cr1* and the antiarrest mutants *tfIIs-cr2* and *tfIIs-cr3*) tested are consistent, evidencing that the antiarrest activity of TFIIS is a must for efficient HSR.

We analysed protein sequence conservation of TFIIS homologs in monocot species by comparing them with gymnosperms, early dicots, and eudicots. A strong conservation of the TFIIS protein sequence and catalytic motifs, including zinc-finger cysteine residues and DE catalytic di-amino acids, was found, hinting at TFIIS antiarrest functionality. A sequence expansion was predicted *in silico* to form additional α-helix structures (between α6 and α7) in monocot species, including barley (coined as α6-b, α6-c). α6-b/α6-c together with α7 helices form a charged surface that may be a platform for protein binding through charge-charge interactions. This remains to be analysed by further work.

Remarkably, *Arabidopsis* and barley are considerably diverged, with a common ancestor estimated to have existed about 140 million years ago. Although further work could uncover commonalities or peculiarities of the pathway within monocot and dicot species, work done in barley and previous work in Arabidopsis provide strong evidence of TFIIS's adaptive role during HSR throughout the entire plant kingdom. This knowledge, combined with the extensive genomic information available, will aid directional selection and breeding programmes to increase productivity and resilience of barley and other important crop species.

In a collaborative work, we have studied and shown that the GW2.1 ubiquitin ligase regulates the trade-off between barley grain yield and qualitative traits. Absence of GW2.1 increased protein content of barley grains without affecting starch or polysaccharide contents. We also found that GW2.1 mRNA is dramatically downregulated during heat stress. This raises the hypothesis that GW2.1 absence may be favourable under adverse/high temperature conditions; notably, in the absence of GW2.1 (like HS-treated plants or *gw2.1* mutant plants), seed set and spike development are decreased, which leads

to fewer but greater grains. Therefore, GW2.1 downregulation could be an adaptive life strategy to maximise seed and seedling viability in the next generation. In sum, our observation puts forward the hypothesis that GW2.1 likely optimises the transgenerational fitness of barley.

Although there is no evidence of a direct connection between the TFIIS and GW2 molecular pathways, these two studies Highlight the complex, intricate regulation of seed development, germination, and grain filling during adaptation to high-temperature conditions.

6. CONCLUSIONS AND RECOMMENDATIONS

In conclusion, the present study provides insights into the transcription-associated regulator TFIIS and protein stability factor GW2.1, both having an indirect effect on barley grain production. First, we demonstrate that TFIIS is essential for reproductive development and qualitative production of germination-competent barley grains; on the other hand, TFIIS is also needed for efficient adaptation of barley plants to elevated temperatures: the absence of TFIIS compromises HS tolerance by impairing the efficient transcriptional elongation of heat-responsive genes, affecting downstream HS-transcript generation that indirectly impacts proteostasis and ultimately plant viability.

Secondly, the loss of GW2.1 increases barley grain protein content without affecting starch or polysaccharide content; however, GW2.1 does not directly regulate canonical heat shock protein induction.

Future research should address the following priorities:

a) The identity of transcripts directly regulated by TFIIS during transcriptional elongation in grain maturation under ambient conditions remains unknown. Targeted ChIP-seq or PlaNET-seq studies could identify TFIIS-dependent elongation hotspots in developing seeds.

b) Transcriptome analyses show a general impact of TFIIS on differentially expressed genes during heat stress response (HSR); however, the mechanism of action remains elusive. Functional mapping of transcriptional regulatory mechanisms associated with distinct structural domains of TFIIS through comparative analysis of the different mutant classes will be an important path to follow.

c) The monocot-specific α6-b/6-c insertion between α6 and α7 helices of TFIIS domain II forms a charged surface potentially serving as an RNAPII-TFIIS protein interaction platform via charge-charge interactions. Future co-immunoprecipitation and structural studies are needed to identify potential alterations between monocot RNAPII-TFIIS binding or so far unknown other binding partners and functional roles involved in TFIIS binding.

7. NEW SCIENTIFIC RESULTS

1. We have identified and characterised a TFIIS homolog in barley (*Hordeum vulgare* L.); we have shown that it shares conserved sequence features with TFIIS proteins from other plant species, while it also displays a monocot-specific characteristic domain II, with additional helices likely involved in RNAPII interactions.

2. Using CRISPR-generated independent mutant *tfIIs* lines, we have shown that although TFIIS is not required for vegetative development, it is needed for proper seed/grain development in barley (*Hordeum vulgare* L.) at ambient temperature conditions.

3. We show that TFIIS is heat-induced and likely autoregulated during HSR.

4. We have shown that TFIIS is required for the timely and efficient accumulation of heat stress–responsive transcripts and proteins, which are central components of heat stress adaptation pathways.

5. In a collaborative work, we have shown that GW2.1 ubiquitin ligase is responsive to high temperatures (repressed during both early and late HSR), but it does not directly regulate HSP production nor affect the HS survival of barley plants.

8. APPENDICES

8.1 List of scientific activities

8.1.1 Conference Presentations and Posters:

i. **Imtiaz Ahmad**, Verma R, István Szadeczky-K, Henrik M Szaker, Hussam S Abbas, András Kis, Aladár Pettkó-Sz, Dániel Silhavy, Zoltán Havelda, Tibor Csorba. Decoding the role of TFIIS in plant resilience to heat stress, GBI Napok, 10-11 December 2024, Gödöllő, Hungary. (1st Prize for oral talk)

ii. **Imtiaz Ahmad**, Verma R, István Szadeczky-K, Henrik M Szaker, Hussam S Abbas, András Kis, Aladár Pettkó-Sz, Dániel Silhavy, Zoltán Havelda, Tibor Csorba, Decoding The roles of transcriptional elongation factors, GBI Napok, 10-11 November 2025, Gödöllő, Hungary

iii. **Imtiaz Ahmad**, Hussam S Abbas, Verma R, István Szádeczky-K, Henrik M Szaker, Tibor Csorba. Ensuring Transcriptome Integrity: Transcriptional Strategies for Coping with Heat Stress in Plants, (Poster) International Conference: Molecular Biology – Current Aspects and Prospects (ICMB2025) Cluj-Napoca, Romania, November 4–7, 2025.

iv. **Imtiaz Ahmad**, Verma R, István Szadeczky-K, Henrik M Szaker, András Kis, Aladár Pettkó-Sz, Dániel Silhavy, Zoltán Havelda, Tibor Csorba. Regulation of Transcriptional Machinery for Heat Stress Adaptation in Monocot Crops, at the 22nd Horizons in Molecular Biology Symposium, 8th -11th September 2025, at the Max Planck Institute for Multidisciplinary Sciences, Göttingen, Germany

v. András Kis, Dávid Polgári, Auwalu Abdu, Ágnes Dalmadi**,** **Imtiaz Ahmad**, Marianna Rakszegi, László Sági, Tibor Csorba, Zoltán Havelda, Utilisation of targeted mutations to alter economically important traits in barley, Plant Biology Europe conference 2025, Budapest, Hungary

vi. **Imtiaz Ahmad**, Verma R, István Szadeczky-K, Henrik M Szaker, Hussam S Abbas, András Kis, Aladár Pettkó-Sz, Dániel Silhavy, Zoltán Havelda, Tibor Csorba, Safeguarding Plant Gene Expression Under Heat Stress: Molecular Mechanisms Supporting Resilience in a Changing Climate, 14th Student Research Conference

Society. Technology. Solutions at Vidzeme University of Applied Sciences, Valmiera, Latvia, on 15 October 2025 (Best poster prize)

vii. **Imtiaz Ahmad**, Verma R, István Szadeczky-K, Henrik M Szaker, Hussam S Abbas, András Kis, Aladár Pettkó-Sz, Dániel Silhavy, Zoltán Havelda, Tibor Csorba, Heat Stress and Transcriptome Stability: Transcriptional Defence Mechanisms in Plants, The 1st International Online Conference on Biology session Plant Biology, 2026.

8.1.2 Publications

1. **Imtiaz Ahmad**, András Kis, Radhika Verma, István Szádeczky-Kardoss, Henrik Mihály Szaker, Aladár Pettkó-Szandtner, Dániel Silhavy, Zoltán Havelda, Tibor Csorba, TFIIS is required for reproductive development and thermal adaptation in barley. Plant Cell Rep 43, 260 (2024).

2. András Kis, Dávid Polgári, Ágnes Dalmadi, **Imtiaz Ahmad**, Marianna Rakszegi, László Sági, Tibor Csorba, Zoltán Havelda, Targeted mutations in the GW2.1 gene modulate grain traits and induce yield loss in barley, Plant Science, Volume 340, 2024, 111968, ISSN 0168-9452