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Understanding the impact of heat stress on crop plants

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1 ABBREVIATIONS

Abbreviation	Definition
AS	Alternative Splicing
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CstF	Cleavage Stimulation Factor
DE	Aspartic Acid–Glutamic Acid Catalytic Motif
GTF	General Transcription Factor
GO	Gene Ontology
GW2	Grain Width And Weight
HSE	Heat Shock Response Element
HSFs	Heat Shock Factors
HSP	Heat Shock Proteins
HSP70	Heat Shock Protein 70
HSP90	Heat Shock Protein 90
HSP101	Heat Shock Protein 101
HSPs	Heat Stress Proteins
HSR	Heat Stress Response
HS	Heat Stress
KO	Knockout
LD	Long-Day Photoperiod
MS	Mass Spectrometry
NMD	Nonsense-Mediated Decay
NT	Non-Treated
ORF	Open Reading Frame
PAS	Polyadenylation Signal
PSII	Photosystem II
PTC	Premature Termination Codon
PVDF	Polyvinylidene Difluoride Membrane
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
QTL	Quantitative Trait Locus
RNAPII	RNA Polymerase II
ROS	Reactive Oxygen Species
RbcL	Ribulose-1,5-Bisphosphate Carboxylase Large Subunit
sHSP	Small Heat Shock Proteins
sgRNA	Single Guide RNA
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TAFs	TBP-Associated Factors
TATA box	TATA-Binding DNA Promoter Element
TBP	TATA-Binding Protein
TFIID	Transcription Factor IID
TFIIS	Transcription Elongation Factor IIS
TFs	Transcription Factors
TGW	Thousand-Grain Weight
TMHT	Thermotolerance To Moderately High Temperature

UPS	Ubiquitin Proteasome System
UTR	Untranslated Region
WT	Wild Type
Zn-finger	Zinc Finger Domain

2 INTRODUCTION

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.

3 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.

4 LITERATURE REVIEW

4.1 Mechanisms and effects of heat stress in agriculture

Plants, as sessile organisms, have developed a complex mechanism by which they cope with the heat stress (HS) to survive and maximise reproductive fitness. The molecular mechanisms that work together to minimise the adverse effects of HS are called the heat stress response (HSR). One main layer of HSR is the transcriptional regulation. This is orchestrated by Heat Shock Factors (HSFs)(Scharf et al., 2012, Charng et al., 2023, Ohama et al., 2017). During the HS, HSFs are activated post-translationally and transcriptionally induce the production of HS transcripts, a ubiquitous process observed in various organisms, including plants. (Scharf et al., 2012, Charng et al., 2023, Ohama et al., 2017). During HS, HSFs are released from the HSP-chaperone complex because HSPs preferentially bind to denatured proteins that accumulate in the cytosol. The released HSFs then form trimers and move into the nucleus, where they bind to specific DNA sequences (nGAAn), called heat shock response elements (HSEs), located in the promoters of heat-responsive genes. The binding activates gene expression at the transcriptional level.(Scharf et al., 2012, Charng et al., 2023, Ohama et al., 2017) (Fig. 1).

HSF protein families are diverse; these were best characterised in *A. thaliana* (Scharf et al., 2012, Charng et al., 2023, Ohama et al., 2017). In *A. thaliana*, there are 21 HSF genes. HSF genes are categorised into 3 classes: A, B, and C, on the basis of structural features and oligomerisation domain with heptad repeats. Among these HsfA classes are activators; HsfA1a acts as the master regulator in tomato. (Mishra et al., 2002); HsfA1a and HsfA1b are required for early induction of HSP (Scharf et al., 2012, Charng et al., 2023, Ohama et al., 2017) whereas HsfA2 and HsfA3 play a role in stress memory (Lamke et al., 2016, Pratx et al., 2024, Pratx et al., 2025). Similarly, barley HS memory rests on the *HvHSEF2* and *HvHSEF3*, which are highly conserved and are needed for the prolonged HS (Lamke et al., 2016, Pratx et al., 2024, Pratx et al., 2025). HsfBs are generally repressors; however, in some cases, these act in complex with HsfAs as activators. The specific roles of HsfCs are unknown. (Scharf et al., 2012, Charng et al., 2023, Ohama et al., 2017). Lily LIHSFC2 coordinates with HSFAs to balance heat stress response and improve thermotolerance (Wu et al., 2024).

The downstream actors of HSR are the HSP proteins. Based on the molecular size, HSPs are classified into HSP60, HSP70, HSP90, HSP101, and small HSPs (Scharf et al., 2012, Charng et al., 2023, Ohama et al., 2017). The HSP chaperone classes cooperate during the HSR. HSP70 is the most

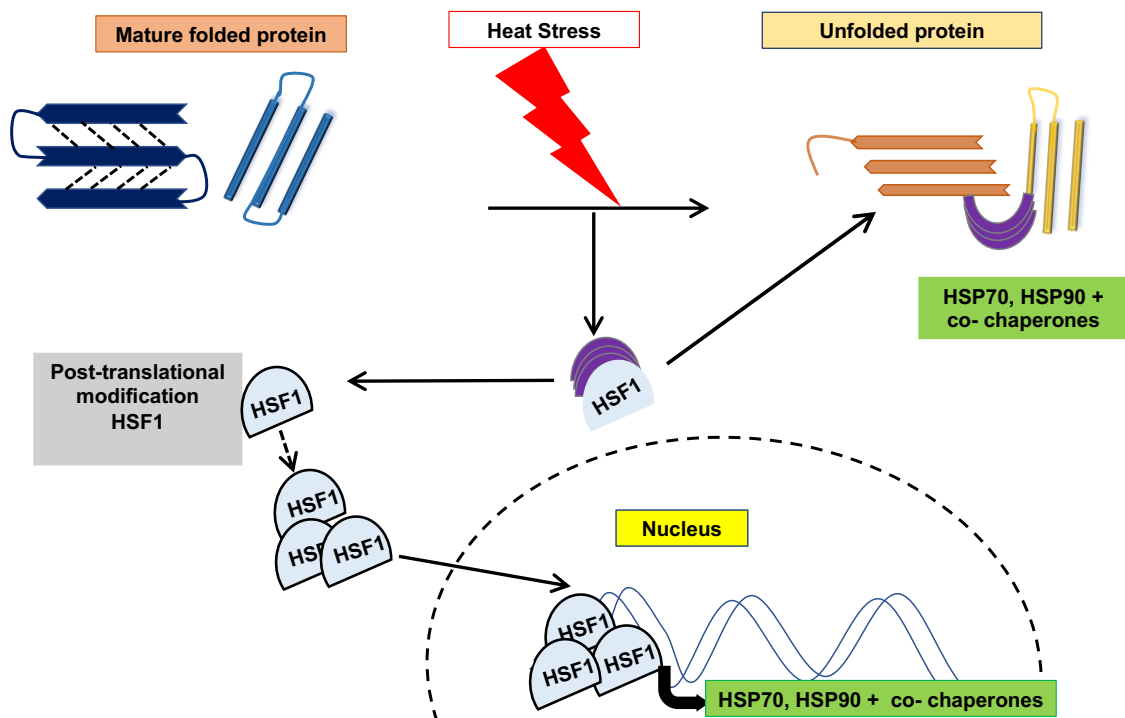
abundant HS protein in eukaryotes. It binds in an ATP-dependent manner to the hydrophobic part of unfolded proteins and prevents protein aggregation (Scharf et al., 2012, Charng et al., 2023, Ohama et al., 2017) and maintains protein homeostasis. It also assists in the folding of nascent polypeptides released from the ribosomes (Hartl and Hayer-Hartl, 2002), protein sorting, etc. HSP 90 chaperone helps in protein sorting, assists in cell signalling and take part in receptor confirmation (Young, 2010, Young et al., 2004). HSP 90 proteins interact with BES1/BZR1 Transcription factors (TFs) and mediate brassinosteroid signalling (Shigeta et al., 2014, Ticha et al., 2020). HSP90 protein interacts with altered forms of proteins, limiting their inactivity under normal conditions and exposing them during stress. Therefore, they establish a mutation buffering system in plants (Murphy et al., 2001, Queitsch et al., 2002, Sangster and Queitsch, 2005). In contrast, HSP100/Clp is a member of the AAA+ ATPase family; proteins regulate the disaggregation and elimination of misfolded proteins (Bosl et al., 2006). The increased expression level of constitutively produced HSP100 genes during HS illustrates their direct involvement in thermotolerance. Whereas sHSP-CI holdsases, acting as ATP-independent chaperones, bind to stress-denatured protein substrate. sHSP-bonded substrates are protected against permanent denaturation and can be processed by ATP-dependent chaperones, specifically HSP70 and co-chaperones, in conjunction with the protein dissociation enzyme HSP100/ClpB, for refolding. (Waters and Vierling, 2020).

Chaperones cooperate and assist in protein folding to be able to have an active functional protein conformation and protein stabilisation. (Bosl et al., 2006). These chaperones help proteins fold, and help them move across membranes. They also protect cells from damage caused by stressful situations. The term "protein quality control" is often used to describe the coordinated interaction between chaperones and proteases.

Expression profiling in barley demonstrated a significant and synchronised activation of various heat shock protein families in response to increased temperatures. Several HSP70 genes, such as *HvHspc70-4*, *HvHsp70Mt70-2*, *HvHspc70-5a*, *HvHspc70-5b*, *HvHspc70-N1*, and *HvHspc70-N2*, exhibited significant upregulation (Chaudhary et al., 2019, Larkindale and Knight, 2002) immediately after both early and prolonged HS treatments, as well as throughout reproductive development under elevated temperature conditions. The continuous expression of these genes signifies that HSP70 proteins are vital in both basal and acquired thermotolerance by stabilising freshly produced peptides, facilitating protein refolding, and preventing irreversible protein aggregation during thermal stress. In addition, HSP90 proteins often collaborate with HSP70. HSP90 is a pivotal regulator of heat-

responsive signalling cascades in barley. Moreover, members of the HSP100 family, *HvHsp100-1* and *HvHsp100-2*, *HvHsp100-3*, and *HvHsp100-4* have shown significantly elevated transcripts after HS in both vegetative tissues and developing seeds. (Chaudhary et al., 2019, Sadura et al., 2020). The expression of these HSP transcripts is highly regulated in a coordinated manner to confer thermotolerance in barley, enabling the plant to withstand extreme heat stress (Mishra et al., 2024). Overexpression of *HvHSFA2* improves the heat tolerance in barley without detectable yield penalties, highlighting HS memory as a promising agricultural strategy for developing HS-resilient crops (Pratx et al., 2025).

A



B

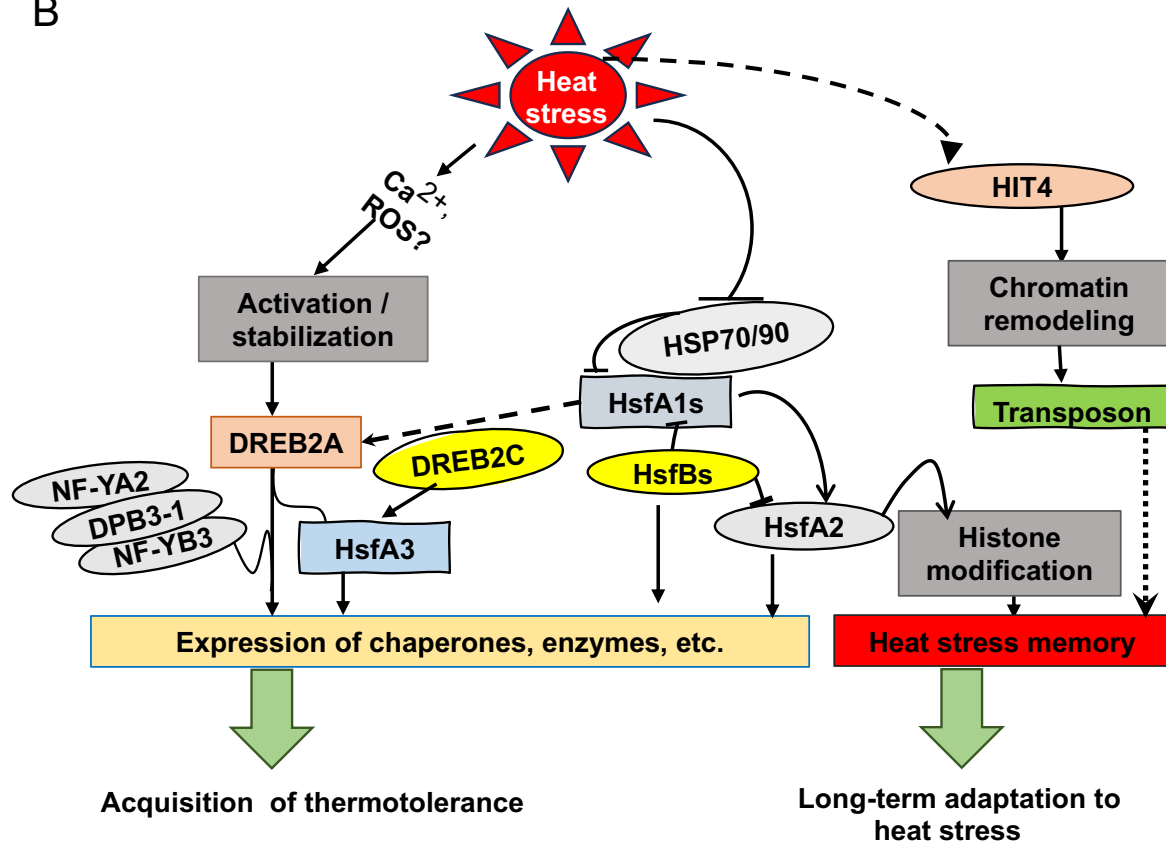


Figure 1: Heat shock response pathway. (A) In response to heat stress, active HSF1 undergoes homotrimerisation and translocates to the nucleus, where it binds to the DNA sequence motif called 'heat shock elements' (HSEs) in the promoter region of targets to induce their transcription. HSPs are the primary mediators in restoring and maintaining protein homeostasis, modified from (Choudhury *et al.* 2020) (B) Heat stress activates heat shock transcription factors to induce chaperone and stress responsive genes, leading to the acquisition of thermotolerance. Chromatin remodelling contributes to heat stress memory, enabling long-term adaptation to repeated HS, modified from (Ohama *et al.* 2017)

4.2 RNA transcription regulation

Transcription is an important pathway that carries the genetic information stored in DNA to the protein production machinery, directing protein synthesis. RNA transcription is a multilayered process that ensures the right genes are expressed at the right time, place, and amount in response to development and the environment. In eukaryotes, this process takes place inside the nucleus, carried out by several DNA-dependent RNA polymerases (RNAPs) with specialised outputs: the primary product of RNA Polymerase I (RNAPI) is the 45S ribosomal RNA (45S rRNA), RNAPII synthesises most mRNAs and many regulatory RNAs like the long non-coding RNAs (lncRNAs), whereas RNAPIII produces

the transfer RNAs (tRNA), 5S rRNA, U6 small nuclear RNA (U6 snRNA) and the 7SL RNA (Osman and Cramer, 2020). In plants, there are two additional polymerases present, the RNAPIV and V; these have evolved from the RNAPII and have roles in the heterochromatic silencing pathway (Haag and Pikaard, 2011). Multi-subunit RNA polymerases IV and V: purveyors of non-coding RNA for plant gene silencing (Haag and Pikaard, 2011, Tan et al., 2012, Borges and Martienssen, 2015).

Transcriptional output of RNAPII-dependent genes is regulated at several levels. At the initiation level, specific transcription factors (TFs) bind to the *cis*-regulatory element of the DNA, interact with co-factors and chromatin regulators, and facilitate the assembly of a functional pre-initiation complex (PIC) at the core promoter site involving activity of general transcription factors (GTFs) and RNAPII core complex and associated factors (Fig. 2). The configuration of the core promoter, such as the presence or absence of the TATA box (TATA box is a short DNA sequence found in the promoter region of many eukaryotic genes) and other motifs, directs the binding of GTFs and RNAPII. The TATA binding protein within TFIID (Transcription Factor II D, which contains TBP (TATA-Binding Protein) + TAFs (TBP-Associated Factors)) is a crucial early player at numerous promoters, and modification within the promoter elements can lead to gene and/or condition-specific expression variations.

Transcriptional elongation is not only simply passive polymerisation but a highly regulated process that can markedly influence the transcriptome. A myriad of transcription elongation factors accompany the RNAPII, including kinase complexes (P-TEFb, DSIF), velocity enhancers (TFIIF, TFIIIS), chromatin remodellers (FACT, SPT6, IWS1), and others (Obermeyer et al., 2023a, Obermeyer et al., 2022, Michl-Holzinger et al., 2022, Grasser, 2020, Grasser and Grasser, 2018, Van Lijsebettens and Grasser, 2014). During elongation, the carboxy-terminal domain (CTD) of RNAPII undergoes dynamic phosphorylation, coordinating transitions during the transcription cycle and modulating its affinity for elongation factors involved in RNA processing and elongation (Leng et al., 2020).

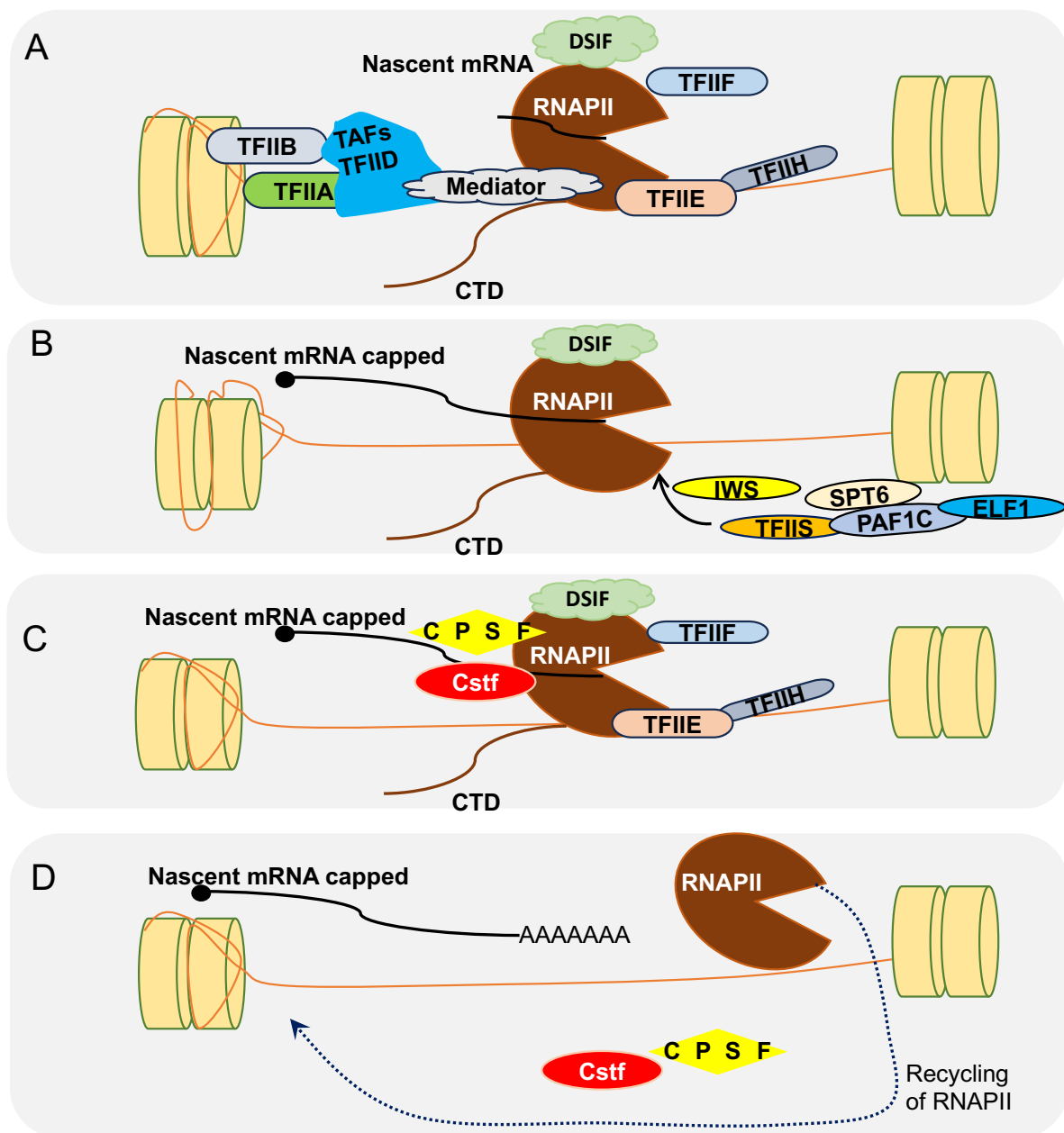


Figure 2: RNA transcription cycle schematic diagram. (A) Initiation: RNAPII can switch to productive initiation mode when all the inhibitory signals/factors are overcome. The nucleosomes flanking the active promoters harbor the histone marks for active transcription. (B) Elongation: Following promoter clearance, RNAPII proceeds for elongating. Meanwhile, TFs such as TFIIB, TFIIF and likely TFIIE fall off. (C) 3' end Processing: Once the RNAPII reaches a pause signal at the gene terminal, 3' end processing and termination specific complexes such as CPSF and CstF are recruited. The region already transcribed by RNAPII is efficiently reassembled into chromatin with the aid of histone chaperones and deacetylases (HDACs). (D) Termination and Recycling: Upon recognition of the polyadenylation signal, the nascent mRNA is cleaved and polyadenylated (*poly A* tail). The RNAPII complex dissociates from the DNA template as the transcription bubble closes. Termination factors (CPSF/CstF) and elongation factors are released, allowing the dephosphorylated RNAPII to be recycled for a new round of initiation.

Transcription termination occurs downstream of the polyadenylation signal (PAS). The transcript is cleaved by the terminator cofactor complex, including Cleavage and Polyadenylation Specificity Factor (CPSF73), Cleavage Stimulation factor (CstF) and Cleavage Factors I and II (CFI and CFII). Nascent RNA is polyadenylated by the poly(A) polymerase complex. Following cleavage, RNAPII is released from the template through the torpedo model, in which the exonuclease Xrn2 degrades the remaining RNA attached to RNAPII, while catching up is destabilising the complex, or through allosteric conformational changes in RNAPII and loss of elongation factors, which promotes dissociation. Current evidence suggests a combination of these mechanisms may widely operate in eukaryotic cells (Skourti-Stathaki et al., 2011, West et al., 2004, Leng et al., 2020).

4.3 Transcription elongation and role of TFIIS during heat stress

Transcription elongation by RNAPII is a crucial regulatory phase of gene expression in plants. During elongation, RNAPII has to cross through the chromatin, overcoming physical obstacles (such as densely packed nucleosomes, DNA secondary structures, head-on transcriptional complexes or DNA replication complexes, etc.), correct polymerisation mistakes, and integrate RNA synthesis with the co-transcriptional RNA processing. These obstacles can induce RNAPII stalling, slowdowns, or backtracking along the DNA template. Nucleotide misincorporations and backtracking events dislocate the 3' end of nascent RNA from the active site, leading to transcriptional halt until the nascent RNA 3' end is repositioned into the catalytic site. This reaction can be performed by the core RNAPII, which may be further boosted by elongation cofactors. A conserved array of transcript elongation factors (TEFs) stabilises elongation of RNAPII and assures transcriptional continuity to ensure efficient transcription (Fish and Kane, 2002).

Among the Transcription elongation factors (TEFs), Transcription elongation factor IIS (TFIIS) has a crucial and vital role. TFIIS is a zinc-finger-containing nuclear protein of 35 kDa in *S. cerevisiae* (Jeon and Agarwal, 1996, Jeon et al., 1994), 45 kDa in *Arabidopsis thaliana*, and 42.9 kDa in *Hordeum vulgare* L (Szadeczky-Kardoss et al., 2022). TFIIS has 3 domains (Kettenberger et al., 2003). The N-terminal part of the TFIIS protein Domain I, comprised of a 4-helix bundle, shows less sequence conservation, but it is important for protein-protein interaction and nuclear localisation. Domain II is the central domain and has 3 alpha (α) helix bundles, responsible for interacting with the core RNAPII. Domain II relates to Domain III with the linker region (Kettenberger et al., 2003, Awrey et al., 1998). Domain III is at the C-terminal part of the protein and has a highly conserved zinc finger motif on a

thin protruding antiparallel beta (β) hairpin; at the tip of Domain III is located the acidic DE di-amino acid important for enhancing the catalytic activity of RNAPII; this is the most conserved part of the protein (Fig. 3).

TFIIS associates with the elongating RNAPII complex and enhances its inherent RNA cleavage activity (Kettenberger et al., 2003, Antosz and Deforges, 2020, Eun et al., 2014). This cleavage eliminates the mismatched segment of nascent RNA, reinstalling the appropriate 3' end that can re-enter the RNAPII active site and permit the continuation of transcription (Kettenberger et al., 2003, Antosz and Deforges, 2020, Eun et al., 2014). TFIIS functions as a rescue factor that prevents prolonged transcriptional stalls and increases transcriptional accuracy. The importance of TFIIS is especially apparent during the HS conditions, when transcriptional problems may be significantly enhanced (Szaker et al., 2025). Increased temperature disrupts the protein-DNA interactions, elevates transcriptional error rates, and intensifies RNAPII pausing and backtracking.

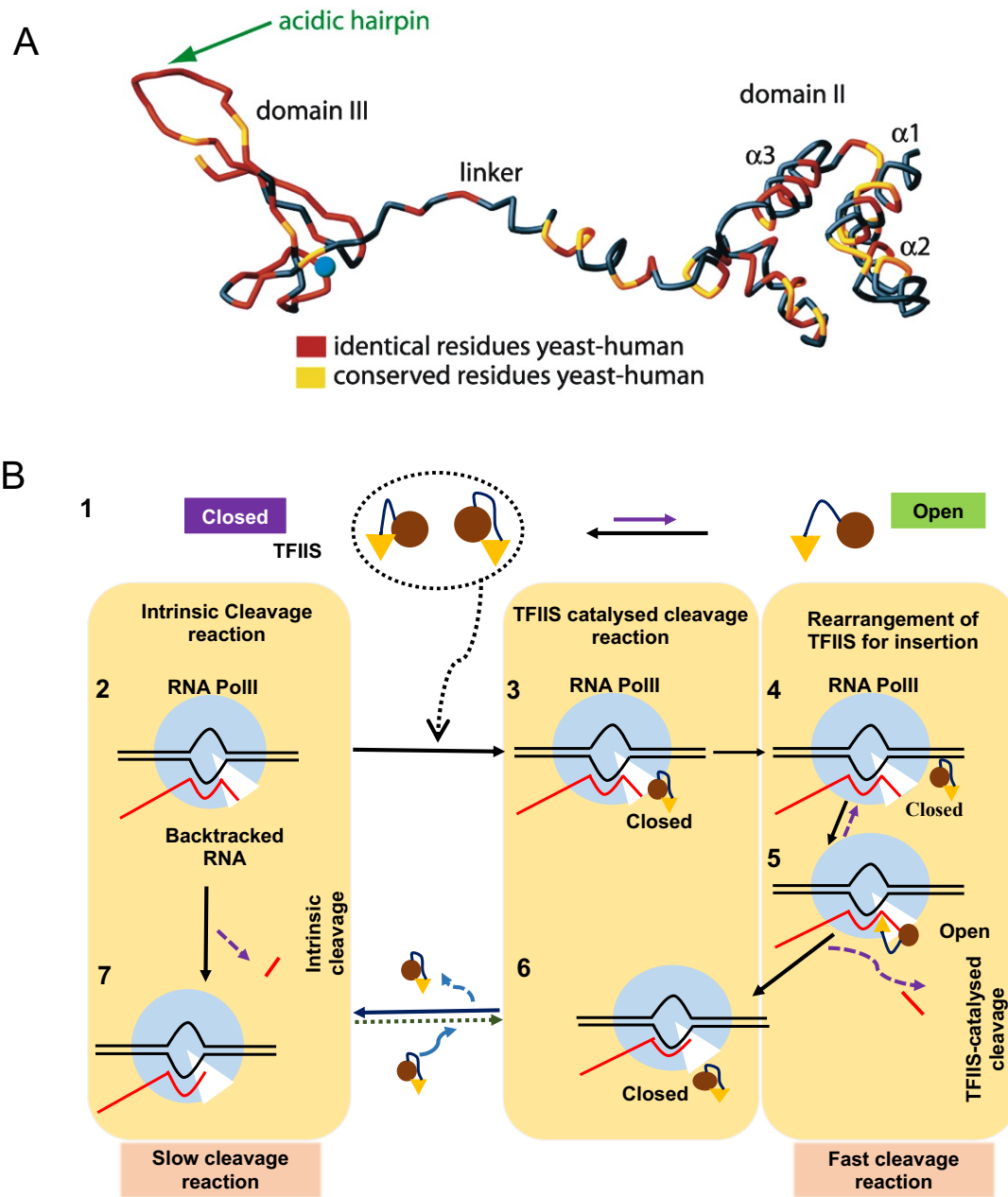


Figure 3: TFIIS structure and regulation. (A) TFIIS structure and conservation. The side view of the structure of yeast TFIIS containing domains II and III. Residues identical and conserved between yeast and human TFIIS are depicted in red and orange, respectively. Acidic hairpin containing invariant aspartic acid and glutamic acid residues is indicated with a green arrow. Picture taken from (Kettenberger *et al.*, 2003b). (B) Schematic diagram of the reactivation process from the arrested state of RNAPII. where (1) TFIIS in solution (2) Backtracked state of RNAPII. (3) State of RNAPII with an initial partially bound TFIIS. (4) State of RNAPII with a partially bound TFIIS for the insertion of the catalytic domain. (5) State of Pol II with a fully bound TFIIS. (6) Elongation state of Pol II with a partially bound TFIIS. (7) Normal elongation state of PolII. Picture modified from (Eun *et al.* 2014)

HS may affect transcription speed and alternative splicing (AS) pattern. TFIIIS regulates both speed and splicing (Dolata et al., 2015, Godoy Herz et al., 2019, Zhu et al., 2020, Szadeczky-Kardoss et al., 2022) and contributes to HS tolerance in Arabidopsis (Szadeczky-Kardoss et al., 2022, Obermeyer et al., 2023b).

4.4 Barley as a model and crop plant

Climate predictions suggest that the average worldwide temperature could rise by 1.5-8.0 °C by 2100. Even a 1°C rise in temperature can diminish the yield of about 6% in crop plants, highlighting the necessity to fully understand the crop reactions to thermal stress. Barley (*Hordeum vulgare* L.) is an important cereal crop plant cultivated all over the world. Globally, it is counted as the fourth most crucial cereal plant.

Barley acts as a model organism for genetic, physiological, and molecular studies due to its diploid genome ($2n = 14$), which can be readily manipulated using molecular or genetic techniques, such as the generation of mutants and/or overexpressors, crossing, and selective breeding (Mascher et al., 2021). It shows significant levels of both natural and cultivated genetic diversity and possesses clearly delineated developmental stages (Cockram et al., 2007, Mascher et al., 2017). In addition, barley shows strong genome synteny with other temperate cereals like rye and wheat, which means that discoveries made in barley can potentially be efficiently transferred to these relatives (Krasileva et al., 2017). Barley can be grown in a multitude of climatic regions and environments. Availability of mutants and diversity in barley varieties has enhanced its application as a model for investigating complex physiological and cultivation features, including stress tolerance, flowering duration, and grain development (Bartlett et al., 2008, Callens et al., 2022, Dawson et al., 2015, Hamar et al., 2020, Harwood, 2019, Jayakodi et al., 2020, Kruszka et al., 2014).

As a crop plant, barley has diverse uses. It is commonly used for animal feed and human food. It is also widely used in the malting industry due to its rich nutrients like proteins, vitamins, fibres, and minerals. Barley shows higher abiotic stress tolerance compared to other grains like rice and wheat, making it an important staple food crop in areas exposed to climate fluctuations and global warming effects. In summary, barley is both a research model plant and an important crop for developing stress tolerant varieties through traditional and modern breeding methods (Bartlett et al., 2008, Callens et al., 2022, Dawson et al., 2015, Hamar et al., 2020, Harwood, 2019, Jayakodi et al., 2020, Kruszka et al., 2014).

4.5 Impact of heat stress on the yield of barley

Many factors affect crop productivity and limit their yields. One of the most adverse factors that affects the yield is HS, which limits crop productivity in barley (Sallam et al., 2018). The severity of HS depends on timing, duration, and intensity, especially during the reproductive stages. E.g. meiosis, flowering, and grain filling periods are particularly vulnerable (Barnabas et al., 2008). Short heat waves with temperature exceeding 30-35°C can severely affect the pollen viability, grain set, and kernel weight, while prolonged exposure to moderately elevated temperature can accelerate development, and by this reduce biomass accumulation and shorten the grain filling period (Reynolds et al., 2009, Talukdar et al., 2022). These physiological disruptions ultimately contribute cumulatively to a significant reduction of both yield and grain quality.

At the molecular level, numerous Quantitative trait loci (QTLs) and regulatory genes that control grain traits have been identified in cereals (Song et al., 2007). One of them, Grain Width and Weight 2 (GW2), encodes a RING-type E3 ubiquitin ligase that functions in the ubiquitin proteasome system (UPS) and has become a key regulator of grain size. In rice, GW2 regulates grain width by ubiquitinating and disrupting proteins associated with cell proliferation, such as WG1 (Hao et al., 2021). GW2 shows functional conservation among cereals, such as maize, rice, wheat, and barley (Zombori et al., 2020, Sestili et al., 2019, Li et al., 2010). It affects grain size by regulating cell proliferation and cell cycle progression, the weight of the grain filling process, which is influenced by the grain's length, width and thickness. Grain number and grain weight are polygenic traits; thus, competition for assimilates during grain filling frequently leads to an imbalance between these yield components.

5 MATERIALS AND METHODS

5.1 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 (Hamar et al., 2020). In heat stress (HS) studies, both wild-type and *tfl1s-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) as shown in (Fig. 4).

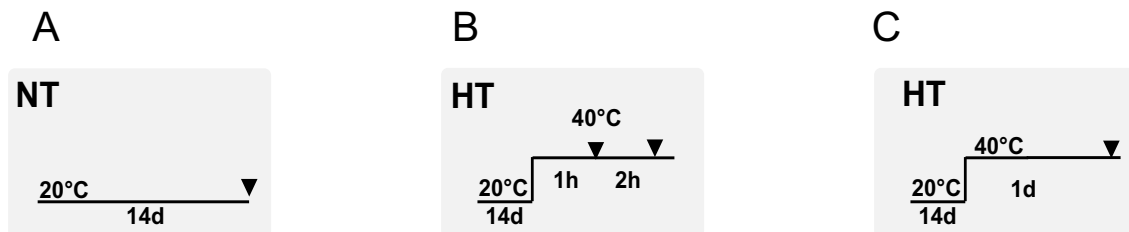


Figure 4: Schematic representation of sampling points. (A) Non-Treated (NT) group: Plants were grown at a constant temperature of 20°C for 14 days. Samples were collected at the end of this period, indicated by the black arrowhead. (B) Short-Term High Temperature (HT) Treatment: Plants were initially grown at 20°C for 14 days and then subjected to a heat stress of 40°C. Samples were collected quickly during this phase at two intervals: 1 hour (1h) and 2 hours (2h). (C) Long-Term High Temperature (HT) Treatment: these plants were grown at 20°C for 14 days and then heat treatment was given at 40°C samples were collected after a longer exposure of 1 day (1d) TMHT.

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.

5.2 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*), as shown by (Ahmad et al., 2024). Potential CRISPR/Cas9 target sites were generated using the CRISPOR program (Concordet and Haeussler, 2018, Kis et al., 2024). 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. The CRISPR-Cas9 vector containing these sgRNAs was constructed (see Fig. 5) according to the methodology described previously (Kis et al., 2024).

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; www.sigma-aldrich.com). 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; www.neb.com). Homozygous mutant lines were chosen and cultivated in greenhouse conditions.

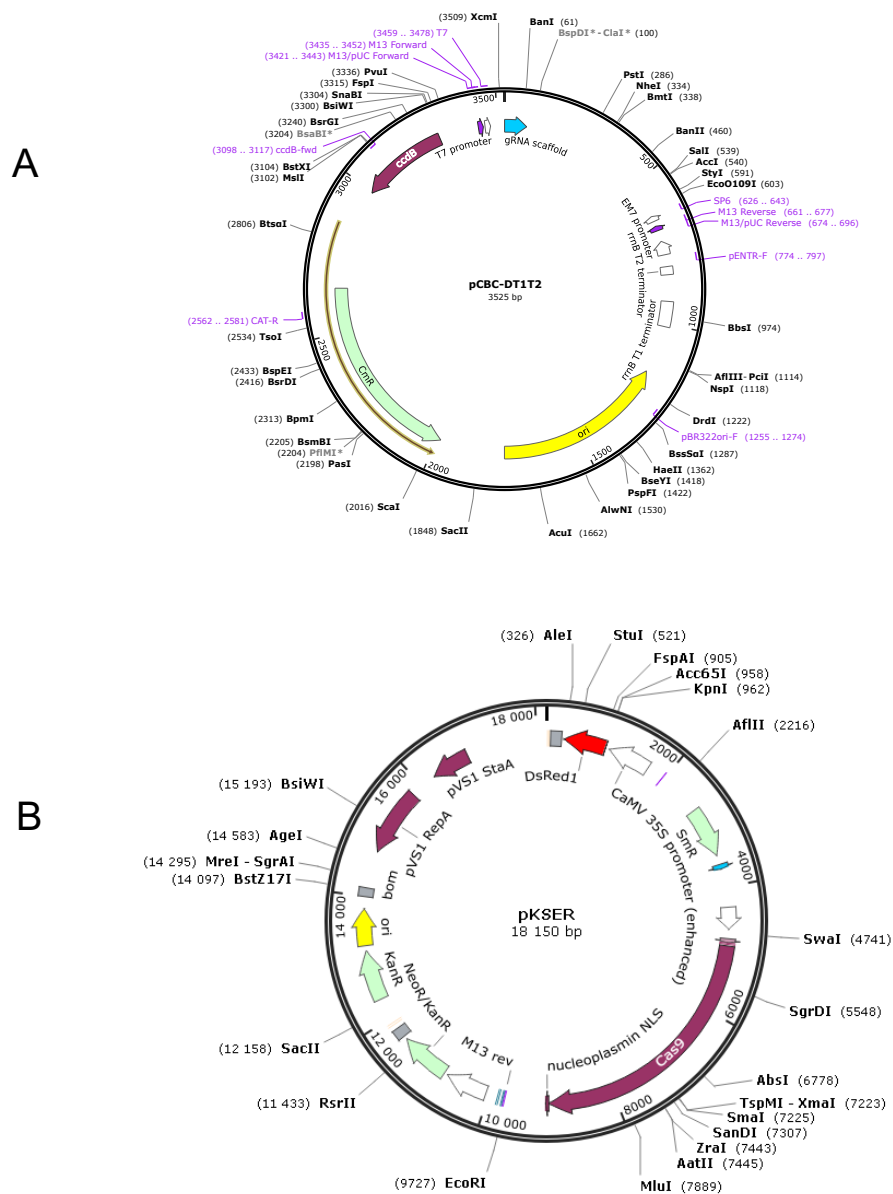


Figure 5: Schematic representation of the CRISPR/Cas9 vector used for barley transformation. (A) Schematic representation of the pCBC-DT1T2 vector (3525 bp) used for sgRNA cloning. The vector contains the BbsI restriction sites for insertion of annealed sgRNA oligonucleotides under control of the U6 promoter and includes the gRNA scaffold sequence. The ccdB cassette enables negative selection in *Escherichia coli*. This intermediate construct was used to generate sgRNA expression cassettes prior to transfer into the pKSER binary vector. (B) Map of the CRISPR/Cas9 binary vector pKSER (18,150 bp) used for targeted mutagenesis of the barley *tfls* gene. The vector harbours the SpCas9 coding sequence fused to a nucleoplamin nuclear localisation signal (NLS) under control of the CaMV 35S promoter. The sgRNA cassette cloned via pCBC-DT1T2 was inserted into the vector backbone. The construct contains the nptII selectable marker for plant transformation and elements required for replication in *Escherichia coli* and *Agrobacterium tumefaciens* strain AGL1. Images created using SnapGene software.

5.3 Germination assessment

Fully developed barley seeds from the wild-type and *tfls-cr1*, *tfls-cr2*, and *tfls-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. The germination outcomes were later shown graphically.

5.4 Embryo rescue

Mature barley seeds from both the wild-type and *tfls-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 Phytigel, as delineated by (Bartlett et al., 2008). 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.

5.5 DNA extraction and Sanger sequencing

Total DNA was isolated from 60 mg of barley leaf tissue in 600 µl 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 (Green and Sambrook, 2017). 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.

5.6 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 (Vennapusa et al., 2020). 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.

5.7 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.

5.8 Purification of protein aggregates

Protein aggregation detection was conducted as previously described. (Ahmad et al., 2024, Szaker et al., 2025). 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 $\times$ 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 $\times$ 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 $\times$ SDS-PAGE sample buffer and cleared via centrifugation at 1500 $\times$ 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.

5.9 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 (Fischer and Kessler, 2015) and evaluated in a single run on the mass spectrometer (Kobayashi et al., 2015). 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.

5.10 Bioinformatics tools and analyses

To examine the sequence conservation of TFIIS homologues, sequences were discovered and retrieved from the Ensembl Plants and UniProt databases (Martin et al., 2023). Protein alignments were conducted using Jalview software [www.Jalview.org](http://www.jalview.org) (Waterhouse et al., 2009). The forecast and depiction of the 3D structure of TFIIS domain II were obtained from and produced by AlphaFold DB (Jumper et al., 2021).

5.11 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 (Ashburner et al., 2000, Yates et al., 2021, The Gene Ontology, 2019). GO enrichment analysis was conducted using the agriGO v2.0 toolkit (Tian et al., 2017). 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 (Supek et al., 2011). The analysis allowed identification of biological pathways and stress-related processes overrepresented under heat stress conditions.

6 RESULTS

6.1 Conservation of TFIIS in monocots

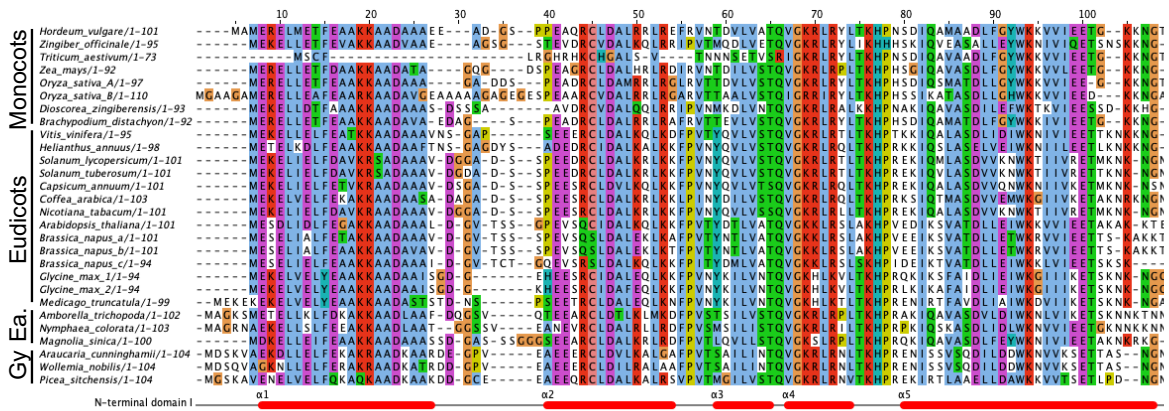
In order to analyse the evolutionary conservation and possible functional importance of the TFIIS protein in cereals, a thorough sequence conservation analysis was carried out. A search for protein sequences for TFIIS homologs was made for the major monocot crops, including barley, maize, wheat, rice and *Brachypodium* and selected gymnosperm, early dicotyledonous, and eudicot species for the purpose of interspecific comparison (Figs 6, 7).

Multiple sequence alignments showed high conservation among the analysed plant groups. Phylogenetic analysis of TFIIS protein sequences revealed a clear separation between monocots, eudicots, and early-diverging plant lineages (Fig. 6C). Barley TFIIS clustered closely with other grass species such as wheat, rice, and maize, which indicates strong evolutionary conservation within monocots. Eudicot TFIIS proteins formed a distinct clade, whereas the gymnosperms and early angiosperms occupied basal positions in the phylogenetic tree (Fig. 6C).

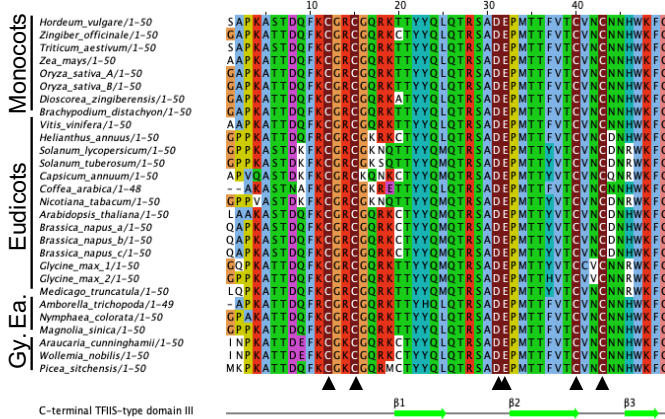
The α -helices of domain I and II, and the β -sheets of domain II were predicted in all TFIIS homologs (Figs 6A, 7A). Apart from some scattered amino acid differences in some lineages, we observed a monocot-specific amino acid stretch inserted in domain II between $\alpha 6$ - and $\alpha 7$ helices; these additional α -helices were named $\alpha 6$ -b and $\alpha 6$ -c, to preserve the nomenclature of the other (Fig. 7A). Based on the three-dimensional structure prediction, the $\alpha 6$ -b and $\alpha 6$ -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 (Fig 6B), 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.

A



B



C

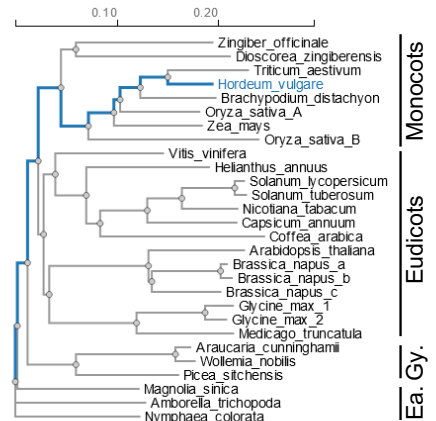


Figure 6: Protein alignment of TFIIS protein homologs. The sequences of gymnosperm (Gy), early dicot (Ea), eudicot and monocot TFIIS protein homologs (as shown on the left) were aligned by the Jalview ClustalO tool, and secondary structure α -helices and β -sheets were predicted (shown below the sequence for TFIIS domain I (A) or TFIIS domain III (B)). The four cysteine residues forming the zinc finger and acidic dipeptide of the TFIIS domain III are shown with arrowheads below. (C) Phylogenetic tree of TFIIS homologs in representative species of the plant kingdom; the length of the branches is indicative of the evolutionary distance between protein sequences.

6.2 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 (*see Materials and Methods for technical details*). 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 (Fig. 8).

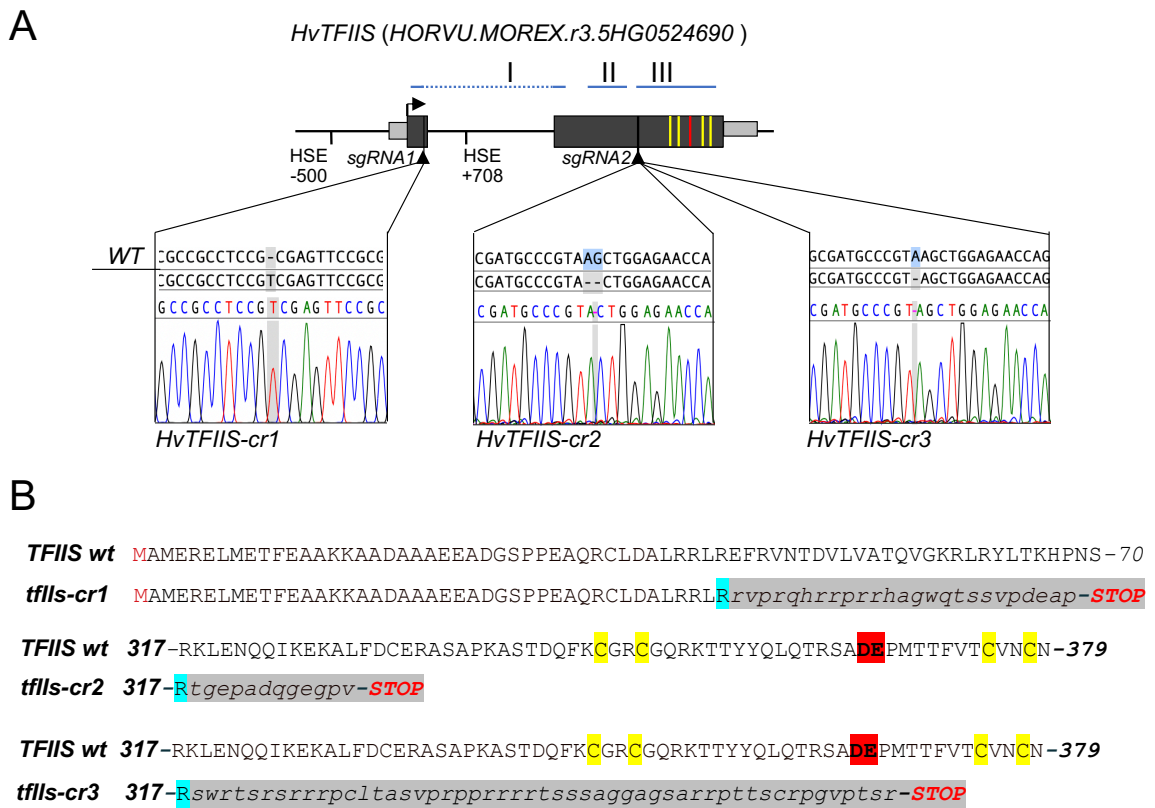


Figure 8: Generation of *tflis* mutant line using CRISPR mutagenesis in barley. (A) Schematic representation of *HvTFIIS* gene locus; protein domain I, II and III locations are shown above; exons are as black boxes, UTRs as grey boxes, Zn finger domain cysteine residues and DE acidic dipeptide motif as yellow and red line, respectively; HSE *cis* elements and location of sgRNA guide sites are shown below; Insertion or deletion mutation within the TFIIS locus in the selected transgenic lines are shown on chromatograms (bottom); (B) *HvTFIIS* protein amino-acid changes with premature termination codon is shown; last correct amino-acid is highlighted with cyan, changed amino acid sequence with grey, Zn-finger motif residues with yellow, catalytic DE dipeptide with red; STOP codons are indicated.

From the progeny of the gene-edited plants, we selected two independent mutant lines for each sgRNA target and successfully obtained homozygous mutants. In the CRISPR/sgrNA1-edited lines, mutations included either an adenine insertion (+A) or a thymine insertion (+T), both resulting in frameshift mutations. In the CRISPR/sgrNA2-edited lines, the mutations consisted of either a dinucleotide (adenine–guanine) deletion (-AG) or a single adenine deletion (-A) (Fig. 8).

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 (Fig 8B). One of the homozygous mutants generated via sgRNA1 (carrying the +A insertion) turned out to be sterile and therefore could not be carried forward for further analysis (*data not shown*). The other sgRNA1-derived line, harbouring a +T insertion, was named *tflIs-cr1*. This line produced seeds, however these were germination incompetent; therefore, this line could be propagated only through embryo rescue methods (see *Materials and Methods*, Fig. 9): embryos were isolated from mature grains, placed on sterile growth media where they successfully developed roots and branches; following this they were placed on soil where the plants ultimately advanced to mature stage, developed flowers and produced seeds. Wild-type plant embryos were processed similarly to provide control material for *tflIs-cr1* for heat stress sensitivity studies or molecular analysis (*see later*).

Besides these, we have tested several additional sgRNA1-mutated plants; no other homozygous viable mutant could be identified (*data not shown*).

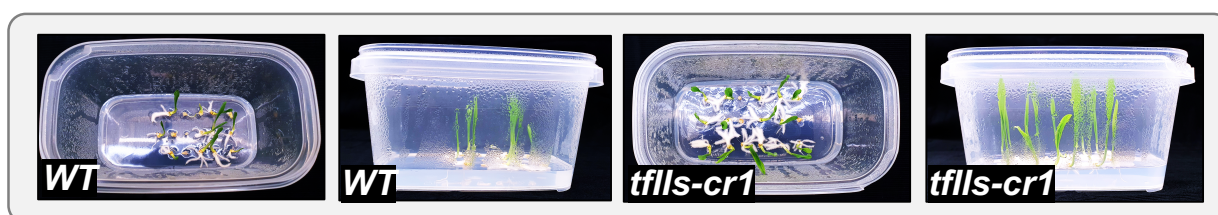


Figure 9: Embryo rescue of *tflIs-cr1* seeds. Wild-type and mutant embryos were extracted from the seeds and placed on sterile germinating agar medium for growth; wt and mutant embryos were treated similarly for generation of plants and TMHT treatment work (*for technical details please see Materials and Methods section*)

In contrast, the sgRNA2-induced mutations resulted in frameshifts near the 3' end of the gene, upstream to the zinc-finger catalytic motif domain (Fig. 8A): 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 (Fig 8B). Importantly, both sgRNA2-derived mutant lines were fertile and produced viable seeds: these were named *tflIs-cr2* and *tflIs-cr3*.

To confirm TFIIS protein absence, we analysed the *tflIs-cr* lines through mass spectroscopy (Fig. 10, *for technical details see Materials and Methods section*), in comparison with wild-type samples. Peptides derived from barley TFIIS were efficiently detected only in the wild type samples (3 biological repetitions, 14% coverage of protein, 4 unique peptides) at both NT and heat treated situation, but we were unable to find these peptides in the samples obtained from barley *tflIs-cr* lines (we tested one sample of *tflIs-cr1*, two samples of *tflIs-cr2*, and one sample of *tflIs-cr3*)(Fig. 10);(to note, TFIIS antibody could not be produced, although we have tried to generate it multiple times). The MS analysis confirmed that CRISPR mutagenesis was efficient; our barley plants likely are unable to produce functional TFIIS.

1 Acc. #: 1 Species: *H vulgare* Name: TFIIS_WT

Version: 1

Protein MW: 42913.0 Protein pI: 9.2 Protein Length: 383 Index: 1

1 MERELMETFE AAKKAADAAA EEADGSPPEA QRCLDALRRL REFRVNTDVL VATQVGKRLR YLTKEHPNSDI QAMAADLFGY
81 WKKVVIEETG KKNCTPANGK SDNSAAKADK PQPMKVEKNS VSAKVIEKN STSATVKTEK SSMSTSVKIE KKGDSIKVEK
161 TENNDSKVQV KVEKVSKEVS RTPDTKKQSS VPNGPPKLTS LVRCNDAARD KYRELLVEAF VKVSKETSED DREEIRDLLN
241 QVNACDPYRV AVTVESALFE RIGRSTGTHK VKYRSILFNL KADNNPDFRR RVLLGEVRPG SLVDMPADEM ASDARKLENQ
321 QIKEKALFDC ERASAPKAST DQFKCGRCGQ RKTYYQLQT RSDPEMTTF VTCVNCNNHW KFC

Search Name	Num Unique	% Cov	Best Disc Score	Best Expect Val
Merged	4	14.1	2.60	1.8e-6

m/z	z	Intensity	ppm	DB Peptide
854.0850	3	0	0.92	VLLGEVRPGSLVDMPADEMASDAR
660.8618	2	34441	0.43	VAVTVESALFER
417.7579	2	29928	0.26	SILFNLK
417.7580	2	324812	0.36	SILFNLK
635.8451	2	1.33e+6	0.50	ADKPQPMKVEK

Figure 10: MS analysis of barley (*Hordeum vulgare* L.) TFIIS protein. Protein molecular weight, pI and length is indicated (top), protein sequence with peptides detected by MS are highlighted in red (middle); m/z, peak intensity and ppm for each peptide detected is shown (bottom);(MS analysis was performed by Proteom Facility (at BRC, Szeged) in collaboration with Pettkó-Szandtner A; *for technical details please see Materials and Methods section*)

All further examinations were carried out in parallel on *tfIIs-cr1*, *-cr2* or *-cr3* in comparison with the wild-type line control.

6.3 Barley requires TFIIS for proper reproductive development

First, we examined the growth of *tfIIs-cr* mutants under standard conditions. The *tfIIs-cr1* plants exhibited mildly reduced growth relative to the wild type (Fig. 11A). 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 (Fig. 11A).

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 (Figs 9, 11B, C). 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 (Fig. 11B, C). 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 (Fig. 11D). 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).

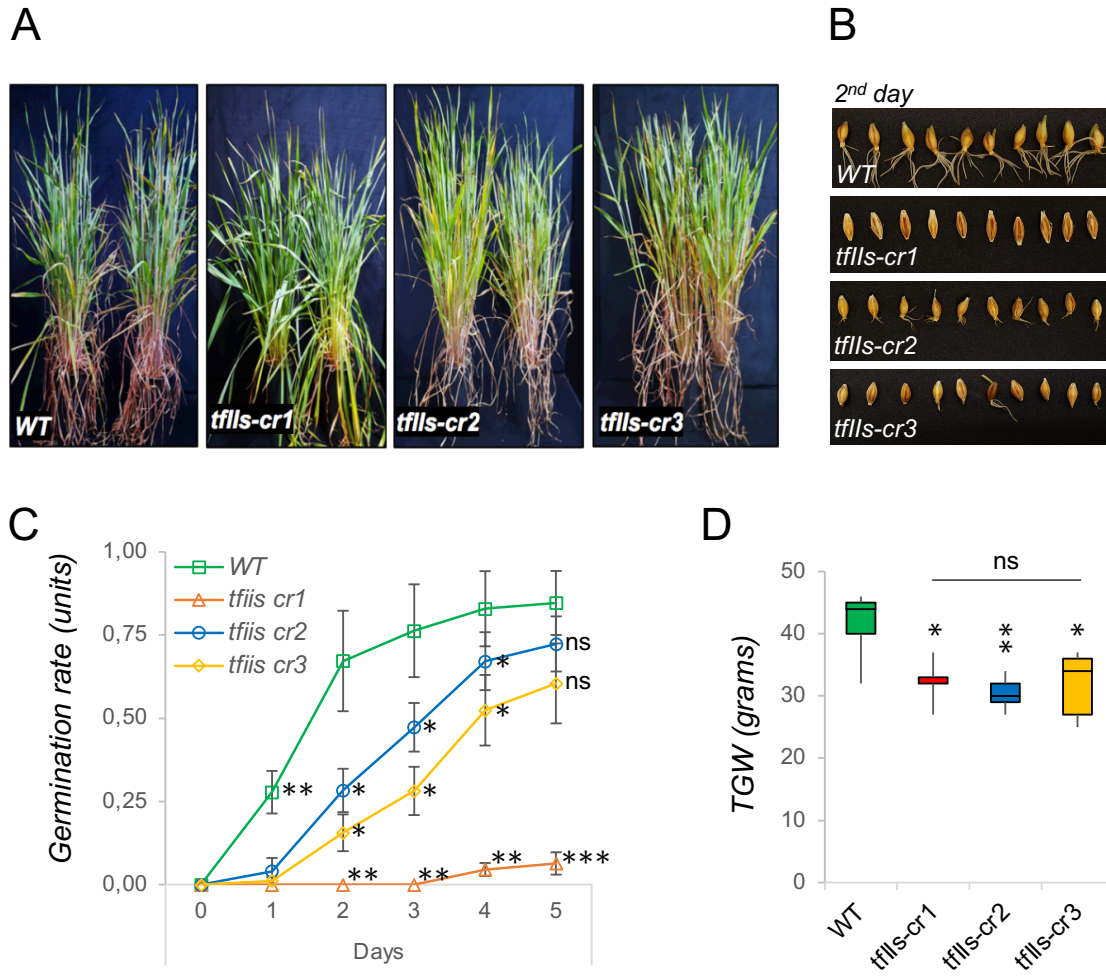
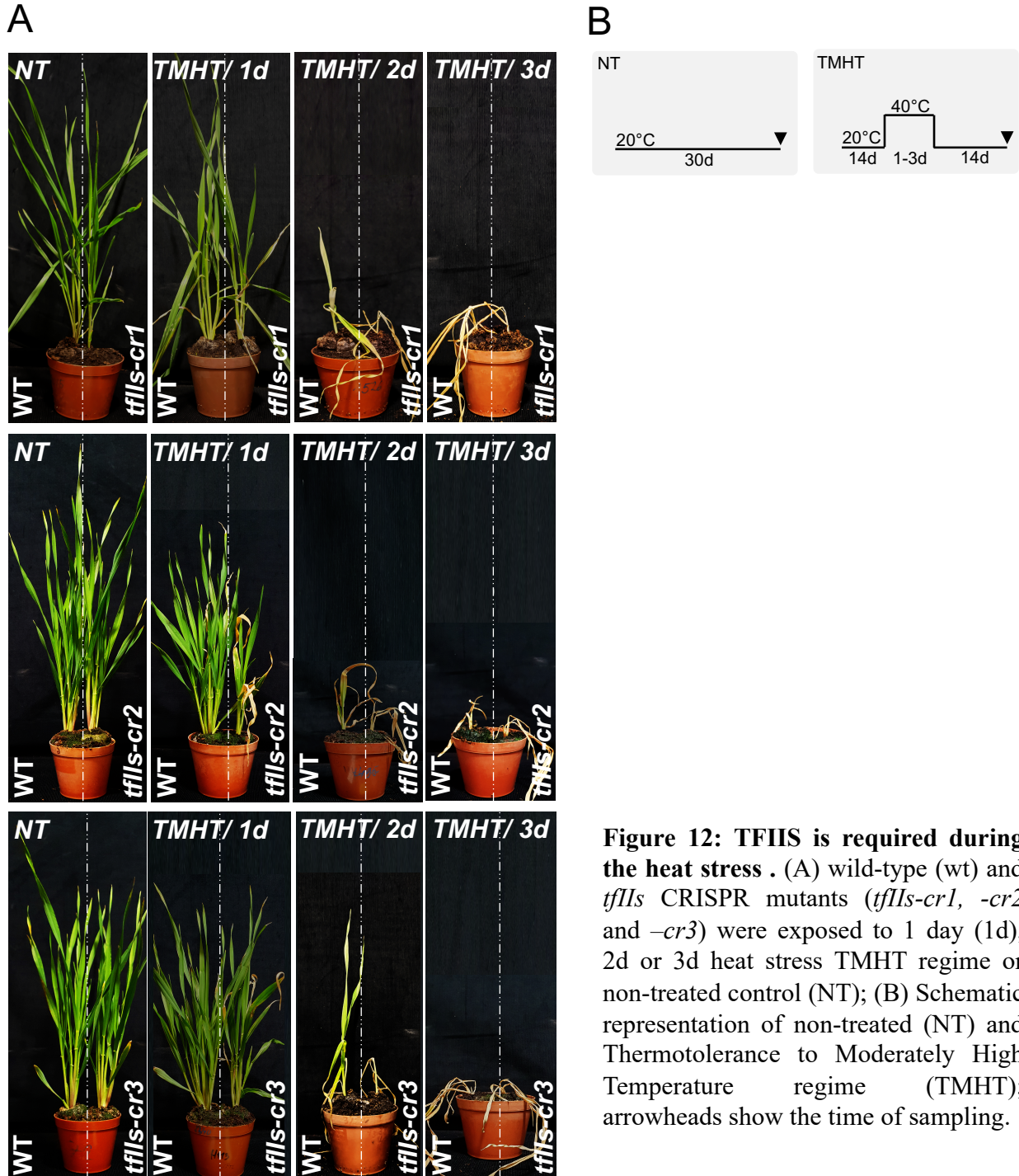


Figure 11: TFIIS is negligible for vegetative growth but needed for reproductive development in barley. (A) *tflls-cr1* mutant plants have slightly retarded vegetative growth compared to wild type plants; *tflls-cr2* and *-cr3* are indistinguishable from wt plants; (B) Pictures of germinating seeds on 2nd day; (C) Germination rate quantification of wild type and *tflls-cr* mutant plants during a timeseries; (D) The calculated thousand grain weight (TGW) of wild type and *tflls-cr* mutant plants; bars represent standard errors based on at least three bio reps; P-values based on two-tailed Student's t-test (*P < 0.05, **P < 0.01, ***P < 0.001, ns; non-significant).

6.4 Barley *tflls-cr* mutants are sensitive to heat stress

It has been shown previously that TFIIS is essential for heat stress tolerance in *Arabidopsis* (Szadeczky-Kardoss et al., 2022, Obermeyer et al., 2023b). To test if TFIIS activity is also needed for HS tolerance in barley, we subjected wild-type control and the *tflls-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 (Fig. 12A). The plants were then heat-treated at 40°C for a 1-to-

3-day time series (Fig. 12B). All the *tflls-cr* lines were heat-sensitive, exhibiting modest symptoms after 1 day and a strong retardation or lethality after 2 days of treatment.



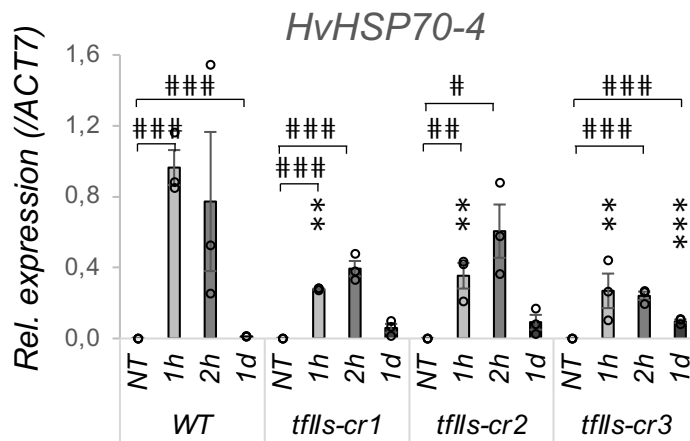
6.5 The TFIIS is an essential component of an effective HSR

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 *tflIs-cr* mutants in comparison with wild-type plants (Fig. 13). Under persistent heat stress (1 day at 40°C), wt plants showed reduced HSP expression, whereas transcript levels remained elevated in the *tflIs-cr* mutants.

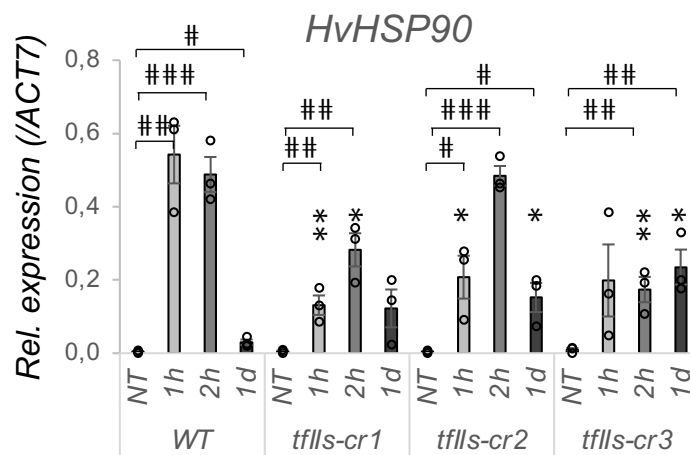
We propose that rapid transcriptional activation of HSPs is essential during the initial stage of heat stress, as observed at TMHT/1-2h. In the absence of TFIIS, elongation efficiency is compromised, resulting in a delayed accumulation of HSPs. This deficit in the early heat period likely leads to a 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 *tflIs-cr* mutants. Wild-type plants exhibited high accumulation of HSP70, HSP90, and HSP101 proteins after early heat stress, whereas *tflIs-cr* mutants accumulated markedly reduced levels of these chaperones (Fig. 14). In later stages of heat stress, the differences between wild-type and mutants were diminished, indicating that *tflIs-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.

A



B



C

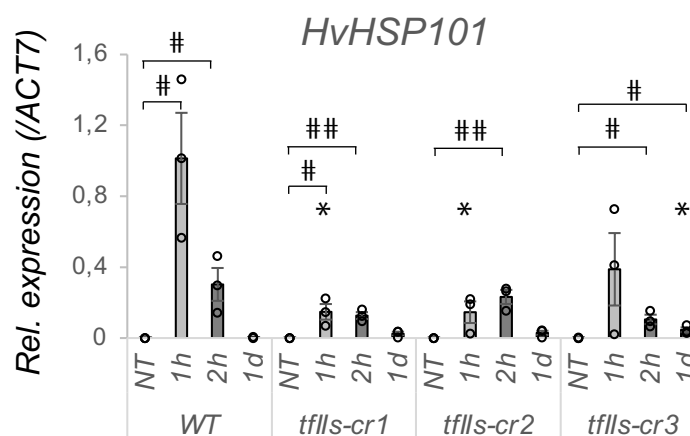


Figure 13: TFIIIS is indispensable for efficient heat stress response. (A-C)

Accumulation of HSR-transcripts is retarded in the absence of TFIIIS: relative expressions of (A) *HSP70-4*, (B) *HSP90*, and (C) *HSP101* mRNA during a timeseries (NT; 1 hour, 1h; 2 hours, 2h; 1 day, 1d); bars represent standard errors based on three bio reps; P-values based on two-tailed Student's t-test * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ show differences between wt and mutants; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ values show differences between NT and heat stress condition within genotypes.

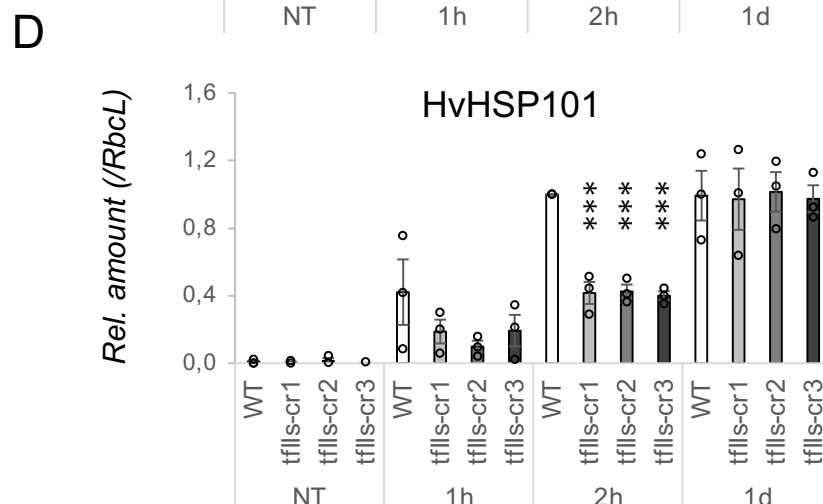
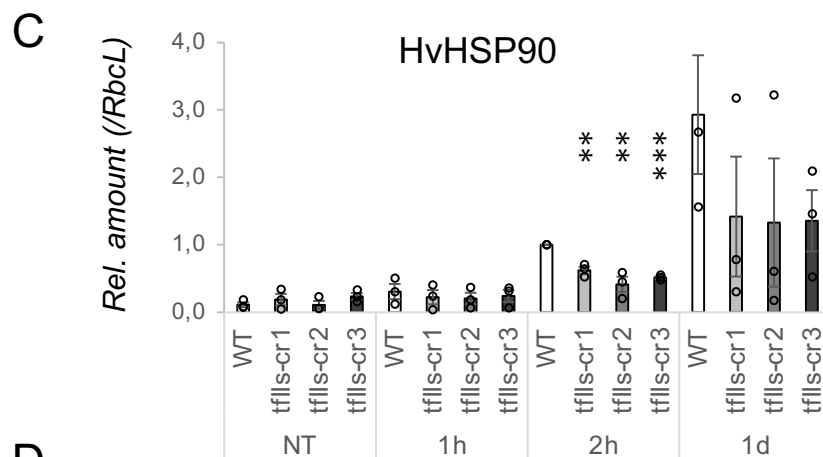
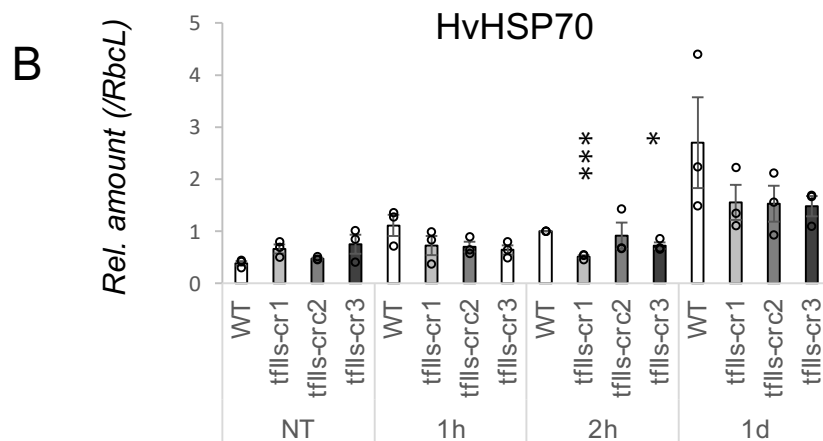
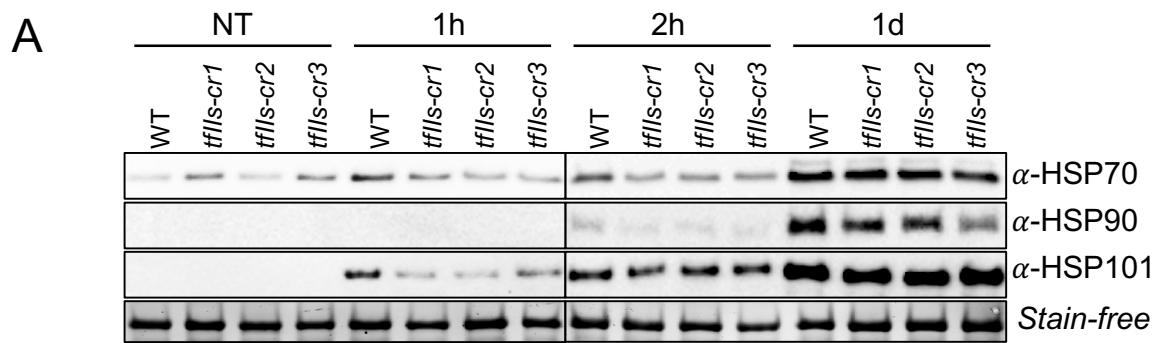


Figure 14: TFIIIS is needed for accumulation of HSP proteins during heat stress in barley. (A) Western blot images HSP70, HSP90 and HSP101 proteins; wild-type (wt) and *tflls-cr1*, *-cr2* and *-cr3*, respectively, were exposed to NT or TMHT for 1 hour (1h), 2h or 1 day (1d), as shown; stain-free picture is shown as a loading control; (A-C) Quantification of protein accumulation of HSP70-4 (B), HSP90 (C), and HSP101 (D) during the same timeseries; P-values based on two-tailed Student's t-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) show differences between wild type and mutant plants.

6.6 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 *tflIs-cr2* plants. A markedly elevated accumulation of insoluble proteins was seen in the *tflIs-cr2* plants under heat stress, indicating a proteotoxic state (Fig. 15). sHSP-CI holdases were present in both the soluble and insoluble fractions, but only in *tflI-cr2* (Fig. 15 bottom). 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 (Waters and Vierling, 2020). Presence of sHSP-CI in *tflIs-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 *tflIs-cr2* plants. Similar quantities of proteins were identified in both lines and treatments (soluble + insoluble proteins) (Fig. 15C). Upon analysing the qualitative and quantitative disparities between wt and *tflIs-cr2*, we observed an elevated number of proteins having higher abundances in the insoluble fraction of the *tflIs-cr2* line (Fig. 15C, D). Gene ontology (GO) analysis of these proteins showed enrichment in RNA/protein metabolism pathways, primarily localised to cytoplasm, organelles, and stress granules (Table 1).

In summary, these data confirm an elevated proteotoxic status of *tflIs-cr2* compared to wt plants in response to heat exposure.

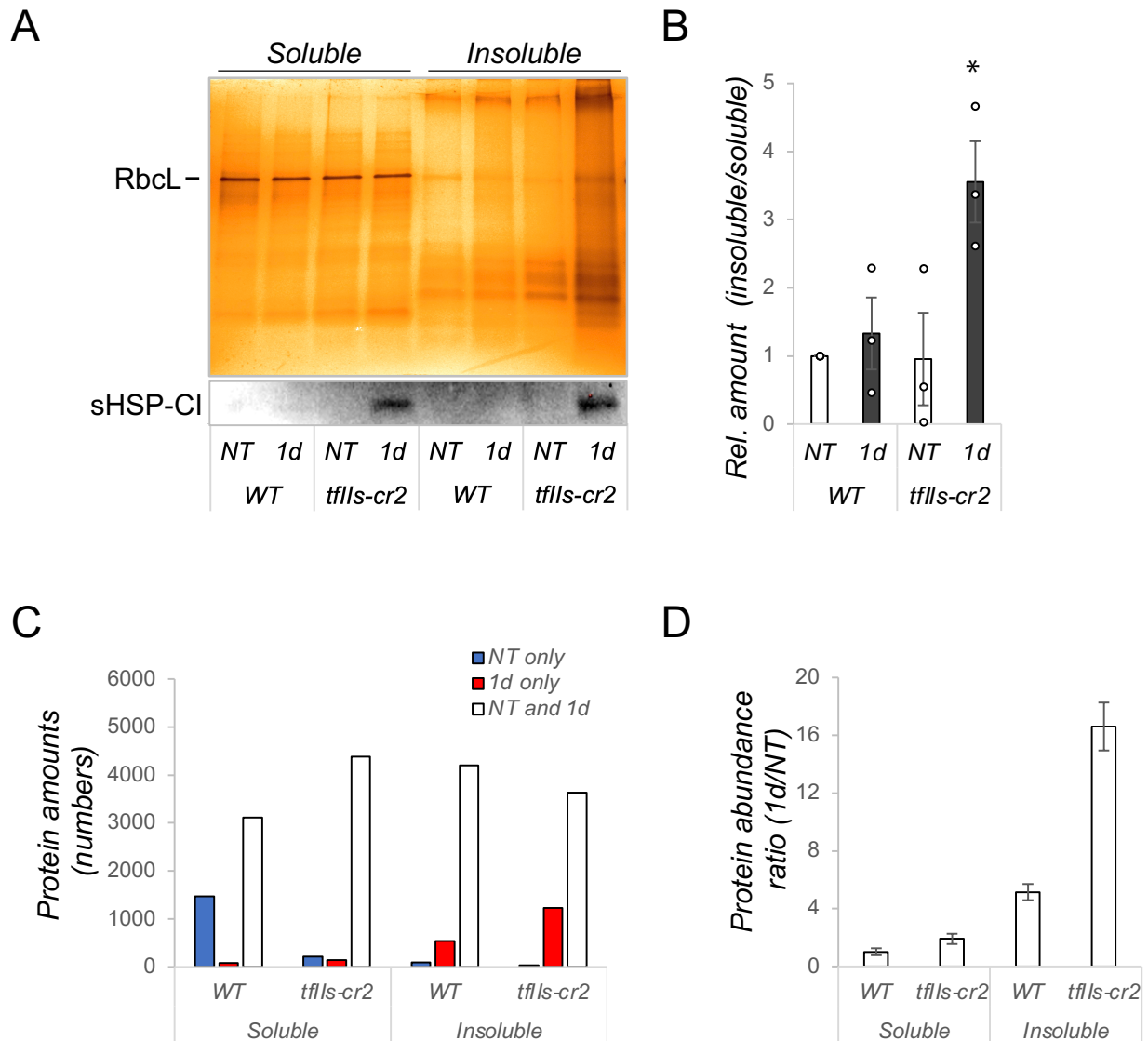


Figure 15: Insoluble protein aggregate accumulation in response to heat stress in wt and *tflIs-cr2* plants. (A) Silver staining gel images of insoluble protein fractions from wt and *tflIs-cr2* plants exposed to non-treated or 1d TMHT heat treatment; Rubisco large subunit (RbcL) is shown as loading control within soluble fraction; treatment conditions are shown; sHSP-CI accumulation is shown at the bottom. (B) Ratio quantifications of insoluble/soluble protein fractions in wild-type and *tflIs-cr2*-mutant plants at non-treated (NT) or TMHT/1d (1 d) stress conditions; P values based on two-tailed Student's t test (*P < 0.05) show differences between wild-type and mutant plants; (C) Mass spectroscopy analysis of soluble and insoluble protein fractions of wt and *tflIs-cr2* mutant barley plants, samples as shown in the legend top-right; (D) Average abundance ratio of proteins found in both non-treated (NT) and heat-treated (1d) samples; error bars are calculated based on protein ratio averages.

sample type	genotype	source	term_name	adjusted_p_value
Soluble	WT	-	-	-
	<i>tflls-cr2</i>	GO:MF	hydrolase activity	0.01333969714671428
		GO:CC	chloroplast	0.004934571568321391
		GO:CC	plastid	0.00921183998888756
		GO:CC	cytoplasm	0.016639822307787412
Insoluble	WT	GO:MF	catalytic activity	0.011496925057547699
		GO:MF	isomerase activity	0.03210566722088539
		GO:BP	small molecule metabolic process	0.00014877636289575496
	<i>tflls-cr2</i>	GO:MF	catalytic activity	0.000007742708525373795
		GO:MF	hydrolase activity	0.0037574918649997983
		GO:MF	hydro-lyase activity	0.018933609394039302
		GO:MF	peptide deformylase activity	0.049842884914428724
		GO:MF	RNA 7-methylguanosine cap binding	0.049842884914428724
		GO:BP	small molecule metabolic process	5.128345925177171e-7
		GO:BP	macromolecule localization	0.004034220640730375
		GO:BP	positive regulation of mRNA splicing, via spliceosome	0.005891156456051293
		GO:BP	positive regulation of RNA splicing	0.005891156456051293
		GO:BP	positive regulation of mRNA processing	0.005891156456051293
		GO:BP	organonitrogen compound biosynthetic process	0.007766806983847455
		GO:BP	protein transport	0.009348219522549382
		GO:BP	nucleobase-containing small molecule metabolic process	0.010188690631060525
		GO:BP	cellular localization	0.016278269311732516
		GO:BP	vesicle-mediated transport	0.017446952242870566
		GO:BP	cellular macromolecule localization	0.018161618120385477
		GO:BP	protein localization	0.018161618120385477
		GO:BP	positive regulation of mRNA metabolic process	0.019717628659807848
		GO:BP	organic substance transport	0.02057106573158788
		GO:BP	organophosphate metabolic process	0.0230152949888641
		GO:BP	aspartate family amino acid metabolic process	0.02418914442169922
		GO:BP	establishment of protein localization	0.035416911413240826
		GO:BP	establishment of localization in cell	0.03608466588374706
		GO:BP	organophosphate biosynthetic process	0.04342420131435845
		GO:CC	cytoplasm	0.000010972201260565011
		GO:CC	plastid	0.01313861563014075
		GO:CC	organelle envelope	0.013690210400966211
		GO:CC	envelope	0.013690210400966211
		GO:CC	organelle inner membrane	0.016908503752418175
		GO:CC	eukaryotic translation initiation factor 4F complex	0.04995258246969349
		GO:CC	cytoplasmic stress granule	0.04995258246969349

Table 1: Gene ontology (GO) terms of MS detected proteins found heat-treated samples of wt and *tflls-cr2* mutant plants.

6.7 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 (Fig. 16A), 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 *tflIs-cr* mutants. In the *tflIs-cr1*, the *TFIIS* mRNA remains at a basal level throughout all treatments (Fig. 16B); notably, in this line, the basal level (at NT situation) is already lower compared to wild-type plants. In the *tflIs-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 (Kerenyi et al., 2008), likely the *tflIs-cr1* transcript is very efficiently targeted for decay.

In the *tflIs-cr2* and *tflIs-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* (Szadeczky-Kardoss et al., 2022), 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.

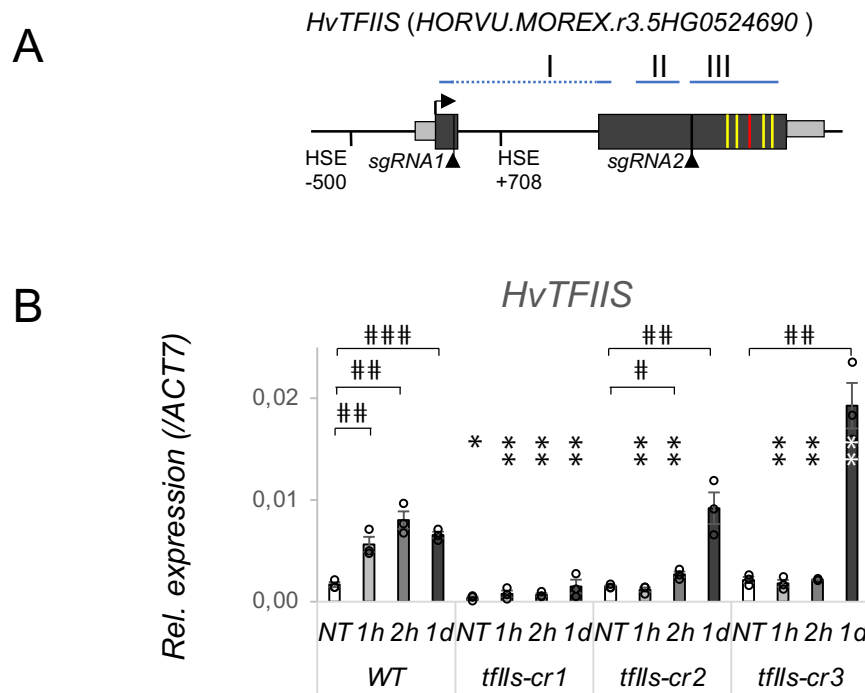


Figure 16: TFIIS is heat- and positively feed-back regulated. (A) Schematic representation of *HvTFIIS* gene locus; protein domain I, II and III location are shown above; exons as black boxes, UTRs as grey boxes, Zn finger domain cysteine residues and DE acidic dipeptide motif as yellow and red line, respectively; HSE *cis* element locations are shown below; (B) *TFIIS* mRNA changes during a timeseries (NT; 1 hour, 1h; 2h; 1 day, 1d); bars represent standard errors based on three bio reps; P-values based on two-tailed Student's t-test *P < 0.05, **P < 0.01, ***P < 0.001 show differences between wt and mutants at the given timepoint; #P < 0.05, ## P < 0.01, ###P < 0.001 values show differences between NT and heat stress condition within each genotype.

Based on these findings, we propose a working model of TFIIS function in barley (Fig. 17). 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 (Fig.17).

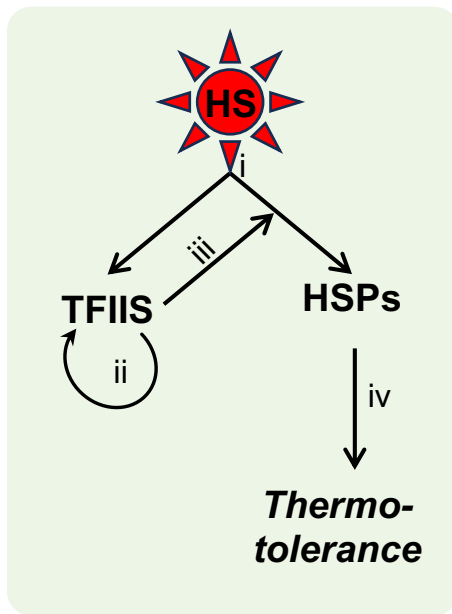


Figure 17: Working model for TFIIS action during heat stress response in barley: (i) Elevated temperature induces expression of HSPs and TFIIS; (ii) TFIIS elongation factor positively feed-back regulates itself and (iii) further enhances expression of HSP transcription; (iv) the timely accumulation of HS-transcripts and proteins ensures efficient thermotolerance in barley plants.

6.8 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 (Kis et al., 2024), 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 (Fig. 18A).

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 (Fig. 18B). 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 (Kerenyi et al., 2008).

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) (Fig. 18B). 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 (Fig. 18C). 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 (Fig. 18D, E). Both proteins accumulated to similar levels following early (1h) or late (1d) heat treatment in both wild-type and *gw2.1* mutant lines.

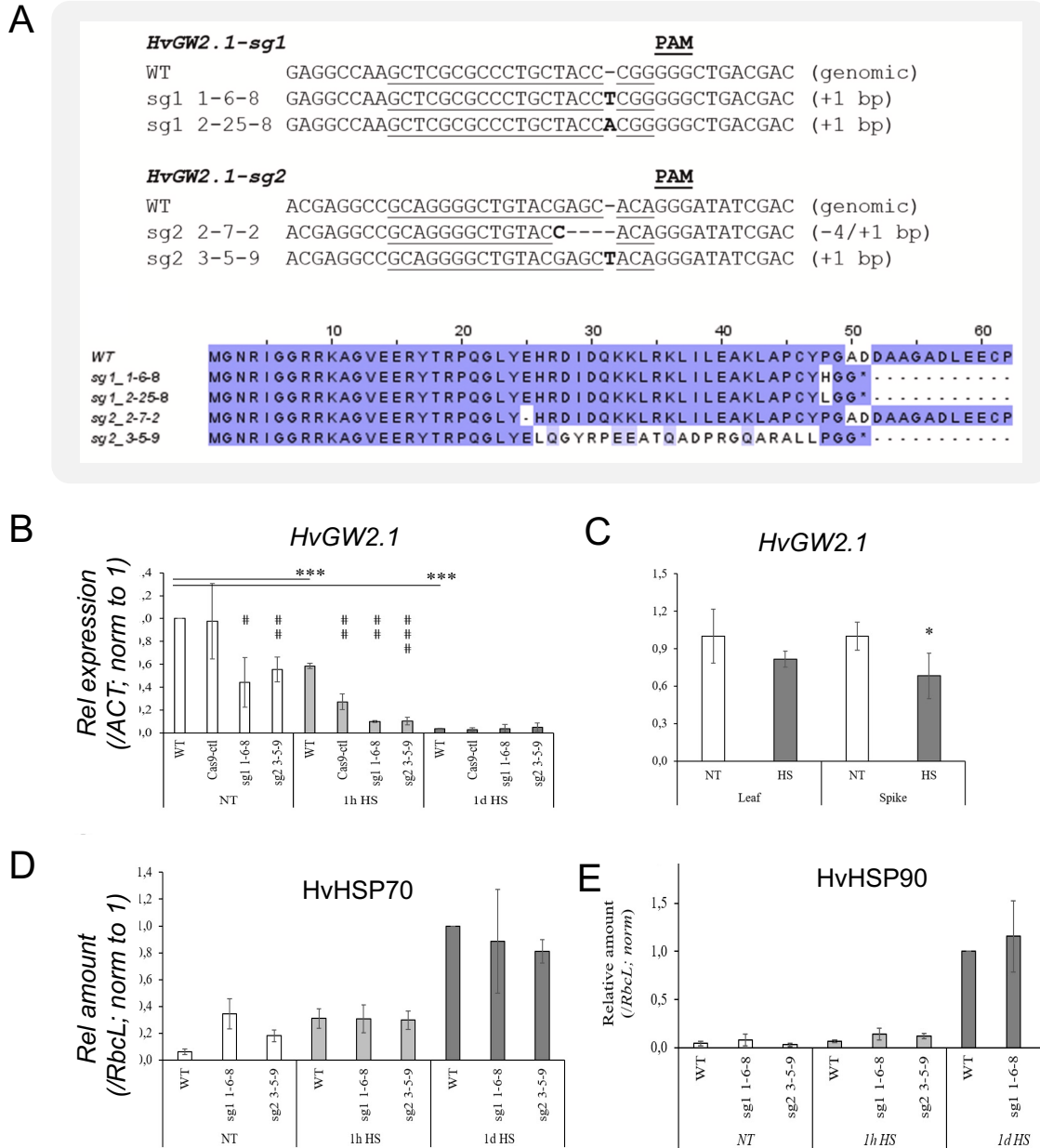


Figure 18: GW2.1 is downregulated by heat stress, but its absence does not directly impact production of HSPs during barley grain filling stage (A) CRISPR-mediated *gw2.1* mutant lines used in the study; independent transgenic progeny plants at T2 derived from sgRNA1- or sgRNA2-mediated Cas9 mutation. The underlined sections represent the target sites of the sgRNAs. Bold letters show the nucleotide insertions. Bottom: *In silico* translation of the *gw2.1* variants. N terminal fragment (62 amino acids) of GW2.1 is shown for each; (B) *GW2.1* mRNA expression changes in response to heat stress treatment as determined by sqPCR in seedlings, and (C) expression of *HvGW2.1* in barley leaf and spike under control (18 °C) and mild heat stress (30 °C, 1 day), showing reduced expression particularly in spike tissue; normalised to *Actin*. (D) HSP70 and (E) HSP90 protein accumulation changes in response to HS (non-treated, NT; 1 hour, 1h HS; or 1 day, 1d HS at 37 °C); normalised to Rubisco large subunit (RbcL). Samples are shown below (wild type, WT, Cas9-control, *gw2.1* mutants sg1_1-6-8 and sg2_3-5-9); P-values based on two-tailed Student's t-test (#P < 0.05, ##P < 0.01, ###P < 0.001 significance against WT control; *P < 0.05, **P < 0.01, ***P < 0.001 significance against NT control).

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 (*data not shown*).

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.

7 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 (Fig 2B), as well documented in dicots and in non-plant organisms such as yeast or mammals (Michl-Holzinger et al., 2022, Obermeyer et al., 2023b, Obermeyer et al., 2022, Szadeczky-Kardoss et al., 2022, Antosz et al., 2017, Cermakova et al., 2021, Conaway and Conaway, 2019, Godoy Herz et al., 2019, Aoi and Shilatifard, 2023). 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* (Liu et al., 2023) and *Arabidopsis thaliana* (Durr et al., 2014). 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 (Liu et al., 2023).

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 (Fig 3B) and is critical for rapid transcriptional reprogramming during heat stress (Szadeczky-Kardoss et al., 2022, Obermeyer et al., 2023b, Szaker et al., 2025). 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 (Fig 6). 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 (Fig. 11A). In *Arabidopsis*, the *tfIIs* mutant plants display essentially normal phenotype (Grasser et al., 2009, Szadeczky-Kardoss et al., 2022). 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*), (Fig. 11B) (iii) mutants originating from sgRNA2 mutagenesis had a significantly decreased germination rate (the *tfIIs-cr2* and *tfIIs-cr3*), (Fig. 11C) and (iv) all *tfIIs-cr* mutants had a significantly reduced TGW value of about 70-75% versus wild-type plants (Fig. 11D). These findings are in accordance with findings in rice (Liu et al., 2023). 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 (Fig. 11C). 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!) (Fig. 11), 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 factor (Obermeyer et al., 2023b, Szadeczky-Kardoss et al., 2022). 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 (Fig. 12). Wild-type plants' higher survival rate is supported molecularly by the quick induction of HS-transcripts and production of defensive HSPs (Fig. 13), 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 (Fig. 16) (Ahmad et al., 2024). 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 (Fig. 6 and 7). 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 (Fig 6B). A sequence expansion was predicted *in silico* to form additional α -helix structures (between $\alpha 6$ and $\alpha 7$) in monocot species, including barley (coined as $\alpha 6$ -b, $\alpha 6$ -c). $\alpha 6$ -b/ $\alpha 6$ -c together with $\alpha 7$ helices form a charged surface that may be a platform for protein binding through charge-charge interactions (Fig. 7B). 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 (Moore et al., 2007). Although further work could uncover commonalities or peculiarities of the pathway within monocot and dicot species, work done in barley (Ahmad et al., 2024) and previous work in *Arabidopsis* (Szadeczky-Kardoss et al., 2022, Szaker et al., 2025, Obermeyer et al., 2023b) 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 (Kis et al., 2024). 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 (Fig. 18B). 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 (Kis et al., 2024). 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 (Ahmad et al., 2024, Kis et al., 2024) Highlight the complex, intricate regulation of seed development, germination, and grain filling during adaptation to high-temperature conditions.

8 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 $\alpha 6$ -b/6-c insertion between $\alpha 6$ and $\alpha 7$ 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.

9 SUMMARY

The present study is built on two parts, investigating an elongation cofactor, TFIIS, and a protein stability regulator, GW2. The two topics are seemingly distant; however, we envision several connection points: (i) the plant system being studied is barley in both cases; (ii) CRISPR mutagenesis is used as a tool for mutant creation; (iii) the impact of mutation of these factors affects grain quality, being an important character to study in crops; and (iv) we analysed HS adaptation of both mutants (although we failed to show any roles of GW2).

Barley is a major cereal and climate-resilient model crop, but heat episodes during flowering and grain filling reduce yield. Plants respond to heat through a heat stress response driven by heat shock factors (HSFs) and heat shock proteins (HSPs), yet most work has centred on transcription initiation. This thesis instead emphasises transcription elongation: RNA polymerase II frequently stalls or backtracks on chromatin, and TFIIS rescues these arrested complexes by stimulating RNA cleavage so elongation can resume, a role that becomes critical when heat increases transcriptional errors and pausing. Sequence analyses across land plants show that TFIIS is strongly conserved, including catalytic zinc-finger cysteines and the DE di-acid motif, with monocots carrying an extra charged helix pair ($\alpha 6$ -b/ $\alpha 6$ -c) that may add interaction capacity. Using CRISPR/Cas9, barley mutants were generated: *tfIIs* mutants, early-exon frameshifts (*tfIIs-cr1*) that abolish the protein and late frameshifts (*tfIIs-cr2/cr3*) predicted to remove the catalytic zinc finger. Mass spectrometry confirmed loss of functional TFIIS in all lines. Under normal conditions, vegetative growth was essentially unaltered. However, reproductive traits were strongly affected: sterile or non-germinating lines from the strongest knockouts, delayed germination in partial-function mutants, and a consistent drop in thousand-grain weight to about 70–75% of the wild type. This shows that while TFIIS is dispensable for most vegetative growth, it is essential for reproductive development, proper grain filling, seed viability and overall reproductive fitness.

Heat stress (40 °C, 1–3 days) revealed that all *tfIIs* mutants are highly heat-sensitive compared with the wild type. Molecular analyses showed that wild-type barley rapidly induced HSP70, HSP90 and HSP101 transcripts and proteins within 1–2 hours of heat, while mutants displayed severely delayed and reduced early induction. After 1 day of heat, HSP transcripts in wild-type declined but remained abnormally high in mutants, consistent with prolonged HSF activation driven by unresolved proteotoxicity. Protein fractionation and mass spectrometry confirmed a marked increase in insoluble

protein aggregates in *tfIIs-cr2* under heat, with stress-related proteins and sHSP-CI accumulating in the insoluble fraction, indicating that chaperone capacity is exceeded. Thus, loss of TFIIS leads to defective, slow HSP synthesis, widespread protein misfolding and aggregation, and failure to maintain proteostasis under heat.

TFIIS itself is strongly heat inducible; the gene locus contains canonical HSEs, placing it under HSF control. In the wild type, its mRNA peaks around 2 hours of heat stress. In *tfIIs-cr1*, the transcript is NMD-sensitive and remains low at all times. In *tfIIs-cr2/cr3* lines, the basal levels of *TFIIS* mRNA are normal, but early heat induction fails to show a strong increase at the later time points. These patterns point to an autoregulatory loop in which functional TFIIS is required for efficient elongation of its own transcript during heat stress, as observed in wild-type vs *tfIIs-cr*; if the TFIIS protein fails to be produced, the transcriptional activation persists, likely as a secondary rescue pathway to replenish the missing factor.

Based on the present data in barley (Ahmad et al., 2024) and earlier observations in *Arabidopsis* (Szadeczký-Kardoss et al., 2022) We proposed a working model, based on which (i) HS activates HSFs (ii) to induce TFIIS accumulation and (iii) and boost HS-transcript production at elongation levels; (iv) accumulation of HS-products ensures proteostasis and (v) ultimately contributes to HS-survival.

During collaborative work (Kis et al., 2024), We were engaged in the examination of GW2.1, a RING-type E3 ubiquitin ligase. GW2.1 regulates grain width, weight and protein content through destabilising proteins via the ubiquitin proteasome system; GW2 is functionally conserved among cereals. Using CRISPR mutagenesis, we have created *crispr-gw2.1* mutants; these show roughly half the normal GW2.1 mRNA level, most likely due to a mutational frameshift making the transcript NMD targets. *crispr-gw2.1* exhibits changes in the balance between grain yield and protein content (vs wild-type plants). Expression studies demonstrate that GW2.1 is strongly downregulated by severe and mild heat, especially in developing spikes, suggesting that it integrates thermal signals into grain development. However, HSP70 and HSP90 accumulation and seedling thermotolerance are unaffected in *gw2.1* mutants, indicating that GW2.1 does not directly participate in the canonical HSR. Instead, GW2.1 appears to tune the trade-off between seed number and grain quality, the mutation resulting in fewer but more protein-rich grains; this may be an adaptation through optimising transgenerational fitness.

Overall, our findings highlight the multi-level complexity of grain development and provide a foundation for future research into this critical stage of crop reproductive biology. Methodologically, this study demonstrates that CRISPR-based mutagenesis is a highly efficient tool for generating functional mutants, offering valuable resources for further molecular investigations into development and stress adaptation in monocot species.

10 PROSPECTS

Building on the current data, we are planning to explore in our future research the alternative pathways that may genetically interact with the TFIIS machinery.

To this end, we are investigating the combined effects of mutations in TFIIS with general RNA quality-control factors and other elongation factors.

Furthermore, we aim to examine various facets of transcriptional regulation under heat stress, specifically fidelity, speed, and processivity, using both *Arabidopsis* and barley as model systems.

These investigations are currently underway.

11 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.

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14 APPENDICES

14.1 List of scientific activities

14.1.1 Presentations related to Thesis

- 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 TFIIIS 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 Szadeczky-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.

14.1.2 Publications related to Thesis

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

14.1.3 DNA Oligo used in the study

Crispr constructs	
HvTFIIIS cr1 F	GGC GGT TGA CGC GGA ACT CGC GG
HvTFIIIS cr1 R	AAA CCC GCG AGT TCC GCG TCA AC
HvTFIIIS cr2 F	GGC GGC GAG CGA TGC CCG TAA GC
HvTFIIIS cr2 R	AAA CGC TTA CGG GCA TCG CTC GC
Genotyping	
HvTFIIIS crispr1 check F	CTG CGC CTA GGG TTT TCA TC
HvTFIIIS crispr1 check R	GCT TTC CGC ACC TCT CAG TC
HvTFIIIS crispr2 check F	ACA GTG GAG TCG GCC TTG TT
HvTFIIIS crispr2 check R	TAC GAA AAA CCG AGC CAA CC
HvTFIIIS cr1 check-qF	GGA GAG GGA GCT GAT GGA GA
HvTFIIIS cr1 check-qR	CGA TAG GGC ACG GAT AGT CTG
qRT-PCR	
HvTFIIIS qF:	TCG CCA CGC AGG TTG GCA AAC G
HvTFIIIS qR:	TTC AAT AAC AAC CTT CTT CCA G
HvActin7 qF	CGT GTT GGA TTC TGG TGA TG
HvActin7 qR	AGC CAC ATA TGC GAG CTT CT
HvHSPc70-4 qF	CAA CAC CGT TTT TGA TGC CA
HvHSPc70-4 qR	ACC ACG ATC ATC GGC TTG T
HvHSP90 qF	AAC TCA TCT GAC GCG CTT GA
HvHSP90 qR	GCT GTC GAT GAT GGA GAG CGT
HvHSP101-1 qF	GTC ATG CAG GAG GTG AGG AGG
HvHSP101-1 qR	CCA CGT CCT TCA TCT GCA G

Supplementary Table 1: DNA oligonucleotide primers used in the study.