HUNGARIAN UNIVERSITY OF AGRICULTURE AND LIFE SCIENCES

GENETICS AND BIOTECHNOLOGY INSTITUTE

DOCTORAL SCHOOL OF BIOLOGICAL SCIENCES

Transcriptional regulation of heat stress response in plants

DOI: 10.54598/007360

DOCTORAL (PhD) DISSERTATION

RADHIKA VERMA

Gödöllő, Hungary
2025

Doctorate School

Name: Doctoral School of Biological Sciences

Science: Agricultural Sciences

Discipline: Plant Biotechnology

Head: Prof. Dr. Erika Csákiné Michéli, CMHAS
Head of Doctoral School
MATE, Hungarian University of Agriculture and Life Sciences
Doctoral School of Natural Sciences

Program Leader: Prof Dr Zoltán Nagy
Professor, DSc
MATE, Hungarian University of Agriculture and Life Sciences
Biological Sciences Program Leader

Supervisor: Tibor Csorba, PhD
MATE, Hungarian University of Agriculture and Life Sciences
Institute of Genetics and Biotechnology

…….. ……..
Approval of the Head of Doctoral School Approval of the Program Leader

..……..
Approval of Supervisor

TABLE OF CONTENTS

ABBREVIATIONS

ATP	Adenosine Triphosphate
BRE	TFIIB Recognition Element
BT	Basal Thermotolerance
CTD	Carboxy-terminal domain
Chd1	Chromatin Remodeller 1
DPE	Downstream Promoter Element
DBD	DNA Binding Domain
DSIF	DRB Sensitivity-Inducing Factor
EC	Elongation Complex
EJC	Exon Junction Complex
eRF1	Eukaryotic Release Factor 1
eRF3	Eukaryotic Release Factor 3
ELF1	Eukaryotic Leucine Zipper Factor 1
ELF7	Early Flowering 7
FACT	Facilitates Chromatin Transcription
GTFs	General Transcription Factors
HS	Heat Stress
HSFs	Heat Stress Factors
HSEs	Heat Stress Elements
HSR	Heat Stress Response
HsfA1	Heat Stress Factor A1
HSPs	Heat Shock Proteins
HSP 101	Heat Shock Protein 101
HSP 90	Heat Shock Protein 90
Inr	Initiator Element
IMP	Inosine Monophosphate
lncRNA	Long non-coding RNA
LAT	Long-Acquired Thermotolerance
mRNA	Messenger RNA
MPA	Mycophenolic Acid
NMD	Nonsense Mediated Decay
ORF	Open Reading Frame
OC	Open Complex
PAF1c	Polymerase Associated Factor 1 Complex
PTC	Premature Termination Codon
PABP1	Poly(A)-Binding Protein 1
PEC	Paused Elongation Complex
PIC	Pre-Initiation Complex
PTM	Post-Translational Modification
rRNA	Ribosomal RNA

RdDM	RNA-directed DNA Methylation
RNAPI	RNA Polymerase I
RNAPII	RNA Polymerase II
RNAPIII	RNA Polymerase III
RNAPIV	RNA Polymerase IV
RNAPV	RNA Polymerase V
Rpb1	RNA polymerase II subunit 1
Rpb2	RNA polymerase II subunit 2
Rpb3	RNA polymerase II subunit 3
Rpb9	RNA polymerase II subunit 9
Rpb12	RNA polymerase II subunit 12
IIS-N	TFIIS- N terminal
IIS-C	TFIIS- C terminal
SAT	Short-Acquired Thermotolerance
sHSP	Small Heat Shock Protein
SSRP1	Structure Specific Recognition Protein 1
SPT4	Suppressor of Ty 4
SPT6	Suppressor of Ty 6
SMG7	Suppressor of Morphogenesis 7
sppRNAs	short promoter-proximal RNAs
sRNA	Small RNA
snRNA	Small nuclear RNA
TATA	TATA Box
TFIIA	Transcription Factor IIA
TFIIB	Transcription Factor IIB
TFIIF	Transcription Factor IIF
TFIIH	Transcription Factor IIH
TFIID	Transcription Factor IID
TFIIS	Transcription Factor IIS
TBP	TATA-Binding Protein
TMHT	Thermotolerance to Moderately High Temperatures
TND	TFIIS N-terminal Domain
TEFs	Transcription Elongation Factors
TEC	Transcription Elongation Complex
tRNA	Transfer RNA
3’UTRs	3' untranslated regions

1. INTRODUCTION

Temperature stress is one of the major abiotic stresses that negatively affects the survival and reproductive fitness of living organisms worldwide. Cells exposed to heat stress activate a series of biochemical and physiological changes that result in a reprioritisation of cellular physiology to aid survival; these processes are collectively called heat stress response (HSR). A central layer of HSR is the transcriptional regulation. While significant efforts have been put into understanding the activity of specific transcription factors that coordinate HSR, and the differentially expressed gene/transcript sets they regulate, *the understanding of general transcriptional machinery regulation and its specific functions during HSR remains scarce*.

In the present work, we aimed to unravel the regulation of RNA polymerase II (RNAPII) core machinery and its associated co-factors. To achieve this, we conducted a heat stress phenotyping screen on selected RNAPII and co-factor mutants and identified the Transcription Factor IIS (TFIIS) as a key player of HSR.

TFIIS is a biochemically and structurally conserved transcription elongation cofactor of RNAPII. While, it has been extensively studied in yeast and metazoan systems, *its regulation and functions in plants, specifically during heat stress responses have not been explored*. Therefore, we characterised the conservation, regulation, functions and molecular mechanisms of TFIIS in plants and its requirements under high-temperature conditions. We demonstrated that TFIIS *enable plants to conduct a timely, qualitative and quantitative reprogramming of transcriptome*, which is vital for heat stress survival. Besides, we have *described* TFIIS *molecular actions on transcriptional fidelity processes at the single nucleotide level* for the first time in plants.

In summary, we believe that our study on transcriptional elongation and the roles of TFIIS will contribute to basic understanding of HSR and will accelerate breeding of heat-tolerant crops crucial for minimizing the threats posed by global warming and climate change.

2. LITERATURE REVIEW

2.1. RNA transcription process in eukaryotes

RNA transcription is a vital cellular process in all living organisms. In eukaryotes, there are three main nuclear DNA-dependent RNA polymerase complexes (RNAPI, II, III). RNAPI produces the ribosomal RNAs (rRNA), the RNAPII transcribes messenger RNAs (mRNAs) and most of the regulatory short and long non-coding RNAs (sRNA, lncRNAs), while the RNAPIII transcribes the transfer RNAs (tRNAs), the 5S rRNA and the U6 small nuclear RNA (snRNA) (*1-3*). Plants have evolved two additional RNA polymerases RNAPIV and V, which are not strictly required for survival; these primarily synthesize regulatory RNAs involved in the RNA-dependent DNA methylation pathway (RdDM)(*4, 5*).

The overwhelming majority of protein-coding transcripts are produced by RNAPII. RNAPII is composed of 12 core subunits, Rpb1-to-12 (*1, 3, 6*). The three largest subunits, Rpb1, Rpb2 and Rpb3 assemble into a horseshoe-shaped structure that embodies the DNA-binding cleft and holds the active catalytic site (*1, 3, 6*). The other subunits (Rpb4-12) are associated with the core on the periphery. The active site components, including the aspartic loop that binds to catalytic metal ion, the bridge helix, and the trigger loop, work together as a flexible racket that drives translocation during RNA chain elongation. The polymerase translocation mechanism is widely conserved. The carboxy-terminal domain (CTD) of Rpb1 plays an important role in coordinating transcriptional regulation during the different stages of the transcription (*1, 3, 6*). The CTD is a tail-like structure that contains heptapeptide tandem repeats with the consensus sequence YSPTSPS (34 heptad units in *A. thaliana*), serving as target to kinases, phosphatases, and a landing scaffold for protein cofactor binding.

The RNA transcription cycle is divided into three main stages: *initiation, elongation* and *termination* (Fig 1). During initiation, specific DNA sequences at the gene promoter, as well as proximal and distant enhancer elements are recognized by specific activators and general transcription factors (GTFs). Together, these factors promote the assembly of the RNAPII complex assembly, known as the pre-initiation complex (PIC). Briefly, the transcription factor TFIID and TBP (TATA-binding protein) bind the promoter of target genes at specific *cis*-elements (including TATA, Initiator Element (Inr), Downstream Promoter Element (DPE), TFIIB Recognition Element (BRE) etc.). This interaction tether other initiation co-factors such as TFIIA, TFIIB, TFIIF and the core RNAPII complex itself. In the next phase,

the promoter DNA is un-winded by TFIIH (RNAPII open complex, OC) and Rpb1 and other cofactors are phosphorylated, so transcription transitions into the early elongation stage.

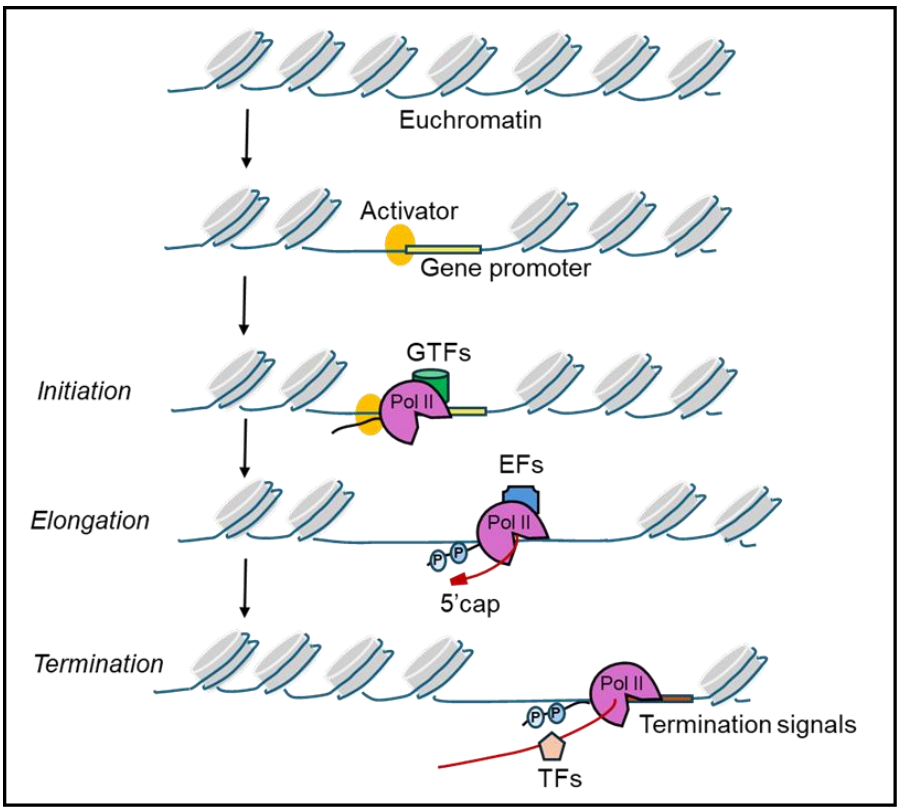

Figure 1: The scheme of key phases of RNAPII transcription process: Initiation, Elongation, and Termination. *Transcription begins with chromatin remodelling. Pioneer transcription factors bind to promoter elements, depleted this of nucleosomes, and initiate core RNAPII and general transcription factors' binding (Pre-initiation complex, PIC). Next, the stabilised RNAPII and bound cofactors escape from promoter and enters into the productive elongation phase (elongation complex, EC) to transcribe the whole locus. Maturation and RNA splicing occurs mostly co-transcriptionally. Upon encountering the termination signal RNAPII complex slows down. Termination factors (TFs) attach to the RNAPII, cleave and eliberate the nascent RNA, while the RNAPII complex is subsequently disassembled and recycled.*

RNAPII is temporarily halted at the promoter-proximal pause region(s) (paused elongation complex, PEC)(*3, 6-8*). This is a quality control step which allows maturation of both RNAPII complex components (e.g. post-translational modification of CTD, co-factor loading, exchange of initiation cofactors to elongation cofactors, etc.) and nascent RNA (e.g. capping, loading of splicing machinery etc.) (*9-11*). The promoter escape of PEC results in either premature termination through the action of the Integrator complex (*12*) or productive elongation (elongation complex, EC). In plant systems, an Integrator-like complex may act to produce the so-called short promoter-proximal RNA transcripts (sppRNAs) (*8*)). As RNAPII transcribes along the gene, most nascent RNAs are co-transcriptionally processed and spliced by splicing complexes associated with RNAPII. (*9, 10, 13*). Finally, transcription is terminated by terminator co-factors, post-translational modifications (PTM) changes of RNAPII subunits,

and mRNA polyadenylation (*14*). Finally, the capped, spliced and polyadenylated mRNA bound by a plethora of RNA-binding protein factors is released from the locus and exported into the cytoplasm to take part in protein translation (*15*).

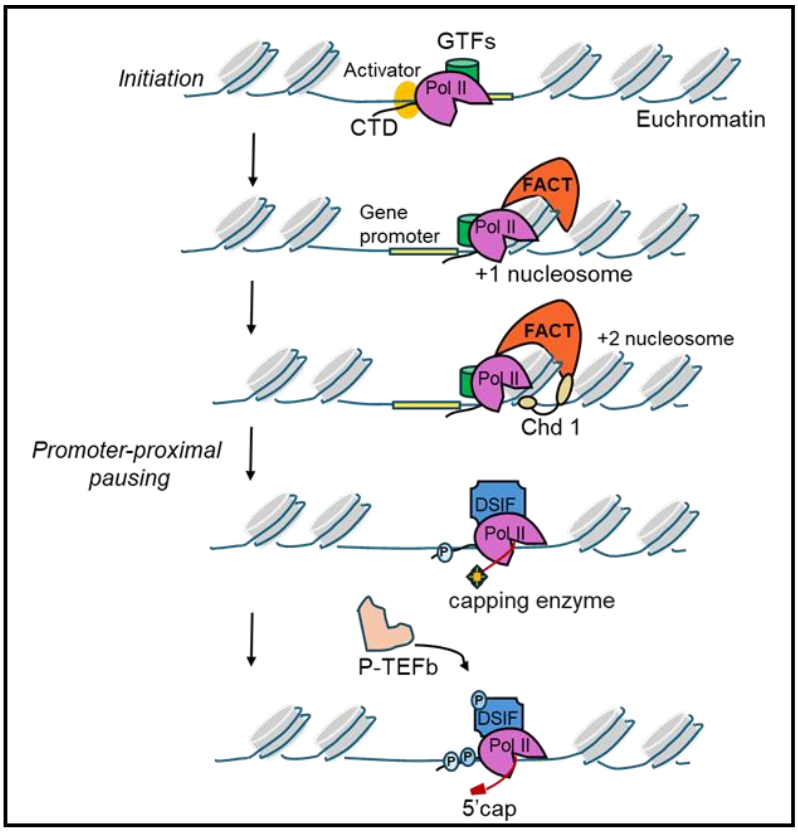

Figure 2: The phases of transcriptional elongation and their associated factors. *Following the initiation phase, the transcription transitions into early elongation. Elongation factors such as FACT and Chd1 help displace nucleosomes, loosening the histone-DNA interactions and enabling RNA polymerase to move through the chromatin: RNAPII naturally pauses promoter-proximally: this allows nascent RNA capping and quality control of the elongation complex through phosphorylation. If RNAPII elongation is validated, transcription will enters productive elongation after the phosphorylation of DSIF and CTD of RNAPII by P-TEFb*

Several recent studies show that the transcriptional elongation phase is one of the rate-limiting steps of gene expression (Fig 2). Elongation is dampened by several conditions (*16*). (i) Before entering productive elongation, at the promoter-proximal pausing halts the RNAPII complexes. This transient stop ensures co-factors validation, including post-translational modification of core RNAPII and cofactors, exchange of initiation factors for elongation factors, and quality control of the nascent RNA including RNA capping. It was proposed that progression into productive elongation (e.g. promoter-proximal escape time) back-regulates the transcriptional initiation rates (*17*). (ii) Downstream, as the RNAPII moves through the gene body, the DNA template is wrapped around nucleosomes, which must be unwrapped for transcription. The chromatin structure slows the transcription and therefore is a rate-limiting

element. The histone chaperone complex, chromatin remodeller 1 (Chd1), , FACT (Facilitates Chromatin Transcription (), and some other factors help RNAPII to transcribe nucleosomal DNA efficiently and overcome this structural impediment (*18*); (iii) During elongation the nascent RNA is co-transcriptionally spliced. The splicing process also impacts elongation and *vice versa*: transcriptional speed regulates alternative splicing (*9, 10*); (iv) For smooth transcription, incorporation of the correct nucleotide is needed: the addition of the template-matching nucleotide promotes forward translocation of EC through the so-called *ratchet* mechanism, whereas ribonucleotide misincorporations, insertions and deletions result in arrests of transcription (*19, 20*).

A wide range of transcription elongation factors have been identified that help the progression of RNAPII complex through the gene body and overcome the various hurdles. The biological functions of plant elongation factors (including PAF1c, FACT, DSIF, SPT6, TFIIS, ELF1, etc.) are now being revealed (*21-29*). It is becoming apparent that plant TEFs are implicated in a wide range of developmental and stress responses. Polymerase-associated factor 1c (PAF1c) is needed for transition to flowering in *A. thaliana* (*30*); *AtFACT* complex subunit mutants show various developmental and reproductive phenotype changes (*31*), OsSPT4, OsSPT5-1, and OsSPT5-2 have roles in vegetative and reproductive growth of rice (*29*), AtPAF1c mutants *elf7* and *elf8* are salt sensitive (*26*); *ssrp1* and *spt16* (FACT mutants) are affected in flavonoid biosynthesis during high-light stress (*32*); FACT complex is needed for progression through first nucleosome under rapid transcriptional induction conditions like heat stress (*33*). *AtTFIIS* mutants have a reduced seed dormancy and are mildly early flowering plants (*22*). Notably, however, the mild developmental phenotype of *tfIIs* mutant plants suggested that RNAPII transcriptional arrests are rare in plant systems. Contrary to this assumption, later studies showed that TFIIS Dominant Negative mutant protein expression causes lethality (*28, 34*), proving that RNAPII arrests do occur and liberation of the transcriptional elongation complex is a vital element of the *A. thaliana* transcriptional elongation process.

2.2. Transcriptional RNA quality control pathway

To ensure the faithful transmission of genetic information from the genome to produce the myriads of cellular components (needed during development and environmental adaptation), transcriptome quality is continuously surveyed and corrected. RNA is carefully

monitored at multiple stages to ensure accuracy, including nuclear (co-transcriptional) and cytoplasmic (co-translational) RNA surveillance (*35, 36*). Errors can occur during transcription, which makes mistakes such as nucleotide substitutions, insertions, and deletions in coding mRNAs. These errors can result in the production of non-functional, dominant negative or malfunctional proteoforms, that consume valuable cellular energy and disrupt protein homeostasis (*35, 37-40*). The RNAPII complex possesses a co-transcriptional fidelity function: the subunits of RNAPII or specific regions (Rpb1 trigger loop and Rpb9 subunit) ensure high transcription accuracy by selecting the correct nucleotide in the pre-polymerization phase (*40-42*). If faulty nucleotides are still incorporated, the RNAPII arrests to rectify these mistakes. Upon arrests, rearrangements of the RNAPII active centre occur. Persistent arrests result in backtracking events, during which the 3' end of the nascent RNA is displaced from the active catalytic core site of RNAPII (*20, 43, 44*).

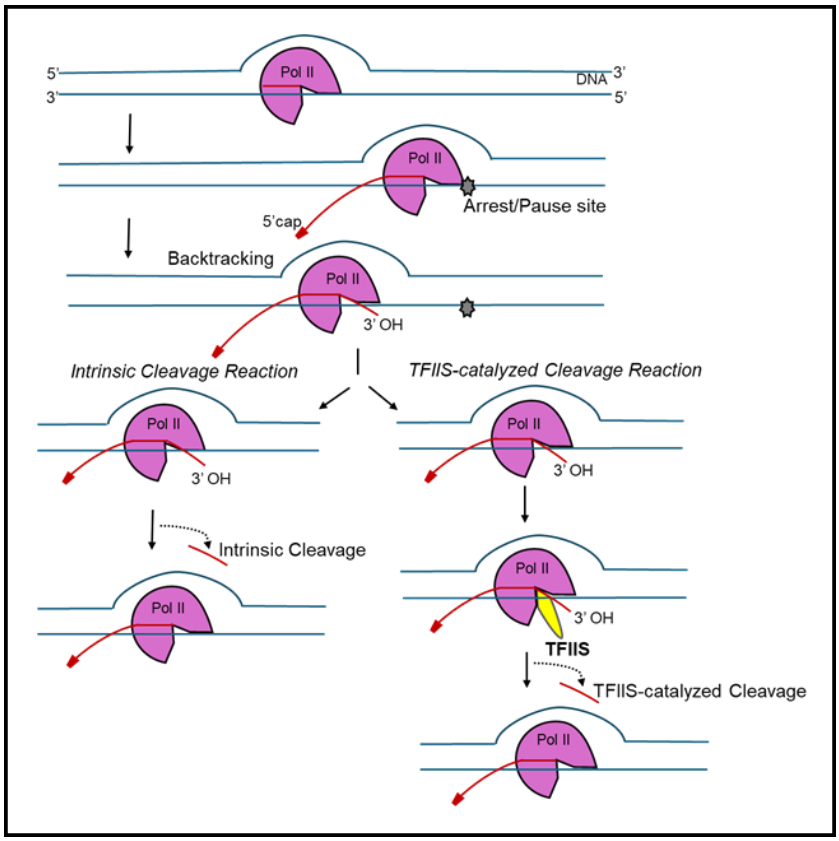

Figure 3: A schematic diagram shows how RNAPII recovers from an arrested state, through intrinsic (TFIIS-independent) and TFIIS-dependent mechanisms, resolving transcriptional arrests and backtrackings. *The intrinsic cleavage reaction involves the cleavage of RNA by RNAPII, but exhibits weak activity; therefore, it is a time-consuming process. In contrast, the TFIIS-dependent mechanism includes the binding of the TFIIS factor, which stimulates fast cleavage of backtracked RNA by RNAPII catalytic core, enabling a fast resolution of the arrested state.*

To resume transcription, the 3' protruding segment of the nascent RNA segment needs to be removed. The catalytic core of RNAPII possesses a weak nucleolytic activity that enables the excision of the wrong base(s). This fidelity control and correction mechanism is slow, therefore, it limits the pace of elongation and the overall transcriptional output. To accelerate the intrinsic nucleolytic activity of RNAPII, a cleavage stimulatory elongation co-factor, TFIIS has evolved. TFIIS binds to the arrested complex and accelerates the cleavage reaction, following which the RNAPII complex is released fast for further elongation (*20, 43, 44*) (Fig 3). TFIIS is a highly conserved co-factor of the polymerase, present in all eukaryotes and there are also functional TFIIS homologs in bacteria and archaea (*44-47*). The TFIIS protein consists of three distinct domains: the N-terminal domain I (IIS-N), the middle domain II (IIS-M) and the C-terminal domain III (IIS-C) (Fig 4). The IIS-N (or TFIIS N-terminal Domain, TND) domain has a five-helical bundle with non-enzymatic functions and is responsible for nuclear localisation and protein interactions (*48, 49*); II-M together with a linker region allows binding to the core RNAPII complex; lastly, IIS-C domain forms a hairpin-like structure that is shaped by a zinc finger domain and holds an acidic dinucleotide (DE) at its tip. The IIS-C with its DE motif reaches inside the RNAPII core close to the catalytic site and stimulates the nucleolytic cleavage of RNA PII.

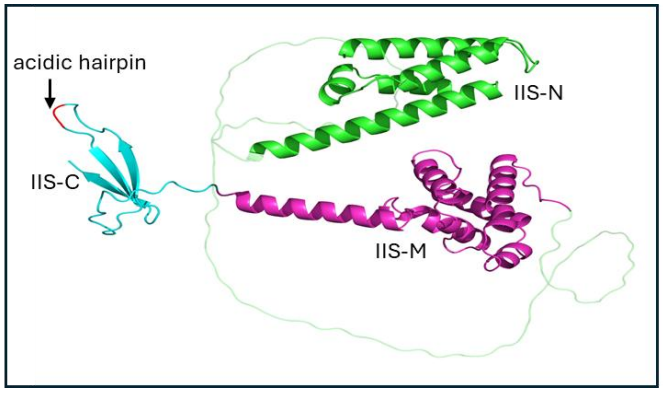

Figure 4: Cartoon representation of the alpha fold model of Arabidopsis TFIIS by PyMol. *The N-terminal domain I (IIS-N) is shown in green, the middle domain II (IIS-M) in magenta and the C-terminal domain III (IIS-C) in cyan, with two invariant acidic hairpin residues in red.*

In *Arabidopsis thaliana*, the dominant-negative mutant form of TFIIS (*TFIISmut*) was created by exchanging acidic hairpin catalytic residues (DE) with alanine (AA); this abolished transcript cleavage, causing severe developmental defects in Col-0 (wild-type background) and lethality in the *tfIIs-1* background. Mutations in other functional domains of TFIIS also cause defects, such as impaired interaction with RNAPII and loss of proper recruitment to

transcription complexes, further depicting that multiple regions of TFIIS are necessary for its role in transcriptional elongation and splicing regulation *(22, 24,31, 81).*

The fidelity roles of TFIIS have been studied in yeast and metazoan, but not in plant systems, so far.

2.3. Cytoplasmic nonsense-mediated RNA quality control (NMD) pathway

In addition to the nuclear fidelity control, post-transcriptional mRNA quality control systems also operate to ensure a high-quality transcriptome and consequently, an accurate proteome. The most detrimental error types are the substitutions that result in STOP codons or 1-2 nucleotide indels that cause open reading frameshifts in mRNAs. The frameshifts often lead to the appearance of premature termination codons (PTC). The PTC-mRNA transcripts would generate truncated proteins and generate/induce proteotoxicity.

Nonsense-mediated decay (NMD) is a cytoplasmic translation-termination coupled, conserved eukaryotic mechanism, that recognizes and degrades PTC-containing aberrant mRNAs, thereby preventing the accumulation of deleterious proteins (*50, 51*). While NMD is dispensable for viability in yeast or *C. elegans*, NMD null mutation is lethal in higher eukaryotes, causing embryo-lethality in fruit-fly (*52*), zebrafish (*53*) and mouse (*54*). Mutations in human NMD genes are associated with intellectual disabilities and cancer (*50, 54*). In the dicot model species *A. thaliana* null mutations in key NMD factors cause seedling lethality, while the hypomorph mutants display a range of developmental abnormalities (*55-57*). NMD identifies PTC-containing mRNAs through the presence of NMD eliciting *cis-elements* as upstream open reading frames (uORF), or unusually long and/or intron-containing 3'UTRs (*50, 51, 58-61*) (Fig 5). Mechanistically, NMD is activated because of non-effective translation termination caused by the NMD-eliciting features present in the mRNA. Whether the mRNA is degraded or released for the next round of translation is decided by the competition between the poly(A)-binding protein 1 (PABP1) and a key NMD factor UP-FRAMESHIFT1 (UPF1) and/or UPF3 for binding to the translation termination complex consisting of eukaryotic release factor 1 (eRF1) and eRF3 (*50, 51, 54*) (Fig 5). Normal eukaryotic 3'UTRs are relatively short and do not contain introns, thus Poly-A Binding Protein (PABP1) can bind to the termination complex and stimulate termination. Long 3'UTRs, however, trigger NMD by physically distancing PABP1 from the translation termination complex (62). Introns located downstream to the STOP codon also trigger NMD (63). mRNAs decorated and targeted for degradation by

NMD trans factors undergo de-capping, de-adenylation and general mRNA decay (50, 51, 54, 62, 64). In plants, NMD efficiency is regulated by various autoregulatory circuits. For instance, NMD targets the UPF3 and SMG7 NMD factors, as well as the eRF1-1 termination factor transcripts (36, 65-67).

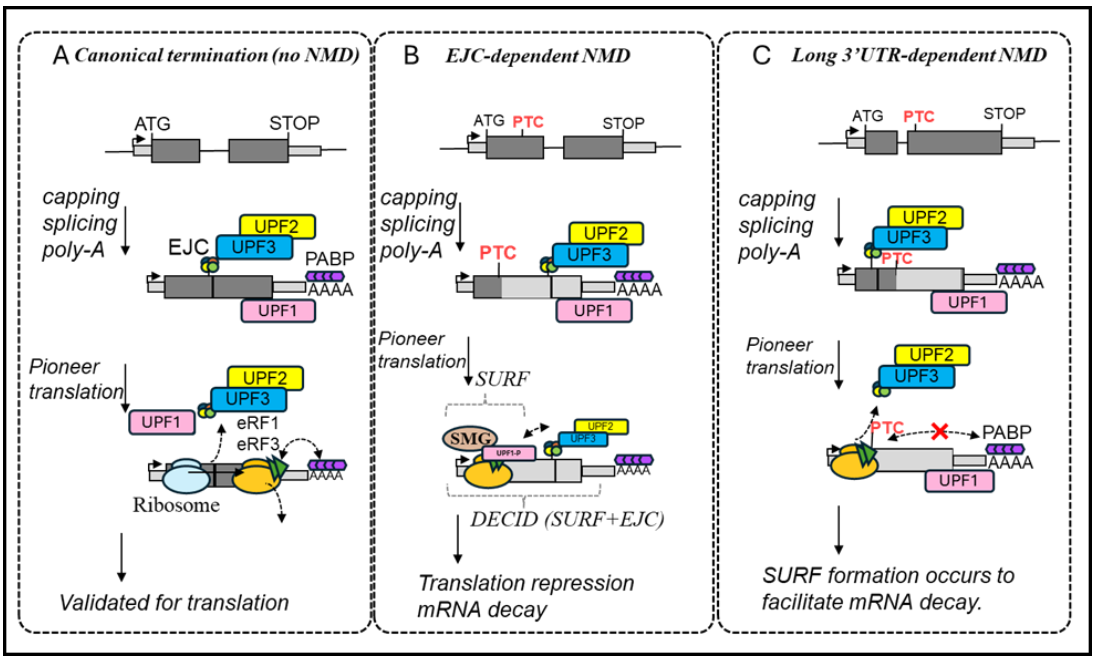

Figure 5: Comparison between the canonical and NMD-eliciting translation termination pathways. *(A) Matured mRNAs (capped, spliced and poly-adenylated) are exported to the cytoplasm for translation. The ribosome translates efficiently and reaches the natural STOP codon in the end of the final exon. Since there is no exon junction complex (EJCs) remaining downstream of the STOP codon within the 3'UTR, eRF1/3 can interact with poly(A)-binding protein (PABP1), therefore the mRNA is considered normal. As a result, translation proceeds to produce functional proteins, and NMD pathway remains in-activated. (B) The mRNA contains a PTC located upstream of EJCs. During translation, the ribosome stalls at the PTC, triggering the recruitment of UPF1, a central NMD factor. The EJC downstream of the PTC interacts with UPF2 and UPF3, forming an active surveillance complex. This recruits SMG proteins, and SURF (SMG1-UPF1-eRF) is formed; lastly, DECID (de-capping or exonucleolytic cleavage) is activated, leading to mRNA degradation via de-capping or exonucleolytic cleavage. (C) The mRNA contains long 3'UTR regions, which trigger NMD due to the inability of direct interaction between the PABP1 and eRF1/3 factors. This disrupts normal translation termination, and subsequently SURF formation occurs to facilitate mRNA decay.*

NMD features may be present in the genome/transcripts or can originate *de novo* from genomic mutations, alternative splicing or transcriptional errors. Frameshifts caused by insertions and deletions or nonsense (STOP-codon generating) substitutions in the ORF, can lead to the formation of PTC. Thus 1-2 nucleotide transcription errors can cause the decay of an mRNA via NMD (*40*). By destabilisation of these aberrant transcripts, NMD prevents the generation of potentially detrimental truncated proteins (*40*). The role of NMD during abiotic stress conditions is understudied (*50, 68*). It was suggested that NMD regulate cytoplasmic protein response (*69*), light signalling (*70*) or salt stress response (*60, 71*).

Whether NMD has roles during heat stress adaptation has not yet been considered.

2.4. Heat stress response pathways in plants

Earth's population is constantly rising. To meet the growing demands, global crop production needs to double by 2050 (*94*). Climate warming has a negative impact on plant survival and productivity (*100-105*). In addition, exposure to extreme heat can damage photosynthetic apparatuses, interfere with reproductive processes, and compromise seed quality, often resulting in substantial reductions in agricultural output. These physiological changes can be regulated through molecular pathways which are evolutionarily conserved across plant species *(73-78).* The ever-increasing demands will require novel technologies. A major limitation is the incomplete knowledge of stress responses; enlarging this wisdom/erudition could help breeders to obtain resilient plant species and varieties.

Excessive environmental heat causes severe damage to plant cells and organisms by impairing photosynthesis, reducing water content, damaging nucleic acids, proteins, and lipid membranes etc, thereby endangering survival and decreasing productivity. Plants cannot avoid exposure to these factors and must adapt morphologically and physiologically. Cells exposed to high temperatures (heat stress, HS) activate cellular changes, collectively referred to as heat shock responses (HSR) (*72, 73*). HSR is universally conserved. Plants possess specific yet overlapping branches of HSR to cope with various aspects of elevated temperature stress such as basal thermotolerance (BT), short and long acquired thermotolerance (SAT and LAT, respectively), and thermotolerance to persisting, moderately high temperatures (TMHT). The branches of the HSR relies on both unique and shared molecular factors, allowing plants to effectively manage heat stress and ensure survival under various thermal conditions (*72, 73*).

A central element of HSR is the transcriptional regulation network. Upon high temperature exposure a wide range of sensors is activated, including those responding to membrane fluidity changes, protein denaturation, oxidative stress etc. The environmental signals are integrated at the level of the specific transcription factors, called heat stress factors (HSFs) that orchestrate the transcriptional response (*72, 73*) Plant HSF proteins share a well-conserved modular structure. The N-terminal DNA binding domain (DBD) of HSF specifically binds to *cis*-elements called heat shock elements (HSEs) in the gene promoters and subsequently activates the transcription of these (*71, 104*). Plant HSFs are classified into three classes, the HsfA, B, and C (*72-75*). The HsfA1 transcription factor family

(HsfA1a, b, d, and e) serves as the "master regulator" of HSR in *A. thaliana* that initiates downstream transcriptional cascades. The chaperone titration model proposes that under normal conditions, HSFs are kept inactive through binding to heat stress proteins (HSPs). When the high-temperature exposure causes misfolding and denaturation of proteins, these are bound more efficiently by HSPs, releasing the HSFs. Subsequently, HSFs oligomerise, translocate to the nucleus and activate the transcriptional cascade that leads to the expression of target genes, including HSPs, secondary transcription factors, antioxidants etc. (*71, 104-112*). Various HSP classes work cooperatively during HSR and are essential to preserve cellular proteostasis (*108-116*). In the early phase of HS, different classes of ATP-dependent chaperones (HSP70 and HSP90) are expressed and play a crucial role in the refolding of denatured/misfolded proteins.

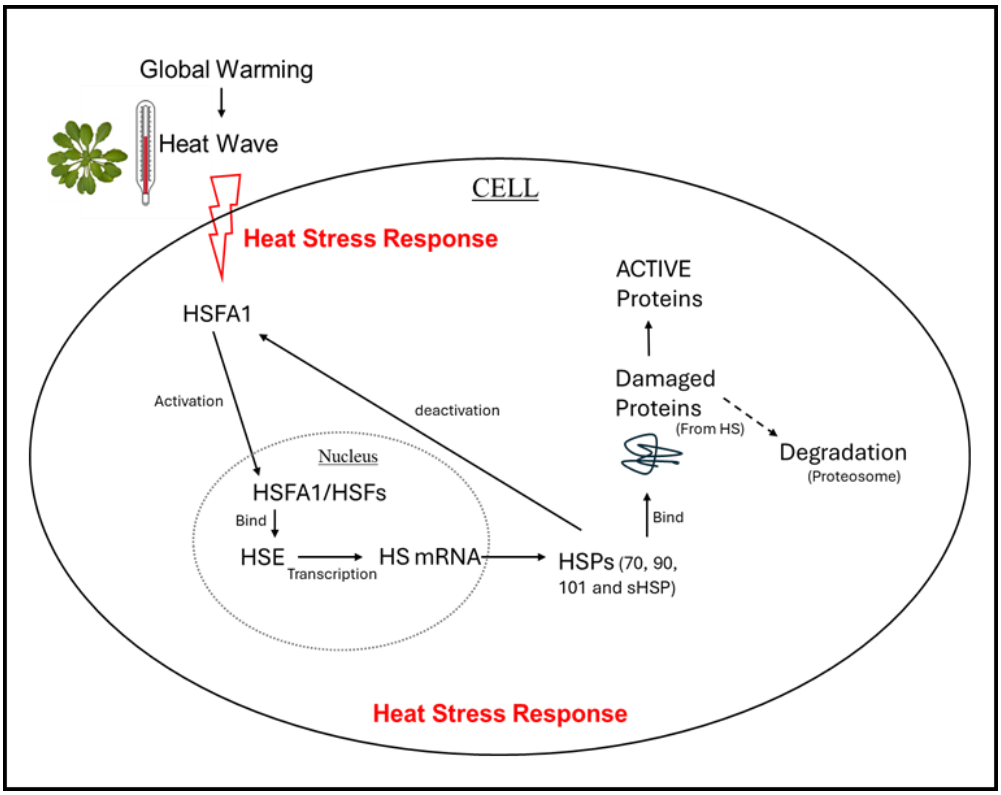

Figure 64: The schematic of heat stress response pathways in plants. *After being exposed to heat stress, plants activate molecular mechanisms to mitigate damage. Heat signals trigger the expression and/or activation of HSF proteins; after being activated, HSFs move into the nucleus and bind to heat shock elements (HSEs) in the promoter regions of heat-responsive genes. This leads to the transcription of Heat Stress Proteins (HSP70, 90, 101 and sHSPs), which function as molecular chaperones. HSPs help in refolding denatured proteins or direct for degradation the damaged proteins through proteosomes or autophagy pathway to maintain proteostasis. In the attenuation phase of HSR, the HSPs bind to HSFs to down-regulate their activity through a negative feedback control mechanism.*

These chaperones use ATP to actively assist in the proper folding and stabilization of misfolded proteins. During prolonged HS (e.g. upon persisting heat stress conditions), small heat shock proteins (sHSPs, 16-42kDa) are produced. These are referred to as holdases or

aggregases because they bind to the unfolded proteins and keep them in a ready-to-refold conformation and form reversible protein aggregates (*71, 81, 117, 118*). For the disassembly of denatured/misfolded protein aggregates the coordinated action of ATP-independent sHSPs and ATP-dependent chaperones (HSP101 and HSP70) is needed. Insoluble aggregates are either dismantled, allowing the proteins to be refolded into their proper structure, or, if refolding is not possible, they are directed for decay via proteasomes or autophagy pathways (Fig 6) (*76-78*).

2.5. Heat stress adaptation of monocot crop species

Cereal crops are cultivated widely across the world, serving as fundamental staples for both animal and human nutrition. Over the past 4 decades, the average air temperature has been rising on average by 1°C and is projected to increase further by 3–5°C by the end of the century (*72, 79, 80*). If the ambient temperature exceeds by >10°C the optimum temperature, it is perceived as heat shock. Rising temperatures are depicted to cause over 60% yield losses in the major grain crops (*79*). In conclusion, understanding the molecular processes that regulate HSR pathways in crops is therefore essential to improving agricultural productivity and creating resilient plant varieties.

The main five crops providing the overwhelming portion of the world's food and feed are wheat (*Triticum sp.*), rice (*Oryza sativa*), maize (*Zea mays*), barley (*Hordeum vulgare*), and sorghum (*Sorghum bicolor*) (*119*). Besides its economic importance, barley now is emerging as a monocot crop model. Studying barley has several advantages: its genome is relatively small (amongst the other crops), has a diploid genome, it is easily manipulated in laboratory conditions, germinates and grows well, can be transformed to create mutant and transgenic lines, has a high genetic variety pool and its genome has been sequenced. Despite its importance, transcriptional machinery regulation in monocot crops remains understudied. The DRB Sensitivity-Inducing Factor (DSIF) complex subunits, SPT4 and SPT5 have been shown to be specifically needed for reproductive development and to influence phytohormone pathway regulation (*29*). In sorghum, Transcription Elongation Factor 1 (TEF1) was implicated in salt stress tolerance (*120*). In wheat, TaTEF-7A was shown to control both vegetative growth and reproductive development (*121*).

The specific functions and regulation mechanisms of several transcription elongation factors during HSR in crop species therefore, remain to be explored.

3. OBJECTIVES

In the present work, we aimed to study the functions of RNAPII, especially focusing on the transcriptional elongation cycle and the roles of TFIIS elongation cofactor during HSR in the model plant *Arabidopsis thaliana* and *Hordeum vulgare* monocot crop plant species.

Specific sub-tasks of the work are:

a) Understanding the regulation of transcriptional elongation during HSR.
b) Validation of the molecular changes coordinated by TFIIS activity, based on RNAseq data showing transcriptional reprogramming from a developmental transcriptome to a heat-stress transcriptome.
c) Understanding the genetic interaction between transcriptional and post-transcriptional RNA quality control pathways.
d) Analyse the consequences of transcriptional errors in plants under ambient and high-temperature conditions.

4. MATERIALS AND METHODS

4.1. Plant materials

Experiments were performed on *Arabidopsis thaliana, Brassica napus, Hordeum vulgare* and *Chlamydomonas reinhardtii*, as described below: *A thaliana* seeds were bleach sterilised for 15 minutes and then plated on Murashige and Skoog (Duchefa M0222, https://www.duchefa-biochemie.com) medium agar plates (0.5× Murashige and Skoog salts, 1% agar, pH 5.7). Plants were routinely grown in a Sanyo MLR-350 growth cabinet under cool white light at 21°C long-day (LD) conditions (16 h light/8 h dark). *Chlamydomonas reinhardtii* (cc-4533) was grown on Murashige and Skoog medium agar plates. *Brassica napus* (RV31) and *Hordeum vulgare* (Golden Promise) were grown on soil. Mutant seeds *of A. thaliana* plants were ordered from NASC (https://arabidopsis.info): *tfIIs-1* (SALK_056755) and *tfIIs-2* (SALK_027259) are two knock-out SALK T-DNA insertion mutants, *upf1-5* (SALK_081178) is a hypomorphic mutant which contains a SALK T-DNA insertion within its 3'UTR region, *upf3-1* is a strong NMD mutant containing a SALK T-DNA insert within its exon 5 (SALK_025175, seeds were donated by K. Riha). Double mutants were generated by crossing these single mutants. For mutant complementation, the *pTFIIS::GSy-TFIIS;tfIIs-1* constructs were generated by Csorba lab and described previously (*81*). All the plant materials (*wild type (Col-0), tfIIs-1, upf1-5, upf3-1 and tfIIs-1;upf1-5*) were grown at 21°, except for *tfIIs-1;upf3-1,* which is sterile at this temperature, therefore grown at 25°C (*Verma, Szaker et al., submitted*).

4.2. Genotyping

For genotyping, genomic DNA was extracted from 30 mg fresh plant material using 100 -µl of Extraction buffer (E7526, Sigma-Aldrich) along with a stainless-steel bead (3 mm, Qiagen Sciences). Homogenisation was done using a mixer mill (Bullet Blender Storm Pro, Next Advance) at speed grade 8 for 1 min; subsequently, the mixture was incubated at 95 °C for 12 min in a dry heat block and placed on ice for 1 min. Finally, 100 µl of Dilution solution (D5688, Sigma-Aldrich) was added, and the supernatant was aliquoted after centrifugation (13000 rpm) for 10 mins. Genotyping PCR was done using DNA Taq polymerase (NEB, M0273S) based on the manufacturer's instructions. Genotyping primer sequences are listed in the *Supplementary Table 1*.

4.3. Stress treatments

We performed 4 types of heat treatments, namely basal thermotolerance (BT), short-acquired thermotolerance (SAT), long-acquired thermotolerance (LAT) and thermotolerance to moderately high temperatures (TMHT). For BT, seedlings were grown on 0.5xMS, 1% agar plates; naive 7-day-old seedlings were exposed to 45°C HS in a water bath for 10–30 min. For SAT, seedlings were pre-grown for 6 days; following these seedlings were heat-treated by a sublethal temperature (37°C for 1 h, acclimation phase), then placed back at 21°C for 2 h for recovery; after the recovery period, the seedlings were challenged by lethal stress temperatures (45°C for 1–3 h, lethal HS). For LAT, 5-day-old seedlings were first acclimated at 37°C for 1h and then recovered for 2 days at 21°C. Lethal HS treatment was applied afterwards in a water bath (45°C, for 20–100 min). For TMHT 7-day-old seedlings were placed in a growing cabinet pre-heated to 37°C and kept for 1–5 days, at long-day conditions (16 h light/8 h dark). All treatments were started at midday (Zeitgeber time, ZT8). Plants were cooled back to 21°C following each treatment.

4.3.1. Heat stress treatments:

For RNA and/or protein sample collections, plant materials were taken immediately after each treatment (after 1 hour, 1h, 4h or one day, 1d, alongside non-treated controls, NT); for recovery samples, materials were collected 2 days of recovery at 21°C following 1 d of TMHT treatment For heat stress phenotyping, seedlings were kept and grown on plates for 1 or 2 weeks back to at 21◦C, LD conditions, and then photographed.

For analysing TFIIS expression in *C reinhardtii* green algae: agar plates containing the C reinhardtii culture were incubated in a water bath for 1 h at 37 °C.

For analysing TFIIS expression in *B napus* and *H vulgare* plants: leaf discs (of 1 cm diameter) of 1-week-old soil-grown plants were placed into hydroponic culture (0.5× MS) and heat stress treated in a water bath for 1 h at 37 °C.

4.3.2. Salt stress treatments:

We prepared salt (0.5xMS) media agar plates containing NaCl concentrations ranging from 0 mM to 200 mM. Seeds were surface sterilised and placed on the agar surface, grown for 3 weeks and then photographed.

4.4. RNA extraction and qRT-PCR

Total RNA was isolated from ~30 mg seedlings in 700 µl extraction buffer (0.1 M glycine–NaOH, pH 9.0, 100 mM NaCl, 10 mM EDTA, 2% SDS) using the phenol–chloroform (pH 4.3) method; the RNA was precipitated in ethanol and resuspended in sterile water. DNase treatment was performed on 5 µg of total RNA (Ambion AM2222, www.thermofisher.com). One microgram of DNase-treated RNA and random primer was used for the first-strand cDNA reaction (NEB, E6300S, www.neb.com). qPCRs were done using the qPCR Master Mix (NEB, M3003S, www.neb.com), in a Light Cycler 96 real-time PCR machine (Roche). At least three independent biological replicas were analysed in each experiment. qRT-PCR primer sequences are listed in *Supplementary Table 2*.

4.5. RNA transcriptome analysis

The HS treatment, RNA extractions for RNA transcriptome and alternative splicing analysis from wild type (Col-0) and *tfIIs-1* mutant at non-treated, one hour (1h) and 1 day were done as described above. Bioinformatic work was done by HM Szaker and published previously (*81*).

4.6. Protein extraction and western blotting

For protein isolation, we took ~30mg of 7d old seedlings (non-treated, NT; heat-treated for 1h, 1d and recovery), homogenised in 100 µl of extraction buffer (150 mM Tris–HCl, pH 7.5, 6 M urea, 2% SDS and 5% -mercapto-ethanol), and the extracts were denatured at 95˚C for 5 mins. The cell debris was removed by centrifugation at 13000 rpm at 4˚C for 10 mins. The supernatants were resolved on 10% SDS-PAGE, transferred to Hybond PVDF membranes (GE Healthcare), and subjected to western blot analysis. Antibodies used for detection: anti-sHSP-CI antibody (AS07 254, Agrisera), anti-HSP90-1 antibody (AS08 346, Agrisera), and anti-HSP101 (AS07 253, Agrisera); as secondary antibody, we used monoclonal HRP-conjugated anti-rabbit (A6154, Sigma-Aldrich). The proteins were visualised by chemiluminescence (ECL kit; GE Healthcare), and quantified by Image Lab 5.1 (Bio-Rad). For quantifications, protein signals have been normalised to Rubisco large subunit (RbcL).

4.7. Protein aggregate purification and detection

Protein aggregates were purified as described before (*76*). Briefly, 0.1 g fresh seedling material of non-treated and heat-treated (1d) in 2.4 ml of isolation buffer [25 mM HEPES, pH 7.5, 200 mM NaCl, 0.5 mM, Na_2EDTA, 0.1% (v/v), Triton X-100, 5 mM ε-amino-N-caproic acid, 1 mM benzamidine] by using a mortar and pestle and then a Cole-Parmer PTFE glass tissue for the grinder. The soluble and insoluble fractions were separated from 2 ml of total extract by centrifugation at 14000 rpm for 15 min at 4°C. The soluble fraction was denatured by adding 0.5 volume of 2x SDS-PAGE buffer and heating for 5 min at 95℃. The insoluble pellet was washed six times repeatedly by resuspension in the isolation buffer containing 0.1 g of quartz sand (Sigma-Aldrich) and vortex. Later, the insoluble pellet was resuspended in 400 ml 2× SDS-PAGE sample buffer and clarified by centrifugation at 1500 rpm for 1 min (insoluble fraction). Samples were separated by SDS-PAGE and stained with the Coomassie Blue Staining method. The whole lanes of insoluble fractions have been quantified by Image Lab 5.1 (Bio-Rad), and ratios to Rubisco large subunit (RbcL) stain free signals were calculated.

4.8. CirSeq library preparation and bioinformatic analysis

For Circle-sequencing (CirSeq) sample preparation, we have used non-treated (NT) and heat-treated (1d) samples of wild type (Col-0), *tfIIs-1* or *upf1-5* single and double mutant plants. CirSeq libraries' preparation and bioinformatic analysis were done as described previously (*39*) in five biological replicates. Amplicon libraries were performed by Novogene Ltd. Sequencing service. Bioinformatic analysis was done by HM Szaker (*Szaker, Verma et al., submitted*).

5. RESULTS AND DISCUSSION

5.1. TFIIS transcription elongation cofactor is needed for proper development and stress response

5.1.1. TFIIS is an RNAPII cofactor specifically needed for heat adaptation

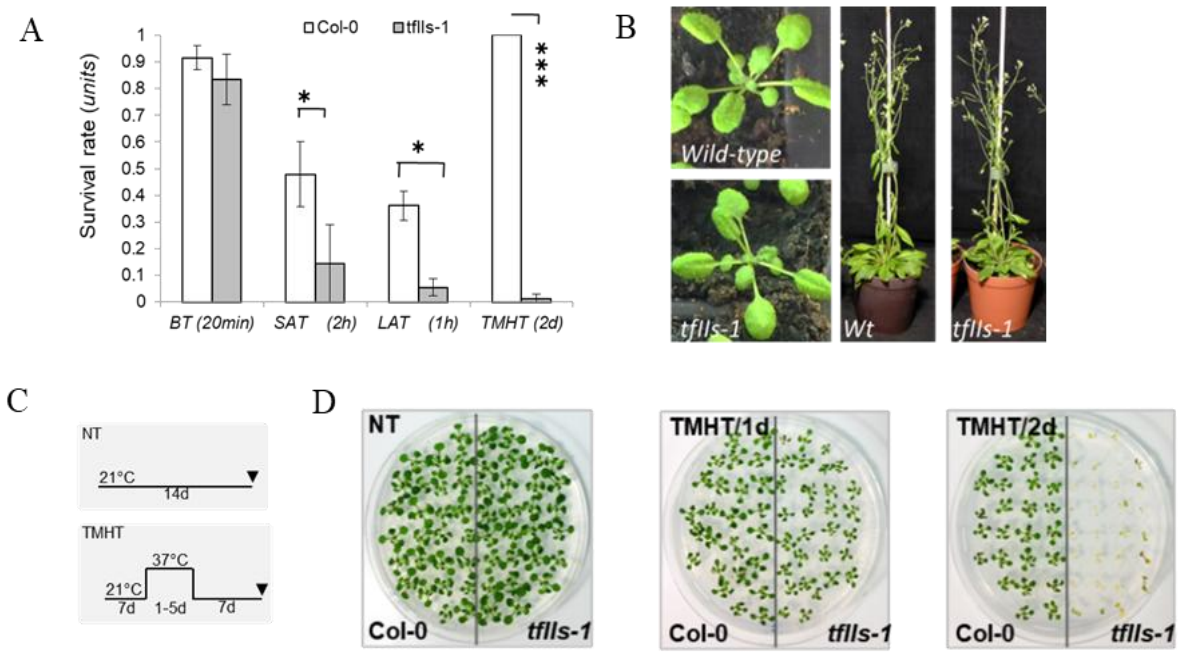

Figure 6: TFIIS is necessary for A. thaliana to adapt to heat stress during each phase of its cycle.
(A) The survival rate of wild-type Col-0 and tfIIs-1 plants under different heat stress treatments: BT, SAT, LAT, and TMHT. (B) The phenotype of wild type (Col-0) and tfIIs-1 under ambient temperature at rosette and flowering stages. (C) Schematic representation of the non-treated (NT) and thermotolerance to moderately high temperature (TMHT) treatment. (D) The heat-sensitivity phenotype of tfIIs-1 mutants compared to wild type (Col-0); TMHT treatment for one day (TMHT/1d) or two days (TMHT/2d).

To unravel molecular players specifically involved in HSR, we conducted a heat stress phenotyping screen; selected RNAPII core and associated cofactors mutant were exposed to BT, SAT, LAT or TMHT heat stress regimes (*see Materials and Methods*) and their stress tolerance was assessed (*data not shown*). Among others, we identified TFIIS mutant line *tfIIs-1* to be sensitive to SAT, LAT, and TMHT (Fig 6A). Under optimal conditions, the development of *tfIIs-1* mutant plants at vegetative and reproductive stages is essentially unaltered compared to the wild type (Col-0) (seedling, rosette stage and flowering stages), with mild differences: *tfIIs-1* plants flower slightly earlier and exhibit reduced seed dormancy (*22*). When exposed to sublethal stress (37 ˚C, TMHT), under which the wild-type plants (Col-0)

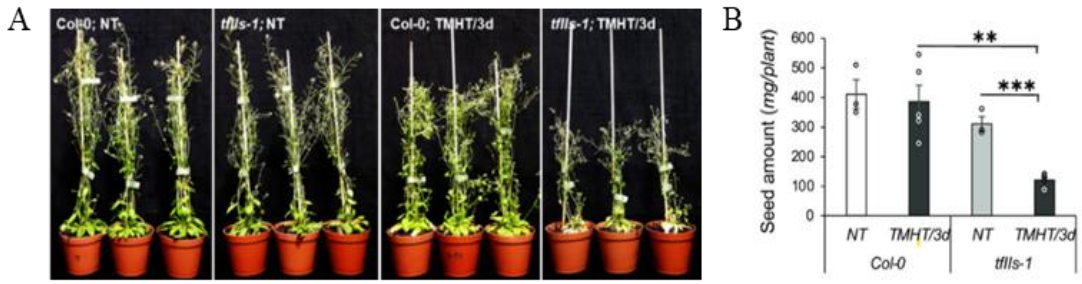

Figure 7: TFIIS is necessary for heat stress adaptation during the flowering stage in A. thaliana.
*(A) Col-0 and tfIIs-1 mutant plants were grown in soil under ambient and TMHT temperatures; TMHT treatment lasted for three days and was applied at flowering stage; (B) quantification of seeds harvested from wild type (Col-0) or mutant plants after TMHT/3d treatments vs non-treated (NT). Bars represent standard errors based on at least three biological replicates; P-values based on two-tailed Student's t-test (**P < 0.01, ***P < 0.001).*

can survive for long (5-7 days), showing developmental phenotypes such as reduced size. In contrast, *tfIIs-1* die after 2 days of exposure (Fig 6C-D). Additionally, TFIIS was needed to withstand moderately high temperatures (37 ˚C), also in soil-grown plants at the

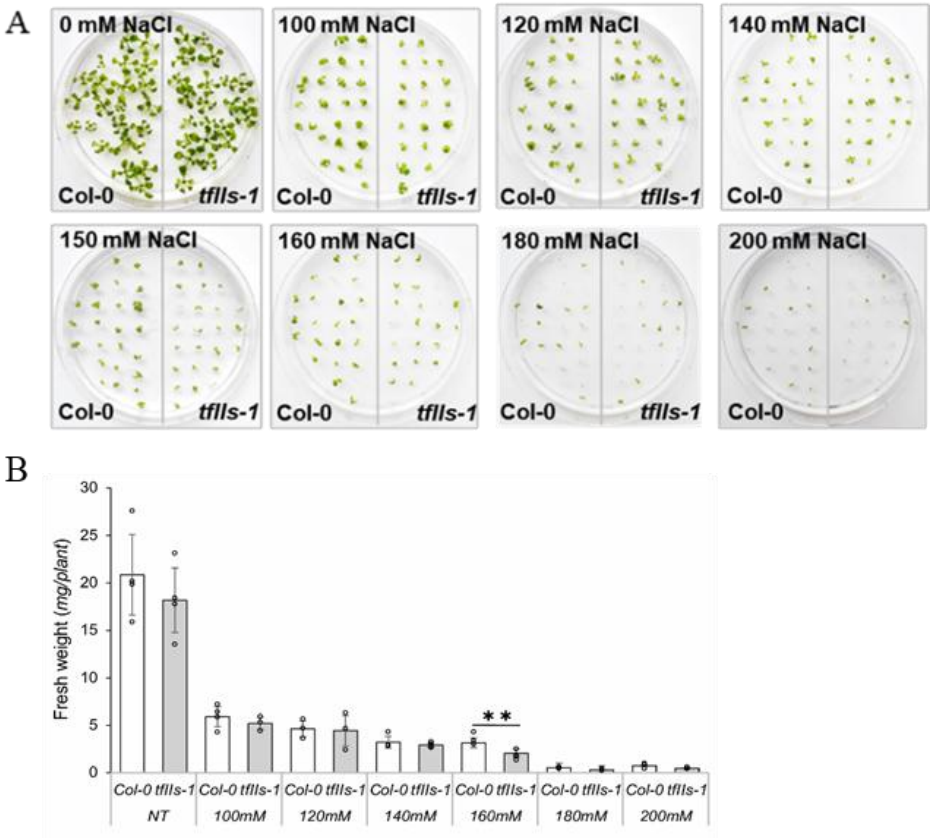

Figure 8: Phenotypic test of salt stress tolerance in wild-type Col-0 and tfIIs-1 mutant plants. *(A) Col-0 and tfIIs-1 plants were grown on media containing different salt concentrations (100-200mM) and photographed after the 14th day of growth. (B) On the 14th day of salt treatment, the fresh weight of green seedlings from both Col-0 and tfIIs-1 plants was measured. Bars represent standard errors based on at least three biological replicates; P-values based on two-tailed Student's t-test (**P < 0.01).*

flowering stage (Fig 7A); the heat-treated *tfIIs-1* produced significantly less amount of seeds (Fig 7B). These findings suggest that TFIIS is a vital component of HSR and is needed throughout the lifecycle of *A. thaliana* for survival and reproductive fitness. To analyse whether the impact of TFIIS mutation has a general impact on stress resilience, or its requirement is specific for HS, we compared the survival rate of *tfIIs-1* with wild-type (Col-0) upon salt stress exposure. The salt sensitivity of wild-type and mutant plants was very similar, showing that TFIIS is not needed for salt stress adaptation (Fig 8A-B).

Complementation assays were performed to confirm that the absence of the TFIIS protein caused the heat-sensitive phenotype of *tfIIs-1*; heat-sensitivity of *tfIIs-1* was reversed by the *pTFIIS::GSyTFIIS; tfIIs-1* transgene (own promoter driven GFP- and Streptavidin-tagged TFIIS transgene in *tfIIs-1* mutant background) in independent stable complementation lines (Fig 10A). In correlation with the mRNA dynamics, TFIIS protein was also accumulated in response to elevated temperature conditions (Fig 10B).

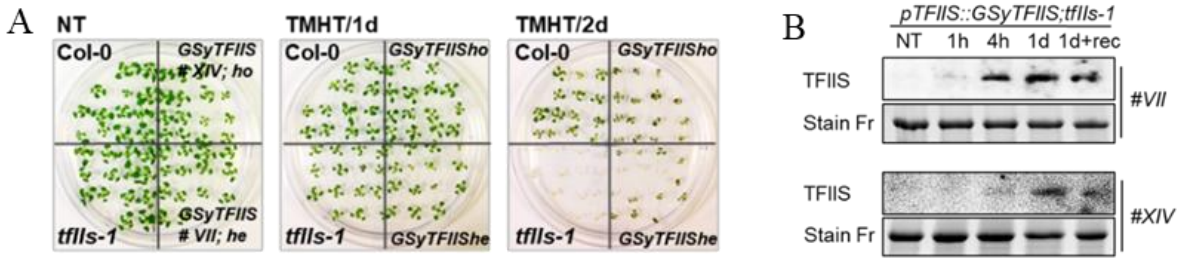

Figure 10: TFIIS complementation assays and TFIIS protein dynamics in response to heat stress. *(A) Heat-sensitive phenotype comparison between wild type (Col-0), tfIIs-1 mutant and GSyTFIIS complemented lines in response to TMHT/1d and 2d (B) Protein expression at different time points following the heat stress NT, 1h, 4h, 1d and rec in two independent complemented lines of GSyTFIIS protein.*

5.1.2. TFIIS transcriptional regulation in response to heat stress

5.1.2.1. *Cis* and *trans* factors of *TFIIS* locus regulation

To gain a deeper understanding of TFIIS regulation, we examined *TFIIS* mRNA changes during the heat-treatment experiment during a HS time course. In the wild type plants, *TFIIS* mRNA was significantly elevated during early HS (1h, 4h), then repressed or attenuated at 1d and in the recovery period (1d+rec) (Fig 9). In the *tfIIs-1* mutant a *tfIIs-tdna* chimera transcript was produced, suggesting that the locus was transcriptionally active and retained HS-inducibility. Notably, the heat-induced accumulation peak of *tfIIs-tdna* RNA was shifted to a later time point (4h-1d). The retarded HS induction of *tfIIs-tdna* transcript suggests that the TFIIS protein itself may be needed, either directly or indirectly, to transcribe the TFIIS locus

efficiently. The *unspliced RNAs* (for both *TFIIS* mRNA and *tfIIs-tdna*) had similar alterations suggesting regulation at the transcriptional level (Fig 9C). To further support this notion, we examined the TFIIS locus and identified at least three heat shock elements (HSE, Fig 9A).

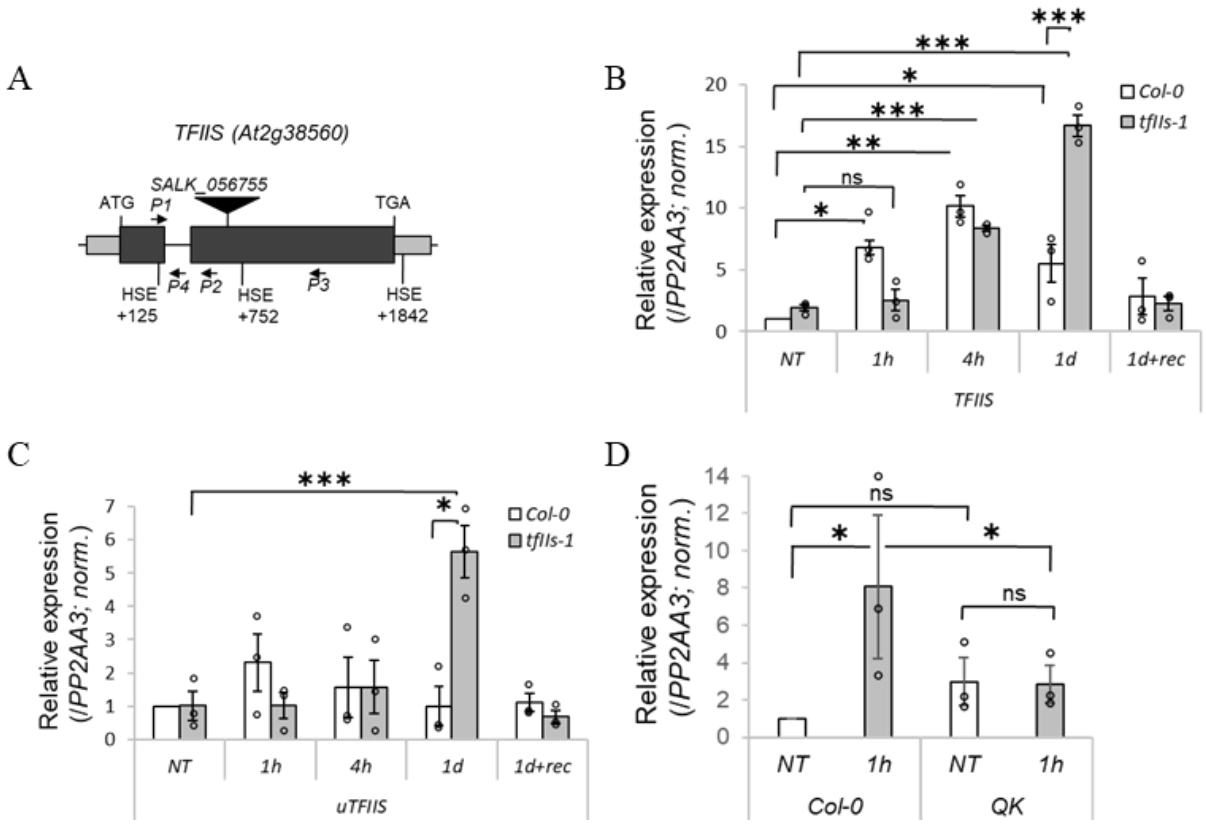

Figure 9. The regulation of TFIIS locus through cis and trans factors. *(A) A schematic diagram of the TFIIS gene locus: exons as black boxes, UTR regions as grey boxes, T-DNA insertion site is indicated above, the positions of HSE cis-elements with the primers used for genotyping (P1, P3) or qRT-PCR (P1, P2, P4) are marked. (B) Expression changes of TFIIS at different time points (non-treated, NT, 1h, 4h, 1d, 1d+rec); values were normalized to NT, wild type (Col-0) plants. (C) Expression changes of unspliced mRNA of TFIIS at different time points; (D) Analysis of relative expression of TFIIS spliced transcripts in hsfa1a;b;d;e quadruple knockout (QK) mutant plants under NT and TMHT/1h. Bars represent standard errors based on at least three biological replicates; P-values based on two-tailed Student's t-test (*P < 0.05, **P < 0.01, ***P < 0.001, non-significant, ns).*

In accordance with these, the accumulation of *TFIIS* mRNA was abolished in the quadruple mutant *hsfA1a;b;d;e* (*quadruple knock-out*, *QK*)(Fig 9D). These findings show that *TFIIS* accumulates during HS through transcriptional initiation by *HsfA1* transcription factor family members and efficient transcriptional elongation through TFIIS protein positive autoregulation.

5.1.2.2. TFIIS heat induction is conserved in the plant kingdom

TFIIS is a highly conserved transcription cofactor of RNAPII (*82*). To see whether its transcriptional regulation during HS is also conserved in the plant kingdom, first we studied

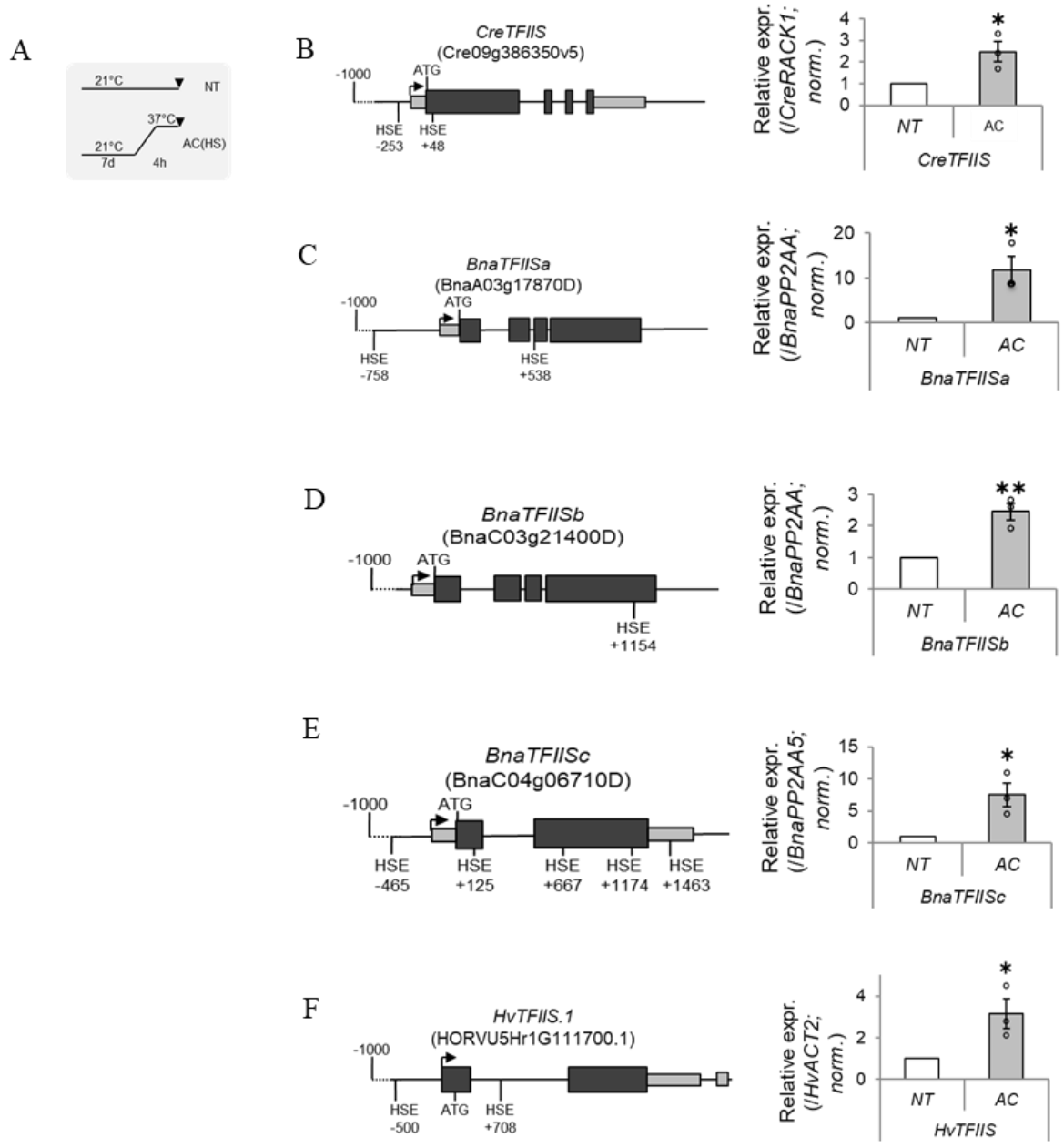

Figure 11: Transcriptional regulation of TFIIS during HS is conserved in the plant kingdom. *(A) Schematic representation of the heat stress regime used; (B-F) schematic depiction of TFIIS homologous genes in C. reinhardtii, B. napus and H. vulgare along with expressional analysis of their RNA transcription, respectively; exons as black boxes, UTR regions as grey boxes, ATG as start codons; HSE cis-elements are shown below. Bars represent standard errors based on at least three biological replicates; P-values based on two-tailed Student's t-test (*P < 0.05, **P < 0.01).*

TFIIS homolog genes/proteins in the green algae *Chlamydomonas reinhardtii*, the *A. thaliana* close relative dicot crop *Brassica napus* and in the monocot *Hordeum vulgare* crop plants (Fig 11). In *C. reinhardtii* and *H. vulgare* we identified *TFIIS* homolog gene loci (named as *CreTFIIS* and *HvTFIIS*), while in the *B. napus* three homologous gene loci (named as *BnaTFIISa*, *BnaTFIISb* and *BnaTFIISc*). All these loci encode a theoretical protein having high similarity to *AtTFIIS* (*CreTFIIS* 37.8%, *HvTFIIS* 57.5%, *BnaTFIISa* 84.1%, *BnaTFIISb* 82.7% and *BnaTFIISc* 80.1%) (*81*). The four cysteine residues within their domain III zinc finger domain and the acidic DE dipeptide are also present (*data not shown*), suggesting these may indeed encode TFIIS elongation factors actively involved in transcription. When we examined the loci, we identified several HSE *cis* elements within these, suggesting that all TFIIS loci may be under the regulation of HSFs, and activated during high temperature exposure. Prompted by these observations, we measured *TFIIS* mRNA changes and detected significant accumulation of all mRNAs following exposure to heat stress (Fig 11B-F). Based on the observations above, multiple independent CRISPR mutants in barley (*hvtfIIs-cr1, -cr2* and *cr3*) were generated in collaboration with my colleagues *(122)*. By studying these, we have shown that TFIIS is needed for proper seed production, seed germination capacity and heat stress tolerance of barley. We have also shown that TFIIS roles in coordination of HSR molecular events are conserved, and also evidenced that *HvTFIIS* locus is autoregulated (*122*). In summary, our studies (*Szádeczky-K, Szaker et al., 2022* and *Ahmad et al., 2024*) show that TFIIS roles during transcriptional regulation of HSR are conserved and are vital for both monocot and dicot species.

5.1.3. TFIIS is needed for transcriptional reprogramming during HSR

5.1.3.1. TFIIS affects qualitative and quantitative aspects of HS transcriptome

To understand the downstream molecular actions of *AtTFIIS* during HSR, we performed RNA transcriptome sequencing analysis on *A. thaliana* samples at different time points of heat treatments (non-treated, NT; TMHT/1h; TMHT/1d and one day recovery, 1d+rec) (*RNAseq data not shown*) (*81*). We have found that TFIIS is needed for qualitative and quantitative transcriptional reprogramming upon heat stress adaptation. As qualitative impacts of TFIIS on the transcriptome, we noticed 6 alternative splicing (AS) variants at NT, 86 AS variants after (TMHT/1h) and 1,760 AS events after (TMHT/1d) of heat stress treatment in *tfIIs-1* compared to the wild type (Col-0). In addition to alternative transcriptional initiations

and alternative terminations as well (*81*); of these, we validated the accumulation of several mRNA AS isoforms through qRT-PCR (Fig 12A-B).

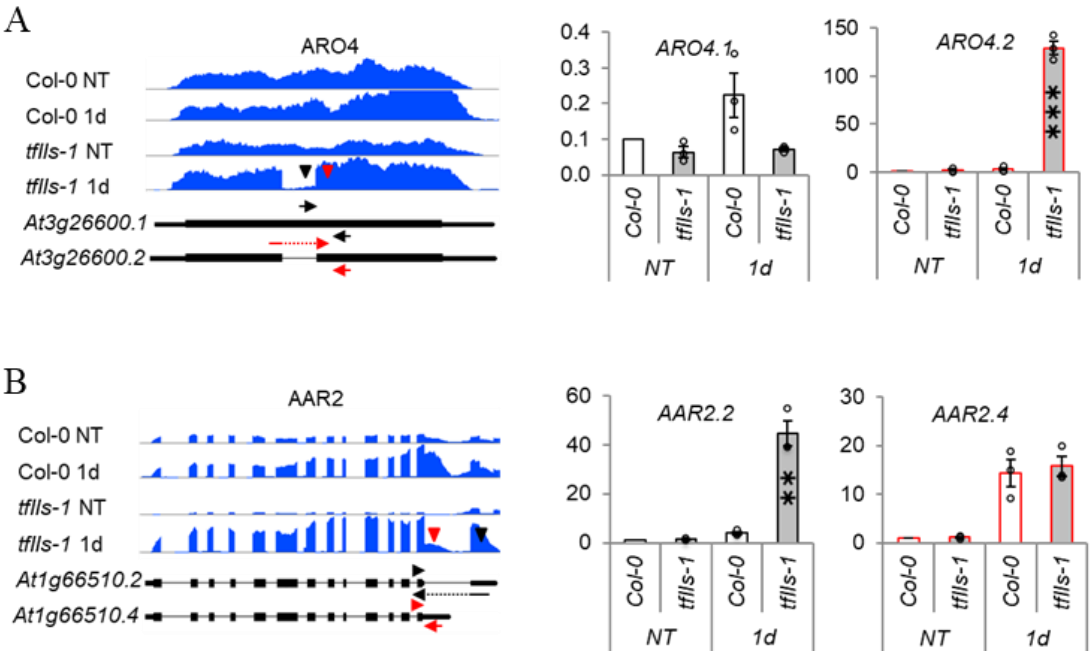

Figure 12: *TFIIS is needed for proper splicing* ***under high temperature.*** *(A-B) On the left, a genome browser image of transcriptome read tracks at selected loci with altered alternative splicing (AS) events (black and red triangles depict the two different AS events within genes/transcripts); gene names, primer's locations, genotypes and temperature conditions are shown. On the right, qrtPCR validation of relative AS RNA isoform amounts as shown; colour codes denote the respective primer shown on left. Bars represent standard errors based on at least three biological replicates; P-values based on two-tailed Student's t-test (**P < 0.01, ***P < 0.001).*

As quantitative effects of TFIIS at the transcriptome level (*data not shown*), we have observed that in *tfIIs-1* plants, the expression of HSR-transcripts (including HSFs, HSPs, and other components) in the early heat response (TMHT/1h) lags compared to wild-type (Col-0), whereas during the late HS phase (TMHT/1d) their expression was inefficiently attenuated (Fig 13) (*81*). The failure of the late HS attenuation of HS transcripts suggests a secondary compensatory effect for replenishing the HSPs. We validated the mRNA alterations of several HS transcripts (such as mRNA dynamics of selected *HSFs* and *HSPs*) in wild-type (Col-0) and *tfIIs-1* mutant plants by qRT-PCR analysis during the HS time course (Fig 13A-D).

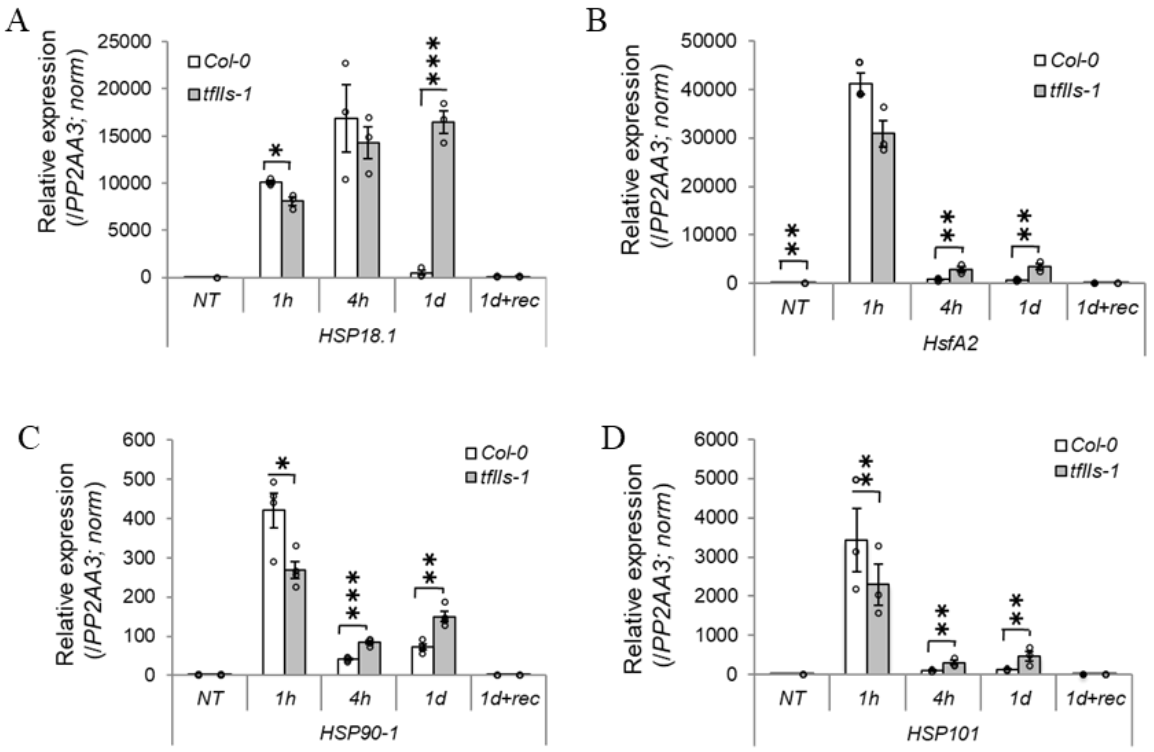

Figure 13: The absence of TFIIS alters the expression of heat stress response transcripts. *(A-D) The expressional changes of different HSR transcripts in tfIIs-1 and wild type (Col-0) during heat stress time series (non-treated, NT; TMHT 1h, 4h, 1d and recovery, 1d+rec). Bars represent standard errors based on at least three biological replicates; P-values based on two-tailed Student's t-test (*P < 0.05, **P < 0.01, ***P < 0.001).*

Based on these, we expected that qualitative and quantitative changes in the transcriptome would affect the HS proteome and compromise the HSR. To demonstrate this, we analysed HSPs' protein accumulations during heat stress in wild-type (Col-0) and *tfIIs-1* plants. Protein accumulation correlated well with mRNA changes. The expression of selected ATP-dependent chaperon families at the early stage of HS was significantly less abundant in *tfIIs-1* mutant plants compared to wild-type (Col-0) (observed at 1h-4h, Fig 14A-B). Whereas the ATP-independent holdase sHSPs were similarly expressed at the early stage of HS, they continue to accumulate massively in the late HS (TMHT/1d), suggesting an elevated level of proteotoxic stress in the *tfIIs-1* mutant plants (Fig 14C). Based on these, we assumed that in the absence of TFIIS the transcriptional elongation process is slow, which leads to retarded expression (and consequently translation) of HSPs chaperones.

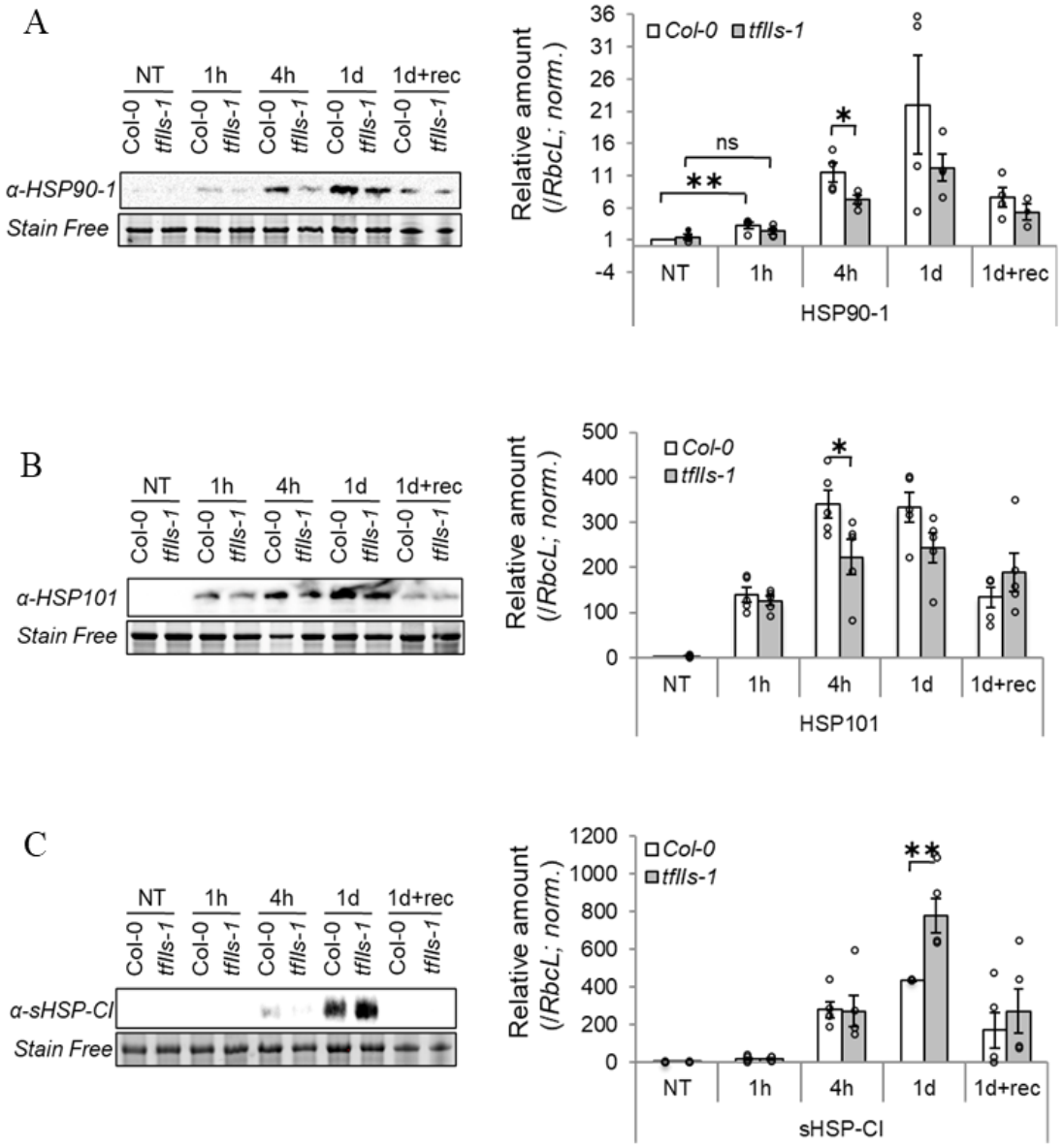

Figure 14: HSP protein accumulation is altered in the tfIIs-1 mutant under heat stress conditions. *(A-C) Western blot analysis of HSP90-1, HSP101 and sHSP-CI proteins during the heat stress time series; western blots on left, quantifications are shown on right for each; stain-free images of RbcL are shown as loading controls. Bars represent standard errors based on at least three biological replicates; P-values based on two-tailed Student's t-test (*P < 0.05, **P < 0.01).*

5.1.3.2. Absence of TFIIS leads to enhanced proteotoxicity during HSR

If our assumptions are true then, we expect to observe increased proteotoxicity in *tfIIs-1* plants. Cellular proteotoxicity is characterized by the presence of abundantly ubiquitinated, sumoylated proteins and peaks in the accumulation of insoluble protein aggregates (*76, 83-85*).

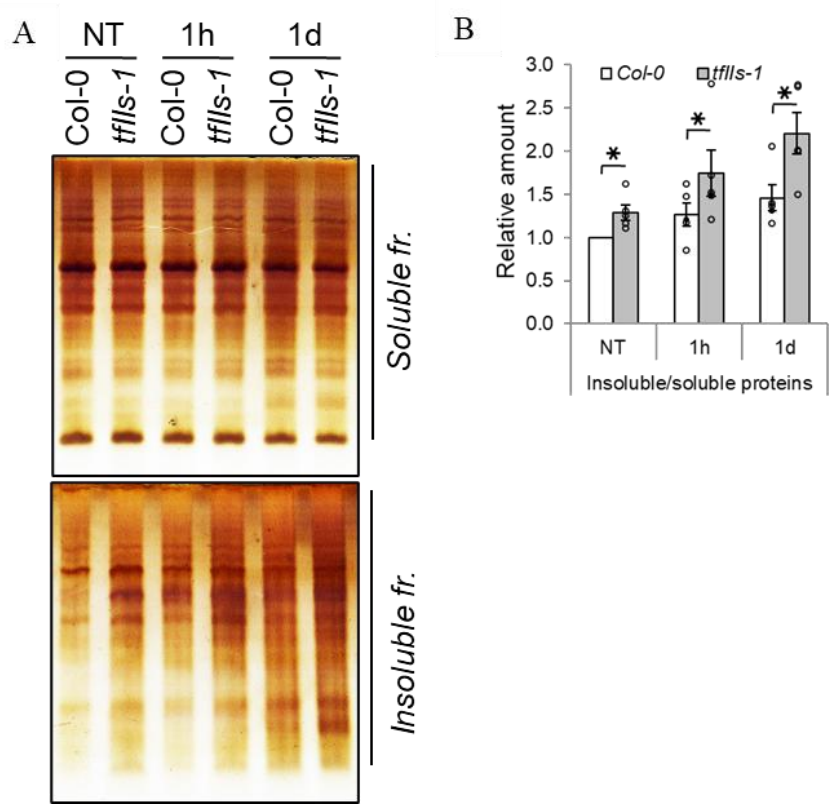

Figure 15: Comparison between insoluble proteins and soluble proteins in wild type (Col-0) and tfIIs-1. *(A) Silver staining gel images of wild type (Col-0) and tfIIs-1 under different time points. (B) Quantifications of the ratios of silver-stained insoluble to soluble protein amounts were calculated. Bars represent standard errors based on at least three biological replicates; P-values based on two-tailed Student's t-test (*P < 0.05).*

To *de facto* demonstrate cellular proteotoxicity, we analysed the levels of ubiquitinated and sumoylated proteins during TMHT at different time points. We found a significant increase in total sumoylated and ubiquitinated proteins in *tfIIs-1* plants, in correlation with HSP chaperon dynamics (*data not shown, (81)*). To further dissect proteotoxicity, we purified the insoluble and soluble protein aggregates and calculated insoluble/soluble protein ratios at NT, TMHT/1h and TMHT/1d, in wt and *tfIIs-1*. Notably, increased insoluble/soluble protein ratios were observed in *tfIIs-1* plants already under non-treated conditions (*34, 81*). In the early and late HS phases, the insoluble protein amount was further increased *tfIIs-1* line (Fig 15A-B).

Based on these findings, we proposed a model of TFIIS actions in plants (*81*), When TFIIS is present, RNAPII arrests are efficiently resolved under both normal and heat stress conditions. We postulate that elongation arrests could be less numerous under normal conditions, so the alternative rescue pathways may act efficiently to compensate for the absence of TFIIS. Under HS conditions, however, either (i) elongation arrests may be more frequent,

(ii) alternative pathways less effective or (iii) a combination of these; therefore, the absence of TFIIS becomes vital (Fig 16).

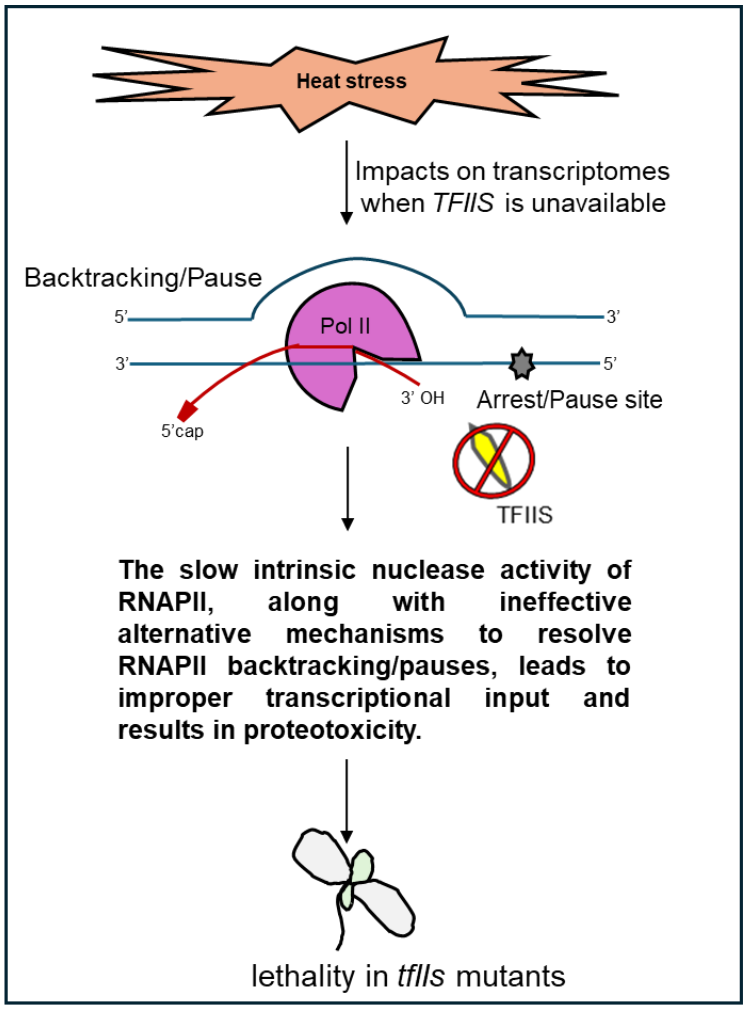

Figure 16: Absence of TFIIS protein results in inefficient transcriptional reprogramming and proteotoxicity under elevated temperature conditions. *Under HS, the likelihood of backtracking or pauses in RNAPII may be increased. Inefficient arrest resolutions in the absence of TFIIS cause qualitative and quantitative alterations of transcriptome, indirectly resulting in proteotoxic stress that finally contribute to plant lethality (see text for details).*

5.2. Mechanistic actions of TFIIS roles during development and HSR

5.2.1. Elevated temperature promotes accumulation of transcription fidelity errors

TFIIS has been described as a fidelity factor in yeast and metazoan (*35, 86, 87*), but its roles as a fidelity factor in plants were not studied. So, we aimed to decipher the transcription error-clearing roles of TFIIS under ambient and HS conditions in *A. thaliana*. The errors within the mRNA transcripts in the absence of TFIIS may be underestimated due to the masking effect of downstream RNA quality control pathways. Erroneous RNAs often contain small insertion

or deletion mutations (1-2 nucleotides), which can cause shifts in the open reading frame. These frameshifts likely introduce premature termination codons (PTCs), leading to translation of truncated proteins (*Szaker, Verma et al., submitted*). The NMD pathway targets erroneous PTC-mRNAs efficiently for degradation. To stabilize the PTC-mRNAs produced in absence of TFIIS quality control, we combined *tfIIs-1* mutant with the cytoplasmic quality control NMD *upf1-5* and *upf3-1* mutant lines.

5.2.2. Interaction between nuclear and cytoplasmic mRNA fidelity pathways

5.2.2.1. NMD is needed for HSR

If NMD is supposed to play a role in the fidelity control during HS, firstly, it must be active under HS. In plants, biotic and abiotic stresses can modify NMD activity (*68, 70, 88*), but the roles of NMD in heat stress adaptation have not yet been analysed in any organism.

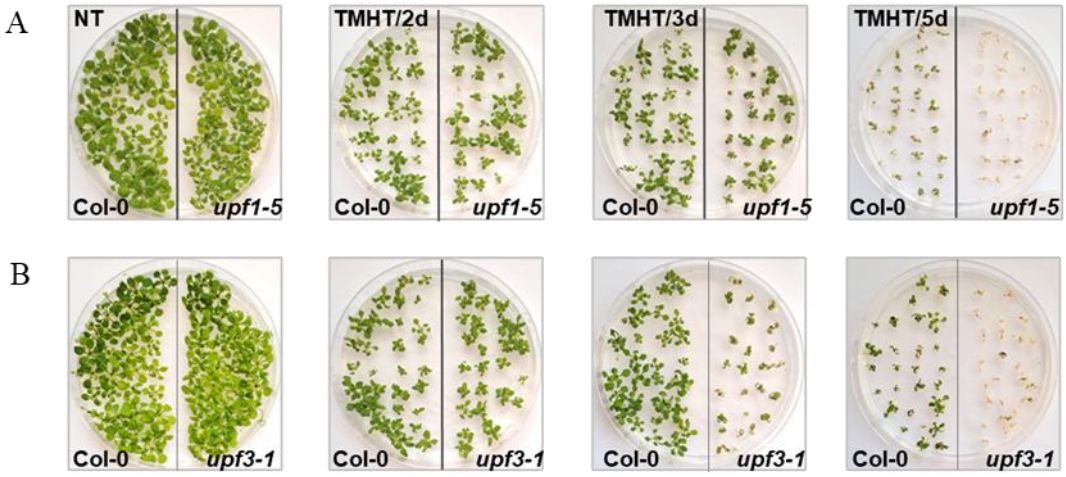

***Figure 17: NMD pathway is needed for efficient HSR**. (A-B) The heat-sensitivity phenotype of upf1-5 and upf3-1 mutants compared to wild type (Col-0) plants; genotypes and temperature regimes are shown.*

Therefore, we tested the HS-sensitivity of *upf1-5* and *upf3-1* lines compared to wild-type (Col-0). NMD mutants *upf1-5* and *upf3-1* were heat-sensitive when exposed to TMHT (Fig 17), suggesting that NMD play roles in temperature adaptation.

5.2.2.2. TFIIS and NMD factors interact genetically

To investigate the roles of NMD and its interaction with the TFIIS pathway, we crossed the single *upf* mutants to generate the *tfIIs-1;upf1-5* and *tfIIs-1;upf3-1* lines for further study. We examined their developmental phenotypes: the single mutant of *tfIIs-1* develops normally,

the *upf1-5* and *upf3-1* exhibit milder phenotypes such as curly leaves; in addition to these, the *upf3-1* shows slightly retarded growth compared to the wild-type (Col-0) (Fig 18). Notably, the double mutants exhibit significant developmental alterations: *tfIIs-1;upf1-5* show elongated curly leaves with serrated margins, besides flowering and seeding normally compared to the single mutants. In contrast, *tfIIs-1;upf3-1* displays strongly retarded growth with a similar leaf shape to *tfIIs-1;upf1-5* (Fig 18). The *tfIIs-1;upf3-1* plants exhibit a significantly delayed transition to flowering, ultimately leading to complete sterility (*Szaker, Verma et al., submitted*).

Figure 18: Genetic interaction between TFIIS and NMD factors*. Developmental phenotype of tfIIs-1, upf1-5, upf3-1, tfIIs-1;upf1-5 and tfIIs-1;upf3-1 mutants compared with wild type (Col-0) under ambient growth conditions (see main text for description).*

The retarded growth of *tfIIs-1;upf3-1* reminded us of immunity mutants (*56, 66*). To see if the autoimmunity pathway is upregulated in these plants, we measured the mRNA level of *PATHOGENESIS RELATED 1* (*PR1*), a marker gene for immunity (*56, 66*). *PR1* levels were several-fold increased in the *tfIIs-1;upf3-1* mutant plants compared to wild type (Col-0) and single mutants, ultimately supporting the hypothesis (*Szaker, Verma et al., submitted*). To further substantiate this, we have grown plants at elevated temperatures, as autoimmunity is suppressed at high temperatures (120). The stunted phenotype of *tfIIs-1;upf3-1* was reverted. However, the leaf shape phenotype persisted, and the plants were able to produce seeds, although in a very low quantity (*Szaker, Verma et al., submitted*). These observations suggest that in *tfIIs-1;upf3-1* plants have at least two independent pathways that are compromised: (i)

the immunity pathway that caused the stunted growth in an NMD-independent manner, (ii) the developmental pathway that causes leaf deformations NMD-dependently.

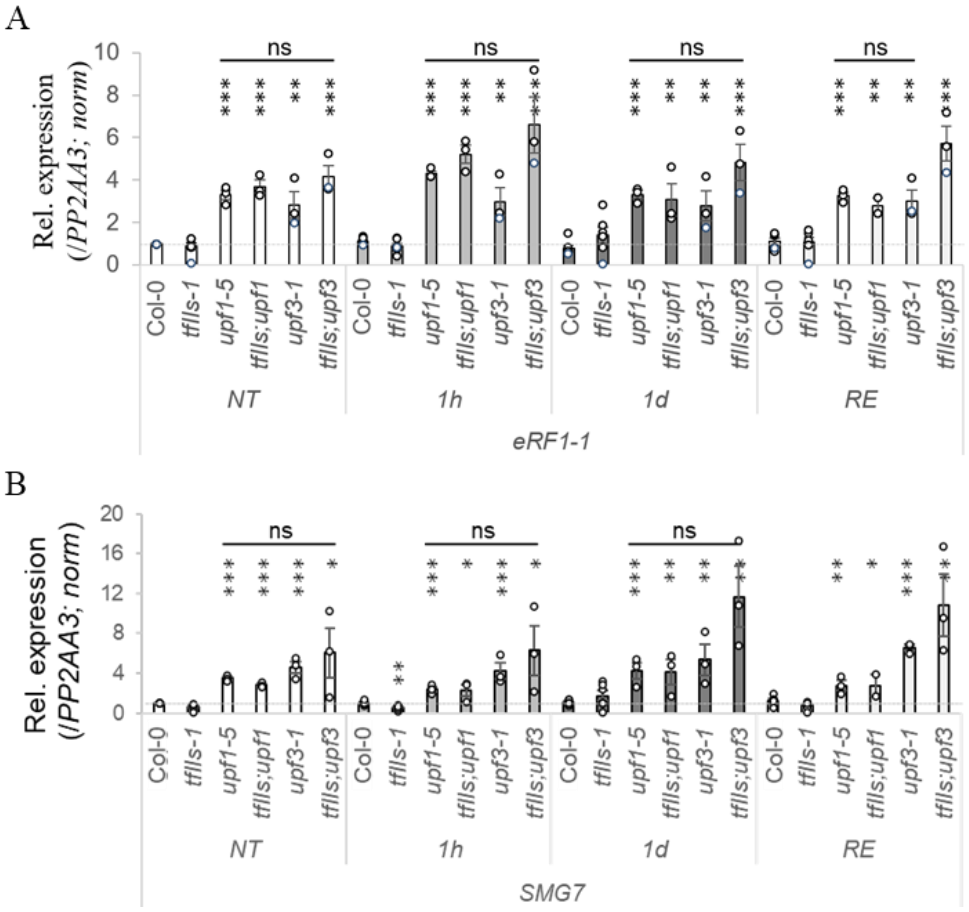

Figure 19: The NMD pathway remains active under heat stress conditions. *(A-B) Expression changes of eRF1-1 and SMG7 in wild type (Col-0), tfIIs-1, upf1-5, upf3-1, tfIIs-1;upf1-5 and tfIIs-1;upf3-1 mutants at different time points (Non-Treated (NT), TMHT/1h, TMHT/1d and recovery (1d+rec). Bars represent standard errors based on at least three biological replicates; P-values based on two-tailed Student's t-test (*P < 0.05, **P < 0.01, ***P < 0.001, non-significant (ns)).*

Next, we analysed the NMD activity at the molecular level under HS conditions in our mutant set, by measuring the transcript levels of *SMG7* and *eRF1-1* autoregulatory components of the NMD pathway (*67*). All transcripts were expressed at significantly higher levels in the NMD mutant background plants *upf1-5, tfIIs-1;upf1-5, upf3-1* and *tfIIs-1;upf3-1*. Importantly, these transcripts remained at low levels in wild-type (Col-0) and *tfIIs-1* compared to the *NMD* mutant background plants throughout all TMHT time points (Fig 19). This indicates that NMD maintains its activity at both ambient and high temperature (37˚C) regimes.

To assess the biological relevance of *TFIIS* and *NMD* pathway interactions, we subjected double mutants to the TMHT regime. The double mutants *tfIIs-1;upf1-5* and *tfIIs-1;upf3-1* plants were more sensitive to TMHT than the corresponding single mutants *upf1-5* and *upf3-1*, and the already sensitive *tfIIs-1* genotype (Fig 20A-B). These data show that the two pathways interact to support efficient HS survival.

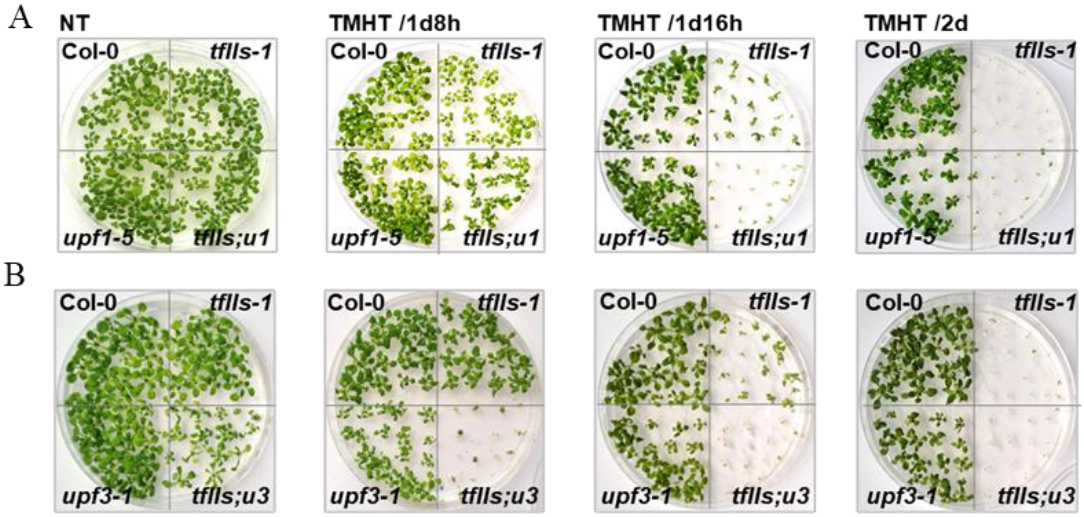

Figure 20: Genetic interaction between TFIIS and NMD is needed for HSR. *Heat sensitive phenotype of tfIIs-1, upf1-5, upf3-1, tfIIs-1;upf1-5 and tfIIs-1;upf3-1 mutants compared to wild type (Col-0) under TMHT; genotypes and temperature conditions are shown.*

5.2.2.3. The impact of combined TFIIS and NMD absence on proteostasis

The faulty transcripts, containing substitutions or indels, may lead to a marked alteration in the proteome (*Szaker, Verma et al., submitted*). Therefore, we aimed to analyse the impact of *TFIIS* and *NMD* mutants along with their combinations on cellular proteotoxicity. It has been proposed that the level of ATP-independent sHSP holdases serves as a readout of proteotoxicity and their protective effect is dose-dependent (*76, 81, 83*). First, we measured the accumulation of *sHSP* class I mRNAs (*sHSP18.2*). The *sHSP* transcripts accumulated at significantly lower levels during early heat stress but were strongly increased after TMHT/1d treatment in *tfIIs-1* backgrounds (Fig 21A). The increase of *sHSP* mRNA levels occurred at a lower extent when *tfIIs-1* was combined with either *upf1-5* or *upf3-1* mutations under HS (Fig 21A), in contrast to expectations . We also analysed changes in sHSP protein accumulation and found that during the late HS response, significantly higher levels of sHSP-CI proteins accumulated in *tfIIs-1, upf1-5, tfIIs-1;upf1-5* and *tfIIs-1;upf3-1* mutant plants compared to wild type (Col-0)(Fig 21B). The accumulation of sHSP-CI was additive in *tfIIs-1;upf1-5* and

tfIIs-1;upf3-1 relative to the single mutant plants *upf1-5* or *upf3-1*. sHSP-CI levels persisted during the recovery period in *tfIIs-1*, *tfIIs-1;upf1-5* and *tfIIs-1;upf3-1* plants, but reached wild type (Col-0) level in the *upf1-5* and *upf3-1* plants (Fig 21B).

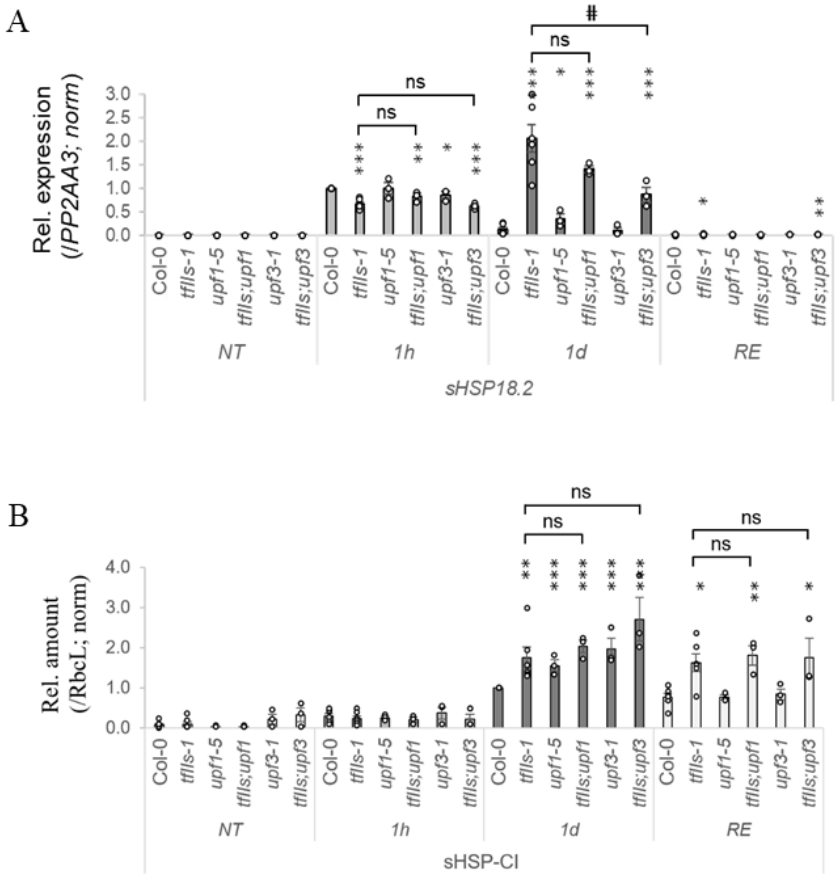

Figure 21: ATP-independent sHSP holdase mRNA and protein level changes in absence of TFIIS and NMD. *(A) Expressional studies of sHSF18.2 transcripts in tfIIs-1, upf1-5, upf3-1, tfIIs-1;upf1-5 and tfIIs-1;upf3-1 and wild type (Col-0). (B) sHSP-CI protein accumulation in tfIIs-1, upf1-5, tfIIs-1;upf1-5, upf3-1 and tfIIs-1;upf3-1 mutants compared to wild type (Col-0). Bars represent standard errors based on at least three biological replicates; P-values based on two-tailed Student's t-test (*P < 0.05, **P < 0.01, ***P < 0.001, non-significant (ns)).*

The elevated level of sHSP holdases in *tfIIs-1* and NMD mutants *upf1-5* or *upf3-1*, and the additive increase in the double mutants suggest that both TFIIS and NMD are required to protect the cells from proteotoxic stress during HS. These data also imply that TFIIS and NMD may protect the cells from proteotoxic stress during HS through different mechanisms.

To *de facto* quantify the cellular proteotoxicity in our mutants during HS, we separated the insoluble protein aggregates from the soluble protein faction and calculated the insoluble/ soluble ratios under non-treated and heat-treated conditions. The *tfIIs-1;upf* plants, and

especially the *tfIIs-1;upf3-1*, had significantly higher amounts of insoluble aggregates compared to the corresponding single mutants and wild type (Col-0) and *tfIIs-1*.

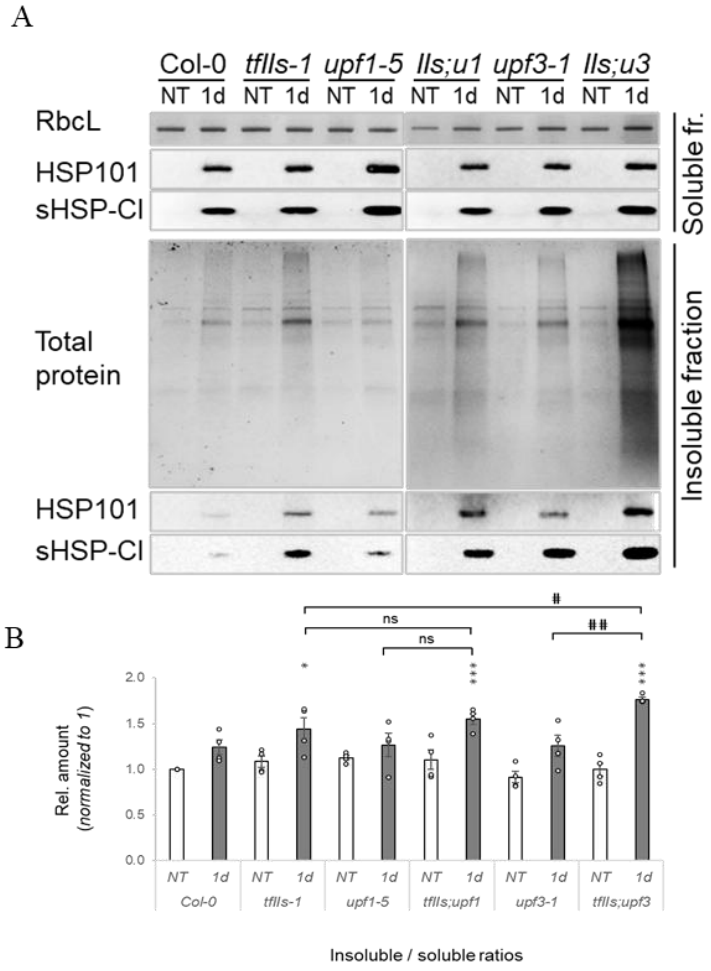

Figure 22: Comparison between insoluble proteins and soluble proteins in wild type (Col-0), tfIIs-1, upf1-5, upf3-1, tfIIs-1;upf1-5 and tfIIs-1;upf3-1 *(A) Coomassie blue staining gel images of single mutants along with double mutants under different time points (non-treated, NT, 1h, 4h, 1d, 1d+rec));(Rubisco large subunit, RbcL as control, HSP101, sHSP-CI are shown below (B) Quantifications of the ratios of Coomassie blue staining insoluble to soluble protein amounts. Bars represent standard errors based on at least three biological replicates; P-values based on two-tailed Student's t-test (*P < 0.05, **P < 0.01, ***P < 0.001, non-significant (ns)).*

The enhanced proteotoxicity within the double mutant genotypes is in correlation with the sHSP changes; the distribution pattern of the HSP101 protein closely resembles that of sHSP-CI. These observations provide further evidence of imbalanced proteostasis in the absence of TFIIS and NMD factors, and upon their combined absence. (Fig 22A-B). Lastly, error-containing mRNAs may contribute to the production of faulty proteins, thereby exacerbating proteotoxicity. We postulate that erroneous transcription in the *tfIIs-1* mutant and combined with the hypo- or non-functional *upf1-5* or *upf3-1*, respectively, underpins the increased proteotoxic stress observed in these plants during high-temperature stress.

5.2.3. TFIIS is a fidelity factor

Besides the retarded and qualitatively altered expression of HS-transcripts, faulty transcription may be another reason for imbalanced cellular proteostasis. As TFIIS was previously described as a transcriptional fidelity factor, we aimed to analyse transcriptional fidelity in its absence and in plants exposed to high temperature stress.

5.2.3.1. TFIIS is needed under imbalanced nucleotide concentrations

First, to demonstrate that accurate transcription is crucial for the HSR process, we created an environment that increased the likelihood of transcription errors. For this, we treated *A. thaliana* wild-type (Col-0) and *tfIIs-1* mutant seedlings with Mycophenolic Acid (MPA) or solvent control (CTL). MPA is a pharmaceutical that inhibits IMP-dehydrogenase, a vital enzyme in the guanosine nucleotide (GTP) synthesis pathway. When GTP levels are reduced, the transcription error rate rises, resulting in the generation of faulty transcripts in the yeast system (*123-124*).

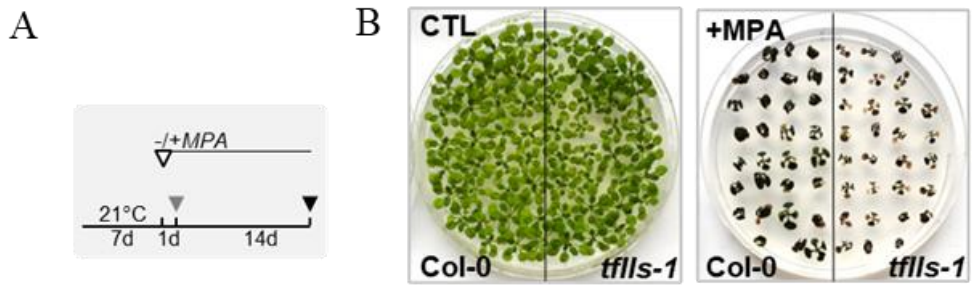

Figure 23: TFIIS is essential for viability under imbalanced nucleotide conditions. *(A) Schematic representation of the MPA treatment; (B) MPA-sensitivity phenotype of wild type (Col-0) and tfIIs-1 mutant plants, photographed after 2 weeks; genotypes and treatment conditions are shown.*

Upon MPA treatment, the *tfIIs-1* mutants showed increased sensitivity, exhibiting slower growth, distorted leaves, and accumulation of anthocyanins; several *tfIIs-1* plants died, while wt plants although have shown strong phenotypic alterations were able to survive (Fig 23A-B). These findings suggest that error correction by the anti-arrest elongation factor TFIIS is crucial. We attempted to combine MPA treatment with heat stress, but the results were inconclusive; therefore, understanding the effects of MPA during HS conditions needs further investigation.

5.2.3.2. Circular Sequencing Methodology

To analyse the impact of transcriptional fidelity at the nucleotide level as a potential underlying cause of HS- and nucleotide imbalance sensitivity in *tfIIs-1* and *upf* single or double mutants, we performed Circle-sequencing transcriptome analysis (CirSeq) (*39*). Notably, CirSeq can distinguish between technical artifacts and *bona fide* biological mRNA errors, which cannot be achieved using conventional RNA-seq analysis (*see below,* and Fig 24.).

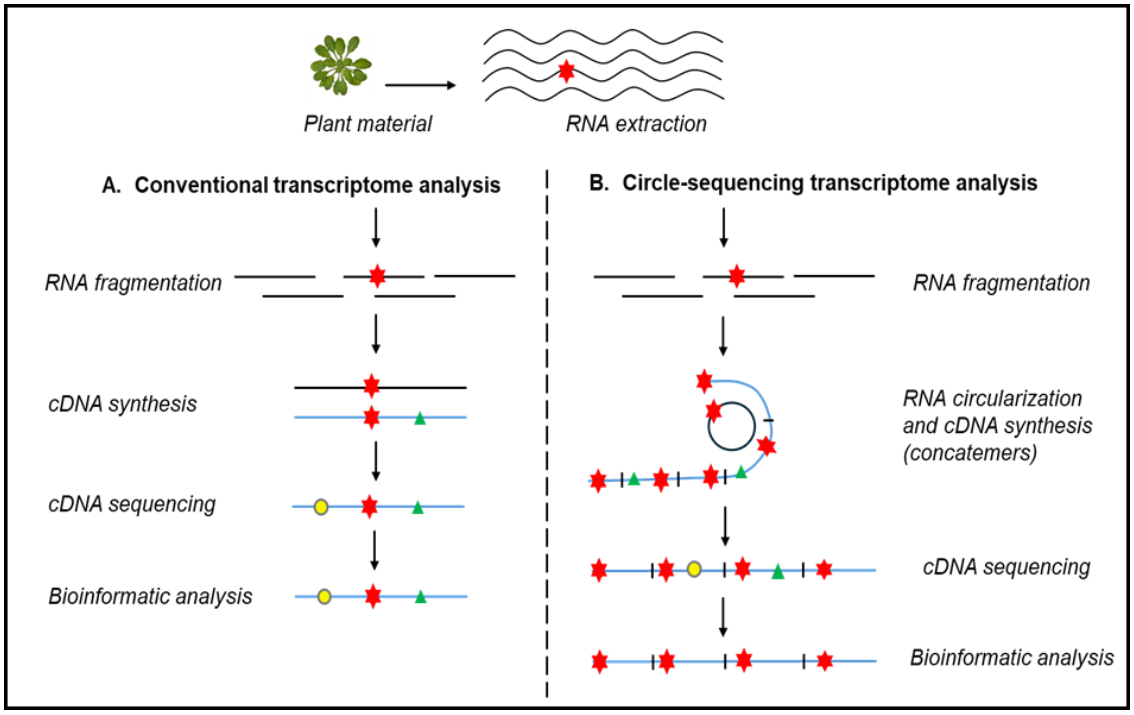

Figure 24: Circular Sequencing Methodology: *Total RNA was extracted from Arabidopsis plants. (A) workflow of conventional RNA sequencing; (B) workflow of CirSeq: the poly-A RNA is purified, fragmented, and circularised, resulting in concatemers through cDNA synthesis. Library construction and high-throughput sequencing, combined with bioinformatic analysis, enables differentiation of errors originating from reverse transcription (RT), library preparation, PCR amplification, or sequencing errors that cannot be filtered out in conventional RNA sequencing (A).*

For CirSeq, total RNA was extracted from *A. thaliana* plants, then polyadenylated RNA was purified to enrich for RNAPII products. The mRNA was then enzymatically fragmented and circularized. Using a rolling cycle reaction with strand-displacing reverse transcriptase, the circular RNAs were reverse transcribed into a long, single-stranded concatenated cDNA molecule. This single-stranded cDNA was converted to double-stranded cDNA, fragments end-repaired, dA-tailed, and later, adapter-ligated for library construction and deep sequencing. If transcription errors are consistently detected at the same position in every copy, they were

likely already present in the mRNA. In contrast, technical errors (arising from reverse transcription, PCR amplification, and sequencing) are distributed randomly across the sequence. Biological errors were distinguished from technical errors through a bioinformatic sorting process (*done by Szaker HM*, Fig. 24).

5.2.3.3. The error landscape of *Arabidopsis thaliana*

We performed CirSeq on Col-0, *tfIIs-1, upf1-5* and *tfIIs-1;upf1-5*, but we were unable to include *tfIIs-1;upf3-1* lines in the analysis, because these plants are sterile at 21˚C. Analysis was done on non-treated (NT) and TMHT/1d samples. The TMHT/1d condition was chosen to assess the effects of heat on transcription fidelity because (i) at this time point, the phenotype of HS-sensitive *tfIIs-1* is almost negligible, therefore does not result in secondary effects, and (ii) there is sufficient time for the production and stabilization of erroneous transcripts.

Using CirSeq, we detected a total of 29,730 transcription errors. These errors originated almost exclusively from RNAPII poly-adenylated transcripts in both wild type (Col-0) and mutant samples, with 97.5% reads in our libraries consisting of RNAPII transcripts. The total error rate of RNAPII transcription in wild-type (Col-0) under NT was 1.6×10^{-5} ($\pm 3.3 \times 10^{-6}$), while the error rate of HS-treated wild-type (Col-0) plants was 1.7×10^{-5} ($\pm 3.4 \times 10^{-6}$), not significantly different from the error rate measured at ambient temperature (21˚C) (Fig 25A). Notably, the error rate increased 2-fold (to 3.6×10^{-5}, $\pm 4.1 \times 10^{-6}$) in the absence of TFIIS under NT, and further elevated by 3.4-fold (to 5.8×10^{-5}, $\pm 3.4 \times 10^{-6}$) during high-temperature conditions (TMHT/1d) (Fig 25A). These observations show that heat stress compromises transcriptional fidelity and establishes TFIIS as a fidelity factor for transcription under both ambient and HS conditions. The total transcription error rate in the *upf1-5* mutant was very similar to the wild type (Col-0) (1.7×10^{-5}, $\pm 2.3 \times 10^{-6}$ under NT, and 1.8×10^{-5}, $\pm 3.4 \times 10^{-6}$ under HS). In contrast, the error rate in the *tfIIs-1;upf1-5* double mutant was similar to the *tfIIs-1* single mutant (3.5×10^{-5}, $\pm 3.8 \times 10^{-6}$ under NT, and 5.7×10^{-5}, $\pm 3.9 \times 10^{-6}$ under HS). These findings suggest that NMD may have a specialized role in fidelity control (Fig 25A). It must be noted that the *upf1-5* is a hypomorph NMD mutant, so its impact is likely underestimated in this analysis.

To unpick the spectrum of errors, we categorised them into groups, such as nucleotide substitutions, insertions, and deletions, and we calculated their rate changes *(39,40)*. Consistent with previous findings (*39*), substitutions were the most prevalent error type (89.2 - 92.5% of

the total errors). The substitution rate was significantly increased in the *tfIIs-1* and *tfIIs-1;upf1-5* mutants and further elevated by heat. In contrast, it remained unchanged in the *upf1-5* plants, indicating that TFIIS serves as the primary fidelity factor controlling substitutions (Fig 25B). The rate of insertion was also increased in *tfIIs-1* background and further enhanced during TMHT/1d condition, and it had a mild increasing trend in *upf1-5*. The effect of *tfIIs-1* and *upf1-5* absence on insertion rate was additive, as observed in the *tfIIs-1;upf1-5* double mutant (1.4- and 1.2-fold increase compared to the *tfIIs-1*, p=0.069 and p=0.143 under NT and TMHT/1d respectively) (Fig 25C) (*Szaker, Verma et al., submitted*).

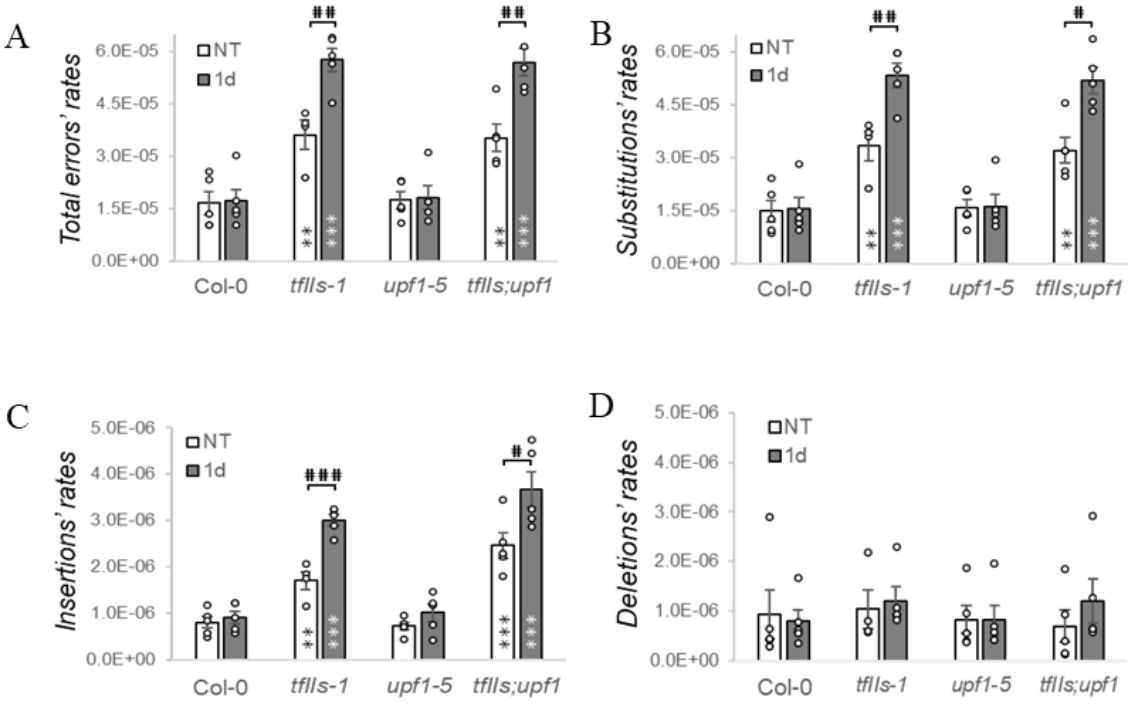

Figure 25: Transcriptional fidelity decreases at high temperatures and in the absence of TFIIS. *CirSeq assay analysis of transcriptional errors was performed on wild type (Col-0), tfIIs-1, upf1-5 and tfIIs-1;upf1-5, plants grown at non-treated (NT) and heat-stress conditions (TMHT/1d). (A) Total error' rates, (B) substitutions' rates, (C) insertions' rates (D) deletions, rates. Bars represent standard errors based on at least four biological replicates; P-values based on two-tailed Student's t-test *P < 0.05, **P < 0.01, ***P < 0.001 are shown for mutant vs wild-type, #P < 0.05, ##P < 0.01, ###P < 0.001 for TMHT/1d vs NT comparisons.*

These data suggest that the two factors (TFIIS and UPF1) may interact to efficiently prevent the accumulation of insertion-containing RNA species. In contrast, the deletions' rate remained unaltered (Fig 25D). These observations indicate that TFIIS prevents the production of all types of transcriptional errors, while NMD may be needed to decay the insertion containing transcripts.

To gain a deeper understanding of RNAPII activity and transcriptional errors, we categorized the substitutions based on their exchange types (Fig 26). Three predominant types of nucleotide exchanges were found: cytosine-to-uracil (C→U), guanine-to-adenine (G→A) and guanine-to-uracil (G→U). The C→U and G→U shifts were rather independent of genotype and temperature, while the G→A were influenced by both genotype and temperature conditions. There were some low-rate mismatches, including the C→A, U→A and U→C changes that were also genotype- and temperature-dependent (Fig 26). These observations suggest that TFIIS efficiently contributes to the eviction of specific transcriptional mistakes, while it is less competent in eradication others.

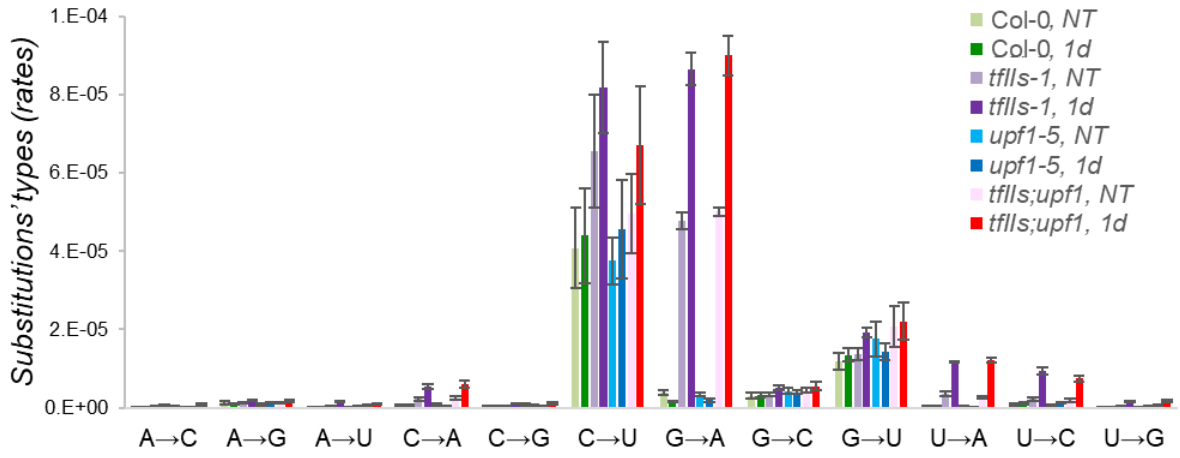

Figure 26: Single substitution type rates analysed by CirSeq assay*; substitution types, genotypes and temperature conditions are shown.*

5.3. A working model: Transcriptional fidelity regulation

Based on our findings, we propose a working model to explore the accuracy of transcription. In wt plants under ambient temperature, RNAPII may occasionally introduce errors; however, these are efficiently corrected by RNAPII intrinsic cleavage boosted by TFIIS activity. High temperatures do not alter transcriptional error rates under these conditions, suggesting either the same error rate or an efficient transcriptional fidelity mechanism.

In the absence of TFIIS (*tfIIs-1*), however, errors are accumulating under NT conditions, showing that TFIIS acts as a fidelity factor. Under elevated conditions, error rates are further increased, showing that high temperatures cause a decrease in RNAPII fidelity. Some of the errors (mainly those consisting of small insertion or deletion mutations of 1–2 nucleotides) are detected and eliminated by NMD; in this respect, NMD emerges as a secondary layer of fidelity safeguard. In the absence of TFIIS or NMD, the erroneous RNA transcripts (infidel RNA) that

enter the cytoplasm will take part in translation and the aberrant/truncated proteins potentially disrupt cellular processes through proteotoxicity (Fig 27).

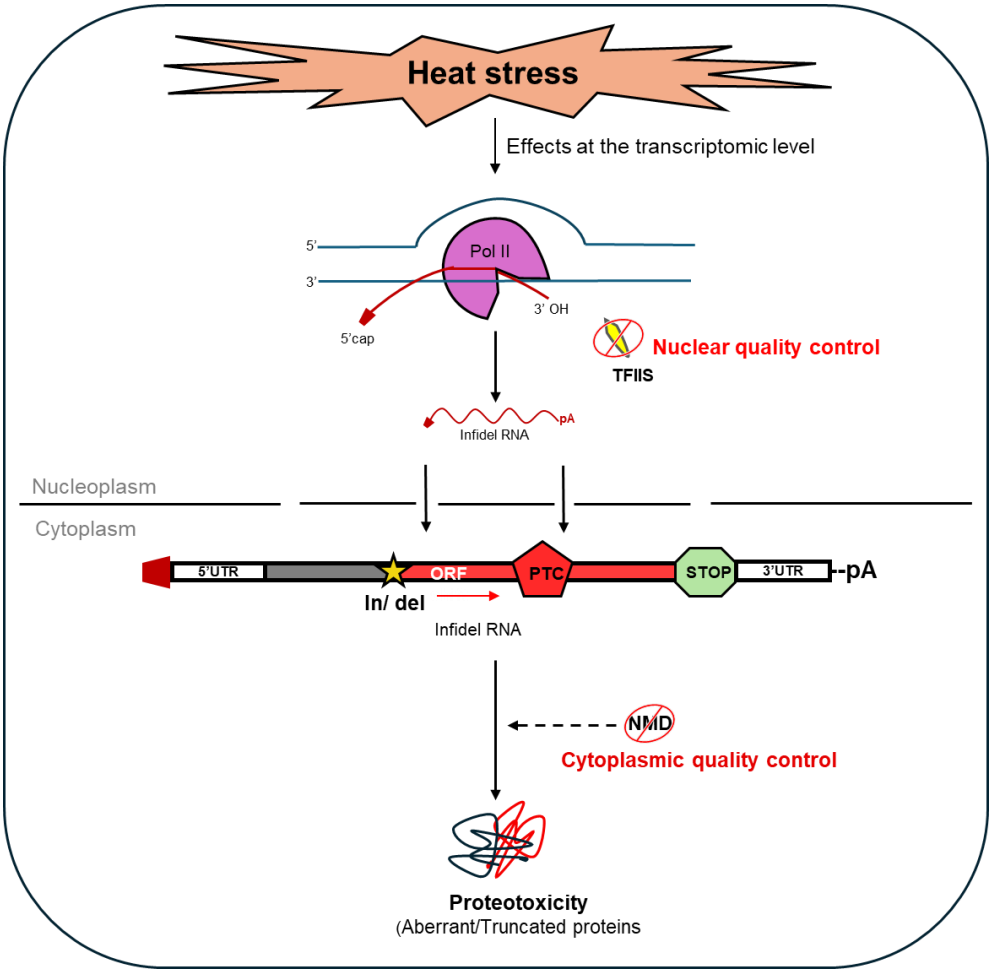

Figure 27: Transcriptional and post-transcriptional pathways interact to ensure transcript fidelity and prevent proteotoxicity, a working model. *During heat stress, the absence of TFIIS leads to the production of faulty/infidel RNAs causing truncated/ aberrant proteins that escape nuclear surveillance. The NMD pathway screens and eliminates the PTC-containing transcripts to prevent proteotoxic stress (see text for details).*

6. CONCLUSIONS AND RECOMMENDATIONS

In summary, through a heat-stress phenotyping screen, we identified TFIIS as a transcription factor involved in the heat stress response (HSR). We have unravelled that TFIIS despite being negligible under optimal conditions, its presence is vital for efficient HS adaptation in *Arabidopsis thaliana* plants. We also demonstrated that TFIIS is positively regulated by HSFs and is self-regulated. TFIIS protein accumulation during HS is conserved across evolutionarily distant species, including the unicellular alga *Chlamydomonas reinhardtii*, dicot *Brassica napus* and monocot *Hordeum vulgare* plants (*81*).
By creating CRISPR mutants in barley, we have shown that TFIIS functionality is necessary for HS survival in barley as well (*117*). Additionally, we investigated the downstream molecular changes regulated through TFIIS activity. TFIIS facilitates efficient transcriptional reprogramming from a developmental program to HS program, during which the timely expression, properly spliced and matured heat-stress transcript production is ensured, which is all needed to enable HS survival.

Finally, to uncover the mechanistic basis of TFIIS transcriptional regulation, we examined its role in transcriptional fidelity in conjunction with the cytoplasmic NMD RNA quality control pathway. We have proved that TFIIS acts as a nuclear fidelity factor; besides we demonstrated that NMD acts as a second layer to eliminate indel-containing transcripts and has a role under HS. Consequently, TFIIS in combination with NMD preserves transcriptome quality and prevents proteotoxic stress (*Szaker, Verma et al., submitted*).
As a future direction for this research, we aim to identify and study alternative pathways that may resolve the arrested RNAPII complex in the absence of TFIIS.

7. **NEW SCIENTIFIC RESULTS**

i. We identified TFIIS, an RNAPII elongation cofactor, to be needed for heat stress adaptation.

ii. We suggest that TFIIS regulation and functions are likely widely conserved in the plant kingdom, based on sequence conservation and expression analysis experiment in *C. reinhardtii, B. napus, A. thaliana, H. vulgare* plants.

iii. Based on RNA transcriptome analysis, we validated the qualitative and quantitative differences in the absence of TFIIS and uncovered the consequences of these alterations at the proteome level.

iv. We have shown that the NMD pathway is required for heat stress adaptation.

v. We demonstrated the interaction between nuclear co-transcriptional and cytoplasmic post-transcriptional RNA fidelity pathways.

vi. We have provided evidence that TFIIS is a fidelity factor under both ambient and high temperature conditions; besides, we have described the error landscape and temperature-dependence in *A. thaliana* for the first time.

8. ACKNOWLEDGEMENTS

Firstly, I would like to thank my supervisor, Dr. Tibor Csorba. He has been a valuable mentor, manager, colleague, and researcher throughout my journey. His unwavering support, vast knowledge, and constant motivation have been instrumental in my work. His remarkable work ethic, generosity, humanity, and passion for science inspire me every day, even during the most challenging times. His unwavering commitment to research and his genuine interest in helping others serve as a constant source of motivation and inspiration.

I would like to thank to the members of the Plant Stress Biology and Epigenetics groups for their support, collaboration, and companionship throughout the years. I am grateful to István Szádeczky-Kardoss, Henrik Szaker, Hussam Abbas Syed, Imtiaz Ahmad, György Szittya, Gyula Péter, Teréz Gorcsa, Tünde Nyikó, Tamás Toth, Anita Sós-Hegedűs, and Szilvia Ráth for their valuable contributions, insightful discussions, and friendship. Their collective assistance and encouragement have been a vital part of my journey.

I would like to extend my sincere thanks to all my colleagues with whom I had the pleasure of working on the publication: Éva Darkó, Aladár Pettkó-Szandtner, Dániel Silhavy, Kis András, Zoltán Havelda and Zoltán Toth. Your collaboration and contributions were invaluable, and I truly appreciate the opportunity to work together.

My deepest gratitude and love go to my family (Jai Ram Verma, Anita Verma and Lalit Kumar Verma), whose unwavering support and strength have been my foundation throughout this entire journey. Their constant presence has provided me with a sense of security and a loving home, offering comfort and encouragement no matter the challenges I faced along the way. Their love has been a guiding light, giving me the resilience to persevere and the confidence to keep moving forward. I am incredibly grateful to my friends for their constant support, encouragement, and companionship throughout this journey. Your kindness, laughter, and unwavering belief in me have made all the difference, especially during the tough times. Thank you for being there to celebrate the highs and lift me during the lows.

Lastly, I extend my thanks to everyone who, though not mentioned, has contributed in any way to my PhD. Your support and assistance have been truly appreciated.

9. APPENDICES

A1: LIST OF TABLES

Supplementary Table 1.

Genotyping	
TFIIS_SALK_056755 for:	ACC AAT TTG GAC ATT ATC CTC TGG A
TFIIS_SALK_056755 rev:	AGC TGC TTT AGC TGG AGC TTT CAT
UPF1-5_SALK_112922_F	ACC CAA AAC ATC CTT ACA ATG GCT
UPF1-5_SALK_112922_R	TGG ACA AGC CCA TAA GCC AAT GAT
UPF3-1 SALK_025175_LP	AGG TGA TTG CAC AAC CTG TTG AGA
UPF3-1 SALK_025175_RP	ACG ATA ATC TGG CTT AGA GCT GCT

Supplementary Table 2.

qRT-PCR	
TFIIS-qF	ACA TAC GAT ACC CTC GTT GCG ACT
TFIIS-qR	TTT ACA ACC GTT AGT GCC TTC GGT
PP2AA3_qF	CCT GCG GTA ATA ACT GCA TCT
PP2AA3_qR	CTT CAC TTA GCT CCA CCA AGC A
HsfA2-qF	TCG TCA GCT CAA TAC TTA TGG ATT C
HsfA2-qR	CAC ATG ACA TCC CAG ATC CTT GC
HSP90-1_qF	ATG GCG GAT GTT CAG ATG GCT
HSP90-1_qR	GGA TCT TGT CAA GAG CAT CAG AAG
uHSP90_qF	ATG GCG GAT GTT CAG ATG GCT
uHSP90_qR	CGA GCA GAG AGA GAT TTG AAG GG
HSP101_qF	CGC TAT AAT CTG CTT GAT TCT CTG C
HSP101_qR	GCT TTT GTA ATC CCT TAA AAC GAT AT
uHSP101_qF	CGC TAT AAT CTG CTT GAT TCT CTG C
uHSP101_qR	ACA AGA TTG TCG CGA TCA TTT ACC T
sHSP18.2_qF	ACA AAC GCA AGA GTG GAT TGG A
sHSP18.2_qR	GCT CCT CTC TCC GCT AAT CTG C
uTFIIS_qF	ACA TAC GAT ACC CTC GTT GCG ACT
uTFIIS_qR	ACA ACA CAA GTC AAT GCA ACG AGA
UPF1-5_qF	GAT CCA CGG AGG CTT AAT GT
UPF1-5_qR	CTC GAC CAA GCA CTC ATG TT
UPF3-1_qF	GATCAACCACTATCTTCAGCAGGAAA
UPF3-1_qR	GTGGAATGGCTCATATATTTAGCCATA
eRF1-1_qF	GAC AGT GAC TTG GCT TTG GA
eRF1-1_qR	CTT CTC CAT CCT CGG AAT CA
SMG7_qF	TGC CCG TGA CAA CTT GAT TGT TG
SMG7_qR	GCT ACC AAG GTC GCA TCT TTC AAT G

Continue….

Supplementary Table 2.

qRT-PCR	
CreTFIIS_qF	GCG GGA AAG CGG CTT AAT AA
CreTFIIS_qR	TTT TGA CGC ACT GCT TCC AT
CreRACK1_qF	CTT CTC GCC CAT GAC CAC
CreRACK1_qR	CCC ACC AGG TTG TTC TTC AG
HvTFIIS_qF:	TCG CCA CGC AGG TTG GCA AAC G
HvTFIIS_qR:	TTC AAT AAC AAC CTT CTT CCA G
HvACTIN_qF	AAT GGA ACC GGA ATG GTC AAG
HvACTIN_qR	CTC GTA GCT CTT CTC AAC TGA GGA G
BnaTFIISa_qF:	GCA CAG GTC AGG AGG TTT CT
BnaTFIISa_qR:	TTC TTC CCT ACC TGA GTC GC
BnaTFIISb_qF:	TGT TAC CTC CTC AAG CCC G
BnaTFIISb_qR:	TCA GCT TCT TCC CTA CCT GG
BnaTFIISc_qF:	GCG ACT CAG GTA GGG AAG AA
BnaTFIISc_qR:	TAG CCT TGG ATG TCG TCT CC
AAR2.2_qR	AGCTATGAAGACAAAGACTGCA
AAR2.4_qR	CTCCCTAAACCGTCAGATAAAA
ARO_qF	TTCACTCTCTTGTTCAGATGAA
ARO_qF	TGGCTTGTGATGAAGATAAGATGAA
BnaPP2AA5_qF	ATC TCT TCA TGG GCG ATT ACG TTG A
BnaPP2AA5_qR	AGC GAA CTT TGA GTG CTA CCA AG

A2: LIST OF SCIENTIFIC ACTIVITIES

A2.1. PRESENTATIONS RELATED TO THE THESIS

- **Verma R**, Szádeczky-Kardoss I, Szaker MH, Pettkó-Szandtner A, Silhavy D, Csorba T: The role of transcriptional elongation during heat stress adaptation in plants, XX Genetikai Műhelyek Magyarországon minikonferencia, Szeged, September 24, 2021.
- **Verma R**, Szádeczky-Kardoss I, Szaker MH, Zoltán T, Csorba T: Efficient transcriptional elongation is needed for heat stress adaptation of plants. ETABS 2022, e-Conference, 14-15 January, 2022. (Best Oral Presentation)
- **Verma R**, Szádeczky-Kardoss István, Szaker MH, Zoltán T, Csorba T. The roles and regulation of TFIIS elongation factor during heat stress response in plants. (Poster) 16th Microsymposium on RNA Biology, Vienna BioCenter, 06 – 08 April, 2022.
- **Verma R**, Szádeczky-Kardoss I, Szaker MH; Silhavy D, Csorba T. Regulation of transcriptional elongation during heat stress response. RNA Mini-symposium, Gödöllő, 2023 June 29. (Best Presenter)
- **Verma R**, Szádeczky-Kardoss I, Szaker MH, Silhavy D, Csorba T: Transcriptional dynamics during heat stress adaptation in plants. GBI Napok Gödöllő, 30 November 2023. (Third price).
- Ahmad I, **Verma R**, Szadeczky-Kardoss I, Szaker MH, Syed Abbas H, Csorba T. Decoding the Role of TFIIS in Plant Resilience to Heat Stress, GBI Napok, 10-11 December 2024, Gödöllő, Hungary.
- **Verma R**, Szaker MH, Szadeczky-Kardoss I, Syed Abbas H, Pettkó-Szandtner A, Silhavy D, Csorba T. Transcriptome fidelity aids development and heat stress response in Arabidopsis. XXIII Genetikai Műhelyek Magyarországon Conference, 06 September 2024, Szeged, Hungary.

A2.2. PUBLICATIONS RELATED TO THE THESIS

- Szádeczky-Kardoss I*, Szaker MH*, **Verma R**, Darkó E, Pettkó-Szandtner A, Silhavy D, Csorba T, Elongation factor TFIIS is essential for heat stress adaptation in plants, Nucleic Acids Research, Volume 50, Issue 4, 28 February 2022, Pages 1927–1950.
- Ahmad I, Kis A, **Verma R**, Szádeczky-Kardoss I, Szaker MH, Pettkó-Szandtner A, Silhavy D, Havelda Z, Csorba T (2024). TFIIS is required for reproductive development and thermal adaptation in barley. Plant Cell Reports,43, 260.
- **Verma R***, Szaker MH*, Szádeczky-Kardoss István, Nóra Gál, Syed HA, Éva Darkó, Pettkó-Szandtner A, Silhavy D, Csorba T. (2025). Transcription fidelity and alternative splicing control contribute to heat stress survival in Arabidopsis (Accepted in Plant Cell) (*authors contributed equally to the work)

A3: REFERENCES

1. S. Osman, P. Cramer, Structural Biology of RNA Polymerase II Transcription: 20 Years On. *Annu Rev Cell Dev Biol* **36**, 1-34 (2020).
2. H. Khatter, M. K. Vorlander, C. W. Muller, RNA polymerase I and III: similar yet unique. *Curr Opin Struct Biol* **47**, 88-94 (2017).
3. M. Girbig, A. D. Misiaszek, C. W. Muller, Structural insights into nuclear transcription by eukaryotic DNA-dependent RNA polymerases. *Nat Rev Mol Cell Biol* **23**, 603-622 (2022).
4. Q. Chen *et al.*, FLOWERING LOCUS T mRNA is synthesized in specialized companion cells in Arabidopsis and Maryland Mammoth tobacco leaf veins. *Proc Natl Acad Sci U S A* **115**, 2830-2835 (2018).
5. Y. Onodera *et al.*, Plant nuclear RNA polymerase IV mediates siRNA and DNA methylation-dependent heterochromatin formation. *Cell* **120**, 613-622 (2005).
6. P. Cramer, D. A. Bushnell, R. D. Kornberg, Structural basis of transcription: RNA polymerase II at 2.8 angstrom resolution. *Science* **292**, 1863-1876 (2001).
7. Y. Lorch, R. D. Kornberg, Chromatin-remodeling for transcription. *Q Rev Biophys* **50**, e5 (2017).
8. Q. A. Thomas *et al.*, Transcript isoform sequencing reveals widespread promoter-proximal transcriptional termination in Arabidopsis. *Nat Commun* **11**, 2589 (2020).
9. L. Caizzi *et al.*, Efficient RNA polymerase II pause release requires U2 snRNP function. *Mol Cell* **81**, 1920-1934 e1929 (2021).
10. S. Zhang *et al.*, Structure of a transcribing RNA polymerase II-U1 snRNP complex. *Science* **371**, 305-309 (2021).
11. G. Garg *et al.*, Structural insights into human co-transcriptional capping. *Mol Cell* **83**, 2464-2477 e2465 (2023).
12. I. Fianu *et al.*, Structural basis of Integrator-mediated transcription regulation. *Science* **374**, 883-887 (2021).
13. D. Zhu *et al.*, The Features and Regulation of Co-transcriptional Splicing in Arabidopsis. *Mol Plant* **13**, 278-294 (2020).
14. C. Baejen *et al.*, Genome-wide Analysis of RNA Polymerase II Termination at Protein-Coding Genes. *Mol Cell* **66**, 38-49 e36 (2017).
15. A. F. Palazzo, Y. Qiu, Y. M. Kang, mRNA nuclear export: how mRNA identity features distinguish functional RNAs from junk transcripts. *RNA Biol* **21**, 1-12 (2024).
16. N. J. Fuda, M. B. Ardehali, J. T. Lis, Defining mechanisms that regulate RNA polymerase II transcription in vivo. *Nature* **461**, 186-192 (2009).
17. S. Gressel *et al.*, CDK9-dependent RNA polymerase II pausing controls transcription initiation. *Elife* **6**, (2017).
18. Y. Xu *et al.*, Architecture of the RNA polymerase II-Paf1C-TFIIS transcription elongation complex. *Nat Commun* **8**, 15741 (2017).
19. Y. A. Nedialkov *et al.*, NTP-driven translocation by human RNA polymerase II. *J Biol Chem* **278**, 18303-18312 (2003).
20. J. F. Sydow, P. Cramer, RNA polymerase fidelity and transcriptional proofreading. *Curr Opin Struct Biol* **19**, 732-739 (2009).
21. J. Durr *et al.*, The transcript elongation factor SPT4/SPT5 is involved in auxin-related gene expression in Arabidopsis. *Nucleic Acids Res* **42**, 4332-4347 (2014).
22. M. Grasser *et al.*, Transcript elongation factor TFIIS is involved in arabidopsis seed dormancy. *J Mol Biol* **386**, 598-611 (2009).

23. P. Michl-Holzinger *et al.*, Phosphorylation of the FACT histone chaperone subunit SPT16 affects chromatin at RNA polymerase II transcriptional start sites in Arabidopsis. *Nucleic Acids Res* **50**, 5014-5028 (2022).
24. S. A. Mortensen, K. D. Grasser, The seed dormancy defect of Arabidopsis mutants lacking the transcript elongation factor TFIIS is caused by reduced expression of the DOG1 gene. *FEBS Lett* **588**, 47-51 (2014).
25. S. Obermeyer *et al.*, TFIIS Is Crucial During Early Transcript Elongation for Transcriptional Reprogramming in Response to Heat Stress. *J Mol Biol* **435**, 167917 (2023).
26. S. Obermeyer *et al.*, Distinct role of subunits of the Arabidopsis RNA polymerase II elongation factor PAF1C in transcriptional reprogramming. *Front Plant Sci* **13**, 974625 (2022).
27. W. Antosz, A. Pfab, H. F. Ehrnsberger, The Composition of the Arabidopsis RNA Polymerase II Transcript Elongation Complex Reveals the Interplay between Elongation and mRNA Processing Factors. **29**, 854-870 (2017).
28. J. Dolata *et al.*, NTR1 is required for transcription elongation checkpoints at alternative exons in Arabidopsis. *Embo j* **34**, 544-558 (2015).
29. J. Liu, W. Jie, X. Shi, Y. Ding, C. Ding, Transcription elongation factors OsSPT4 and OsSPT5 are essential for rice growth and development and act with APO2. *Plant Cell Rep* **42**, 1217-1231 (2023).
30. X. Yu, S. D. Michaels, The Arabidopsis Paf1c complex component CDC73 participates in the modification of FLOWERING LOCUS C chromatin. *Plant Physiol* **153**, 1074-1084 (2010).
31. K. D. Grasser, The FACT Histone Chaperone: Tuning Gene Transcription in the Chromatin Context to Modulate Plant Growth and Development. *Front Plant Sci* **11**, 85 (2020).
32. A. Pfab, M. Breindl, K. D. Grasser, The Arabidopsis histone chaperone FACT is required for stress-induced expression of anthocyanin biosynthetic genes. *Plant Mol Biol* **96**, 367-374 (2018).
33. S. Obermeyer, L. Schrettenbrunner, R. Stockl, U. Schwartz, K. D. Grasser, Different elongation factors distinctly modulate RNA polymerase II transcription in Arabidopsis. *Nucleic Acids Res* **51**, 11518-11533 (2023).
34. W. Antosz, J. Deforges, Critical Role of Transcript Cleavage in Arabidopsis RNA Polymerase II Transcriptional Elongation. **32**, 1449-1463 (2020).
35. M. Vermulst *et al.*, Transcription errors induce proteotoxic stress and shorten cellular lifespan. *Nat Commun* **6**, 8065 (2015).
36. Z. Kerenyi *et al.*, Inter-kingdom conservation of mechanism of nonsense-mediated mRNA decay. *EMBO J* **27**, 1585-1595 (2008).
37. C. Chung *et al.*, Evolutionary conservation of the fidelity of transcription. *Nat Commun* **14**, 1547 (2023).
38. C. Chung *et al.*, The fidelity of transcription in human cells. *Proc Natl Acad Sci U S A* **120**, e2210038120 (2023).
39. C. Fritsch, J. P. Gout, M. Vermulst, Genome-wide Surveillance of Transcription Errors in Eukaryotic Organisms. *J Vis Exp*, (2018).
40. J. F. Gout, W. Li, The landscape of transcription errors in eukaryotic cells. **3**, e1701484 (2017).
41. C. Jeon, K. Agarwal, Fidelity of RNA polymerase II transcription controlled by elongation factor TFIIS. *Proc Natl Acad Sci U S A* **93**, 13677-13682 (1996).
42. C. Walmacq *et al.*, Rpb9 subunit controls transcription fidelity by delaying NTP sequestration in RNA polymerase II. *J Biol Chem* **284**, 19601-19612 (2009).

43. E. Nudler, RNA polymerase backtracking in gene regulation and genome instability. *Cell* **149**, 1438-1445 (2012).
44. H. Kettenberger, K. J. Armache, P. Cramer, Architecture of the RNA polymerase II-TFIIS complex and implications for mRNA cleavage. *Cell* **114**, 347-357 (2003).
45. D. Kulish *et al.*, The functional role of basic patch, a structural element of Escherichia coli transcript cleavage factors GreA and GreB. *J Biol Chem* **275**, 12789-12798 (2000).
46. W. Hausner, U. Lange, M. Musfeldt, Transcription factor S, a cleavage induction factor of the archaeal RNA polymerase. *J Biol Chem* **275**, 12393-12399 (2000).
47. R. C. Conaway, J. W. Conaway, The hunt for RNA polymerase II elongation factors: a historical perspective. **26**, 771-776 (2019).
48. K. Cermakova *et al.*, A ubiquitous disordered protein interaction module orchestrates transcription elongation. *Science* **374**, 1113-1121 (2021).
49. K. Cermakova, V. Veverka, H. C. Hodges, The TFIIS N-terminal domain (TND): a transcription assembly module at the interface of order and disorder. *Biochem Soc Trans* **51**, 125-135 (2023).
50. T. Kurosaki, M. W. Popp, L. E. Maquat, Quality and quantity control of gene expression by nonsense-mediated mRNA decay. *Nat Rev Mol Cell Biol* **20**, 406-420 (2019).
51. M. Ohtani, A. Wachter, NMD-Based Gene Regulation-A Strategy for Fitness Enhancement in Plants? *Plant Cell Physiol* **60**, 1953-1960 (2019).
52. P. Avery *et al.*, Drosophila Upf1 and Upf2 loss of function inhibits cell growth and causes animal death in a Upf3-independent manner. *RNA* **17**, 624-638 (2011).
53. N. Wittkopp *et al.*, Nonsense-mediated mRNA decay effectors are essential for zebrafish embryonic development and survival. *Mol Cell Biol* **29**, 3517-3528 (2009).
54. J. Hwang, L. E. Maquat, Nonsense-mediated mRNA decay (NMD) in animal embryogenesis: to die or not to die, that is the question. *Curr Opin Genet Dev* **21**, 422-430 (2011).
55. L. Arciga-Reyes, L. Wootton, M. Kieffer, B. Davies, UPF1 is required for nonsense-mediated mRNA decay (NMD) and RNAi in Arabidopsis. *Plant J* **47**, 480-489 (2006).
56. N. Riehs-Kearnan, J. Gloggnitzer, B. Dekrout, C. Jonak, K. Riha, Aberrant growth and lethality of Arabidopsis deficient in nonsense-mediated RNA decay factors is caused by autoimmune-like response. *Nucleic Acids Res* **40**, 5615-5624 (2012).
57. P. Bulankova, N. Riehs-Kearnan, M. K. Nowack, A. Schnittger, K. Riha, Meiotic progression in Arabidopsis is governed by complex regulatory interactions between SMG7, TDM1, and the meiosis I-specific cyclin TAM. *Plant Cell* **22**, 3791-3803 (2010).
58. J. Rehwinkel, I. Letunic, J. Raes, P. Bork, E. Izaurralde, Nonsense-mediated mRNA decay factors act in concert to regulate common mRNA targets. *RNA* **11**, 1530-1544 (2005).
59. K. Hori, Y. Watanabe, UPF3 suppresses aberrant spliced mRNA in Arabidopsis. *Plant J* **43**, 530-540 (2005).
60. G. Drechsel *et al.*, Nonsense-mediated decay of alternative precursor mRNA splicing variants is a major determinant of the Arabidopsis steady state transcriptome. *Plant Cell* **25**, 3726-3742 (2013).
61. M. Kalyna *et al.*, Alternative splicing and nonsense-mediated decay modulate expression of important regulatory genes in Arabidopsis. *Nucleic Acids Res* **40**, 2454-2469 (2012).
62. S. Kertesz *et al.*, Both introns and long 3'-UTRs operate as cis-acting elements to trigger nonsense-mediated decay in plants. *Nucleic Acids Res* **34**, 6147-6157 (2006).

63. H. Le Hir, E. Izaurralde, L. E. Maquat, M. J. Moore, The spliceosome deposits multiple proteins 20-24 nucleotides upstream of mRNA exon-exon junctions. *EMBO J* **19**, 6860-6869 (2000).
64. E. Conti, E. Izaurralde, Nonsense-mediated mRNA decay: molecular insights and mechanistic variations across species. *Curr Opin Cell Biol* **17**, 316-325 (2005).
65. T. Nyiko *et al.*, Plant nonsense-mediated mRNA decay is controlled by different autoregulatory circuits and can be induced by an EJC-like complex. *Nucleic Acids Res* **41**, 6715-6728 (2013).
66. J. Gloggnitzer *et al.*, Nonsense-mediated mRNA decay modulates immune receptor levels to regulate plant antibacterial defense. *Cell Host Microbe* **16**, 376-390 (2014).
67. E. Degtiar *et al.*, The feedback control of UPF3 is crucial for RNA surveillance in plants. *Nucleic Acids Res* **43**, 4219-4235 (2015).
68. R. Luha, V. Rana, A. Vainstein, V. Kumar, Nonsense-mediated mRNA decay pathway in plants under stress: general gene regulatory mechanism and advances. *Planta* **259**, 51 (2024).
69. A. Sugio, R. Dreos, F. Aparicio, A. J. Maule, The cytosolic protein response as a subcomponent of the wider heat shock response in Arabidopsis. *Plant Cell* **21**, 642-654 (2009).
70. C. Shi, I. T. Baldwin, J. Wu, Arabidopsis plants having defects in nonsense-mediated mRNA decay factors UPF1, UPF2, and UPF3 show photoperiod-dependent phenotypes in development and stress responses. *J Integr Plant Biol* **54**, 99-114 (2012).
71. K. Vexler *et al.*, The Arabidopsis NMD Factor UPF3 Is Feedback-Regulated at Multiple Levels and Plays a Role in Plant Response to Salt Stress. *Front Plant Sci* **7**, 1376 (2016).
72. N. Ohama, H. Sato, K. Shinozaki, K. Yamaguchi-Shinozaki, Transcriptional Regulatory Network of Plant Heat Stress Response. *Trends Plant Sci* **22**, 53-65 (2017).
73. K. D. Scharf, T. Berberich, I. Ebersberger, L. Nover, The plant heat stress transcription factor (Hsf) family: structure, function and evolution. *Biochim Biophys Acta* **1819**, 104-119 (2012).
74. S. Kotak *et al.*, Complexity of the heat stress response in plants. *Curr Opin Plant Biol* **10**, 310-316 (2007).
75. S. Kotak, M. Port, A. Ganguli, F. Bicker, P. von Koskull-Doring, Characterization of C-terminal domains of Arabidopsis heat stress transcription factors (Hsfs) and identification of a new signature combination of plant class A Hsfs with AHA and NES motifs essential for activator function and intracellular localization. *Plant J* **39**, 98-112 (2004).
76. F. McLoughlin, M. Kim, R. S. Marshall, HSP101 Interacts with the Proteasome and Promotes the Clearance of Ubiquitylated Protein Aggregates. **180**, 1829-1847 (2019).
77. P. Wang *et al.*, Plant Autophagy: An Intricate Process Controlled by Various Signaling Pathways. *Front Plant Sci* **12**, 754982 (2021).
78. Z. Xie *et al.*, Proteasome resides in and dismantles plant heat stress granules constitutively. *Mol Cell* **84**, 3320-3335 e3327 (2024).
79. S. Pandey, S. Divakar, A. Singh, Genome editing prospects for heat stress tolerance in cereal crops. *Plant Physiol Biochem* **215**, 108989 (2024).
80. M. Akerfelt, R. I. Morimoto, L. Sistonen, Heat shock factors: integrators of cell stress, development and lifespan. *Nat Rev Mol Cell Biol* **11**, 545-555 (2010).
81. I. Szadeczky-Kardoss *et al.*, Elongation factor TFIIS is essential for heat stress adaptation in plants. *Nucleic Acids Res* **50**, 1927-1950 (2022).
82. K. Sekimizu, N. Kobayashi, D. Mizuno, S. Natori, Purification of a factor from Ehrlich ascites tumor cells specifically stimulating RNA polymerase II. *Biochemistry* **15**, 5064-5070 (1976).

83. F. McLoughlin *et al.*, Class I and II Small Heat Shock Proteins Together with HSP101 Protect Protein Translation Factors during Heat Stress. **172**, 1221-1236 (2016).
84. E. R. Waters, E. Vierling, Plant small heat shock proteins - evolutionary and functional diversity. **227**, 24-37 (2020).
85. T. C. Rytz *et al.*, SUMOylome Profiling Reveals a Diverse Array of Nuclear Targets Modified by the SUMO Ligase SIZ1 during Heat Stress. *Plant Cell* **30**, 1077-1099 (2018).
86. H. Koyama, T. Ito, T. Nakanishi, N. Kawamura, K. Sekimizu, Transcription elongation factor S-II maintains transcriptional fidelity and confers oxidative stress resistance. *Genes Cells* **8**, 779-788 (2003).
87. H. Koyama, T. Ito, T. Nakanishi, K. Sekimizu, Stimulation of RNA polymerase II transcript cleavage activity contributes to maintain transcriptional fidelity in yeast. *Genes Cells* **12**, 547-559 (2007).
88. Y. Kurihara *et al.*, Genome-wide suppression of aberrant mRNA-like noncoding RNAs by NMD in Arabidopsis. *Proc Natl Acad Sci U S A* **106**, 2453-2458 (2009).
90. H. Koyama, T. Ito, T. Nakanishi, K. Sekimizu, Stimulation of RNA polymerase II transcript cleavage activity contributes to maintain transcriptional fidelity in yeast. *Genes Cells* **12**, 547-559 (2007).
91. Y. Kurihara *et al.*, Genome-wide suppression of aberrant mRNA-like noncoding RNAs by NMD in Arabidopsis. *Proc Natl Acad Sci U S A* **106**, 2453-2458 (2009).
92. A. K. Srivastava, Y. Lu, G. Zinta, Z. Lang, J. K. Zhu, UTR-Dependent Control of Gene Expression in Plants. *Trends Plant Sci* **23**, 248-259 (2018).
93. B. Pucker *et al.*, A chromosome-level sequence assembly reveals the structure of the Arabidopsis thaliana Nd-1 genome and its gene set. *PLoS One* **14**, e0216233 (2019).
94. K. Adelman and J.T. Lis, Promoter-proximal pausing of RNA polymerase II: emerging roles in metazoans. Nat. Rev. Genet. **13**,720–31(2012).
95. R. Belotserkovskaya and D. Belotserkovskaya. FACT and Chd1: accomplices in chromatin. Nature Reviews Molecular Cell Biology. **5**, 374-381(2004).
96. K. Luger, M. L. Dechassa, and D. J. Tremethick. "New insights into nucleosome and chromatin structure: an ordered state or a disordered affair?" Nature Reviews Molecular Cell Biology. **13**, 436-447 (2012).
97. S. Venkatesh and J. L. Workman. Histone exchange, chromatin structure and the regulation of transcription. Nature Reviews Molecular Cell Biology, **16**, 178-189(2015).
98. K. Struhl, E. Segal. Determinants of nucleosome positioning. Nat Struct Mol Biol. **20**, 267–273 (2013).
99. D. K. Ray, N. D. Mueller, P. C. West, J. A. Foley. Yield Trends Are Insufficient to Double Global Crop Production by 2050. PloS one. **8**, e66428 (2013).
100. K. Richter, M. Haslbeck, J. Buchner. The heat shock response: life on the verge of death. Molecular cell. **40**, 253-266 (2010).
101. A. Grover, D. Mittal, M. Negi, D. Lavania. Generating high temperature tolerant transgenic plants: Achievements and challenges. Plant science: an international journal of experimental plant biology. **205-206**, 38-47 (2013).
102. A. L. Qu, Y. F. Ding, Q. Jiang, C. Zhu. Molecular mechanisms of the plant heat stress response. Biochemical and biophysical research communications. **432**, 203-207 (2013).
103. X. Ma, M. Mau, T. F. Sharbel. Genome Editing for Global Food Security. Trends in biotechnology. (2017).
104. A.J. Caplan, S. Jackson, D. Smith. Hsp90 reaches new heights. EMBO Rep. **4**, 126–130 (2003).

105. S.K. Calderwood. Regulatory interfaces between the stress protein response and other gene expression programs in the cell. Methods. **35**, 139–148 (2005).
106. J.R. Buchan, R. Parker. Eukaryotic stress granules: the ins and outs of translation. Mol. Cell. **36**, 932–941(2009).
107. D. Mittal, D.A. Madhyastha, A.Grover. Genome-wide transcriptional profiles during temperature and oxidative stress reveal coordinated expression patterns and overlapping regulons in rice. PLoS One **7.** e40899 (2012).
108. S. K. Baniwal, K.Bharti, K. Y.Chan, M. Fauth, A. Ganguli, S Kotak, et al. Heat stress response in plants: a complex game with chaperones and more than twenty heat stress transcription factors. J. Biosci. **29**, 471–487(2004).
109. H. Sakurai, and Y.Enoki. Novel aspects of heat shock factors: DNA recognition, chromatin modulation and gene expression. FEBS J. **277**, 4140–4149 (2010).
110. S.V. Kumar, P.A. Wigge. H2A.Z-containing nucleosomes mediate the thermosensory response in Arabidopsis. Cell, **140**, 136–147. 32 (2010).
111. T. Yoshida, N. Ohama, J. Nakajima, S. Kidokoro, J. Mizoi, K. Nakashima, K. Maruyama, J.M. Kim, M. Seki, D. Todaka, et al. Arabidopsis HsfA1 transcription factors function as the main positive regulators in heat shock-responsive gene expression. Mol. Genet. Genomics. **286**, 321–332 (2011).
112. N. Andrási, A. Pettkó-Szandtner, L. Szabados. Diversity of plant heat shock factors: regulation, interactions, and functions. J Exp Bot. **2**,1558-1575 (2021).
113. S. Kotak, J. Larkindale, U. Lee, P. von Koskull-Doring, E. Vierling, K.D. Scharf. Complexity of the heat stress response in plants. Curr. Opin. Plant Biol. **10**, 310–316 (2007).
114. J. K. Björk, L. Sistonen. Regulation of the members of the heat shock factor family. FEBS Journal, **277**, 4126-4139 (2010).
115. N. Ohama, H. Sato, K. Shinozaki, K. Yamaguchi-Shinozaki. Transcriptional Regulatory Network of Plant Heat Stress Response. Trends Plant. Sci. **22**, 53-65 (2017).
116. R. Gomez-Pastor, E.T. Burchfiel, D.J. Thiele. Regulation of heat shock transcription factors and their roles in physiology and disease. Nat. Rev. Mol. Cell Biol. **19**, 4–19 (2018).
117. F. McLoughlin, E. Vierling. Chaperone-mediated protein disaggregation in plants." Journal of Experimental Botany. **70**, 217-233 (2019).
118. A. Mogk, B. Bukau, H. H. Kampinga. Cellular strategies for controlling protein aggregation. Nature Reviews Molecular Cell Biology. **19**, 715-727 (2017).
119. E.H. Baillo, R.N. Kimotho, Z. Xu. P. Zhang. Transcription Factors Associated with Abiotic and Biotic Stress Tolerance and Their Potential for Crops Improvement. Genes. **10**, 771(2019).
120. L. Chang, T. Lei, Y. Wenbo, et al. Natural variation in SbTEF1 contributes to salt tolerance in sorghum seedlings. Journal of Integrative Agriculture, 2095-3119, (2024).
121. J. Zheng, H. Liu, Y. Wang, L. Wang, X. Chang, R. Jing, C. Hao, X. Zhang. TEF-7A, a transcript elongation factor gene, influences yield-related traits in bread wheat (Triticum aestivum L.), Journal of Experimental Botany. **65**, 5351–5365, (2014).
122. I. Ahmad, A. Kis, R. Verma, et al. TFIIS is required for reproductive development and thermal adaptation in barley. Plant Cell Rep. **43**, 260 (2024).
123. H. Koyama , T. Ueda, T. Ito, K. Sekimizu. Novel RNA polymerase II mutation suppresses transcriptional fidelity and oxidative stress sensitivity in rpb9Delta yeast. Genes Cells. **15**, 151-9 (2010).
124. N. Kovinich, G. Kayanja, A. Chanoca, M.S. Otegui, E. Grotewold. Abiotic stresses induce different localizations of anthocyanins in Arabidopsis. Plant Signal Behav. **10**, e1027850 (2015).