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FINAL REPORT

Transcriptional and epigenetic regulation during heat stress in *Brassicaceae*

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Abbreviations:

A. thaliana: *Arabidopsis thaliana*
B. napus: *Brassica napus*
H. vulgare: *Hordeum vulgare*
HSR: heat stress response
HSE: heat shock element
HSF: heat stress factor
HSP: heat stress protein
BT: basal thermotolerance
SAT: short acquired thermotolerance
LAT: long acquired thermotolerance
TMHT: thermotolerance to moderately high temperatures
RNAPII: RNA polymerase II
wt: wild-type
mRNA: messenger RNA
DCL: Dice-like protein
AGO: Argonaute protein
RDR: RNA-dependent RNA polymerase
NDX: Nodulin Homeobox
RNAseq: RNA transcriptome sequencing
ChIPseq: chromatin immunoprecipitation sequencing

Summary

Climate change negatively affects the yield of agriculturally important crops all over the world. Plants being sessile organisms are more exposed to the temperature fluctuations, specifically to high temperature as a direct consequence of global temperature rising and heat waves. High temperature stress (heat stress, HS) is one of the major abiotic stresses affecting the distribution and productivity of agriculturally important plants worldwide. Heat stress responses (HSR) is best known in *Arabidopsis thaliana*, but much less studied in crop plants like the rapeseed *Brassica napus* or others. The central element of heat stress response is the transcriptional regulation. In the present project we have planned to understand the heat stress-specific transcriptional complex composition and regulation in *A. thaliana* and *B. napus* systems.

Findings of the work:

(i) To uncover regulators of HSR transcriptional response we have conducted a comprehensive mutant screen to detect HS-tolerant or HS-sensitive cellular regulators. From this screen we recovered TFIIS, an RNA polymerase II -associated elongation factor as being vital for the proper heat stress tolerance. While is non-essential under ambient conditions, ***TFIIS ensures the qualitative and quantitative transcriptome program switch during HSR, that is vital for HS survival***. TFIIS is physically recruited to HS loci (and depleted at the same time from housekeeping loci) to assist RNAPII transcription, using its anti-arrest activity during the elongation phase of transcription[1]. To prove the agricultural relevance of our findings, we generated the *tfIIs*-mutant *H. vulgare* plants by CRISPR technology, using two independent sgRNA guides (*hvtfIIs-cr1* and *-cr2*, respectively). *hvtfIIs*-mutant plants are heat sensitive; the basis of heat sensitivity and similarities and/or differences between dicot *A. thaliana* and monocot *H. vulgare* are being analysed (e.g., induction of HSFs, HSPs at RNA and protein levels) (*in prep*).

(ii) We have studied the impact of heat stress on transcriptional regulation of RNA silencing pathway components in *H. vulgare* system[2]. RNA silencing is a central regulator of development and stress responses. We have shown that ***transcriptional regulation controls the expression of RNA silencing's trans factors in barley***[2,3].

(iii) We have studied transcriptional regulator protein called Nodulin homeobox (NDX). NDX is a nuclear protein originally described as a regulator vernalization (prolonged cold temperature sensing) and ABA hormonal signalling. Surprisingly however, we have shown that NDX is primarily a heterochromatin-bound factor (rather than euchromatic regulator), that functions in pericentromeric regions to control siRNA production and non-CG methylation. Inactivation of NDX leads to differential siRNA accumulation and DNA methylation, of which CHH/CHG hypomethylation colocalizes with NDX binding sites. Intrachromosomal interactions decreased at peri-centromere regions, where NDX is enriched. In conclusion ***NDX is a positive regulator of heterochromatin compaction required for peri-centromeric and centromeric genome structure maintenance***[4]. Furthermore, *ndx* mutant plants have floral organ developmental phenotypes that are suppressed at high ambient temperatures. The molecular basis of this observation is being analysed (*in prep*).

(iv) We have completed a comprehensive analysis of miR824/AGAMOUS-LIKE16 (AGL16) module changes in response to heat stress. We have shown that miR824 accumulates in response to HS in *A. thaliana* and several *Brassicaceae* crops including *B. napus*: miR824 induction is dependent on HSE *cis*-elements and HSFA1 and HSFA2 transcription factors. We have further shown that miR824 acts as a "***post-transcriptional memory factor" to extend the acute impact of environmental fluctuations in the post-stress period and fine-tune flowering time regulators***[5].

(v) We have published a ***book chapter*** on summarizing the regulation of high-temperature stress response by small RNAs, and wrote a ***spotlight review*** on APOLO, a regulator of root development in response to auxin hormone and cold temperature conditions[6].

In summary, the findings of this research proposal have been presented on international and national conferences comprising ***5 conferences, 5 scientific publications and 1 book chapter***.

Content

I. Introduction

Heat stress is one of the most severe abiotic stresses, causing serious losses of agriculturally important crop plant worldwide and affecting natural habitats as well[7]. Understanding heat stress response is of uttermost importance especially in the light of global warming and heat waves[8,9].

The extreme environmental heat causes severe damage to the plant cells and organism: it impairs photosynthesis, reduce water content, damages nucleic acids, proteins, and lipid membranes etc., endangering survival and decreasing productivity. Plants cannot avoid the exposure to these factors therefore need to adapt morphologically and physiologically. Cells exposed to high temperatures activate heat shock responses (HSR). HSR is a universally conserved pathway that involves transcriptional and epigenetic changes[8,9]. The HSR is regulated at multiple levels, transcriptional regulation being one of its most important pillars[9-11].

The 'master transcriptional regulator' of HSR is HSFA1 family. Plant HSR is best known in *A. thaliana*. In *A. thaliana* the HsfA1 family consist of 4 members (AtHSFA1a, b, d and e), having highly redundant activities[12,13]. AtHSFA1s are constitutively expressed at low levels since heat shock proteins (HSPs) limit their activation through protein-protein interactions. Upon HS, HSPs dissociate from HSFA1s, the monomers of HSFA1s homo-/hetero-oligomerize, translocate to nucleus and bind to DNA *cis* element known as the heat shock element (HSE) to triggers activation of their targets, such as secondary transcription factors, HSFs, HSPs, antioxidant enzymes etc. HSFA1s binding to target loci indirectly initiate RNA polymerase II (RNAPII) binding, however the link between the specific transcription factors (e.g. HSFs) and the general transcriptional machinery (RNAPII and its cofactors) is elusive. Several studies have been performed on HSFs' activities, however regulation of RNAPII core components during HSR is much less investigated[8,9,11,14].

B. napus (rapeseed) is becoming an important crop model system as its genome has been recently published[15]. The large size of BnaHSF family (*BnaHSF1-64*) suggests the need for adaptation to diverse climatic regions[16]. The mechanistic detail of BnaHSFs' activities and downstream target genes is largely elusive. Barley (*Hordeum vulgare L.*) is the fourth most important crop in the world. Due to its obvious economic importance and relatively easy manipulation of its diploid genome, barley is becoming a monocot crop model, useful for understanding stress responses and gathering data for increasing crop resilience[17].

II. Objectives

The objectives of the proposal were to (i) understand the mechanism of transcriptional regulation of heat stress response, (ii) investigate the composition of HS-transcriptional complex and (iii) to characterize the interplay between HS-specific transcriptional complexes and chromatin features in *Brassicaceae* model and crop plants.

II. Results

1. The study of RNAPII-associated factor TFIIS' roles during heat stress response.

To unravel players involved in coupling the specific heat stress transcription factors (e.g. HsfA1s) directly or indirectly to general RNA Polymerase II transcription machinery (e.g. RNAPII subunits and associated co-factors) during heat stress response, we performed HsfA1a and HsfA1d immunoprecipitation coupled to mass spectrometry (in collaboration with the Proteomics group, BRC, Szeged). From the common HsfA1a and A1d putative interactors (*not shown*) we have selected several genes/proteins (such as RNAPII subunits, Mediator subunits, transcriptional initiation or elongation cofactors, DNA and RNA binding proteins, epigenetic regulators etc.) for further tests. We obtained the *A. thaliana* mutant lines of these candidates (NASC, *arabidopsis.info*) and assayed their heat sensitivity in different heat stress regimes, including basal thermotolerance (BT), short acquired thermotolerance (SAT), long acquired thermotolerance (LAT) and thermotolerance to moderately high temperatures (TMHT)[14]. Amongst others, transcription elongation factor TFIIS mutant (*tfIIs-1*) were found to be heat-sensitive even when exposed to sub-lethal high temperatures. Remarkably, the requirement of TFIIS was most apparent when exposed to persistent moderately high heat (TMHT), the regime that mimics the natural conditions of global warming effects. Heat sensitivity of TFIIS mutant was confirmed in a second SALK line *tfIIs-2.* Based on these findings we decided to further analyse TFIIS activities during HSR.

In eukaryotes RNAPII is responsible for transcription of most mRNAs. Diverse circumstances may halt and cause backtracking of RNAPII[18]. During backtracking, the 3'-end of the nascent RNA is displaced from the polymerase active core. To resume transcription, the cleavage of the protruding 3'-end and reestablishment of the correct base pairing between the nascent RNA and template DNA is needed at the polymerization site. RNAPII core possesses a weak nuclease activity, which can perform the cleavage although at a rather low efficiency. TFIIS is an associated to RNAPII and assists the transcriptional elongation process. Upon transcriptional arrests TFIIS reaches through a pore inside RNAPII in the proximity of its active site and boosts the nuclease activity of the polymerase to release it from the arrested sites[18]. Besides, TFIIS binding induces structural changes of RNAPII complex to aid reactivation following transcriptional arrests. Therefore, the TFIIS anti-arrest activity makes transcriptional elongation highly efficient[18,19].

In *A. thaliana* TFIIS is encoded by a single-copy gene, and is expressed throughout the whole plant body[20]. Under standard conditions TFIIS activity seems negligible in *A. thaliana* plants since only very mild phenotypes can be observed on *tfIIs* mutant plants[20,21]. This suggests that RNAPII or alternative pathways can cope with arrests and backtracking events at ambient conditions.

To study TFIIS in *Arabidopsis* we have prepared TFIIS complemented lines: consistent with its RNAPII regulatory role, GSy-TFIIS fusion protein (GFP-Streptavidin double-tagged protein having yellow fluorescence under UV light) was localized to the nucleus but excluded from

nucleoli (the site of RNAPI and III action) (Fig 1). TFIIS localization was dynamically altered in response to heat: upon HS exposure TFIIS chromocenters were dissolved but formed a transient perinucleolar ring structure (at 1h), that could not be observed upon longer heat exposures (*unpublished*, Fig 1.). These cytological observations suggest an active regulation of this factor and anticipate a biological significance of TFIIS activity during HSR.

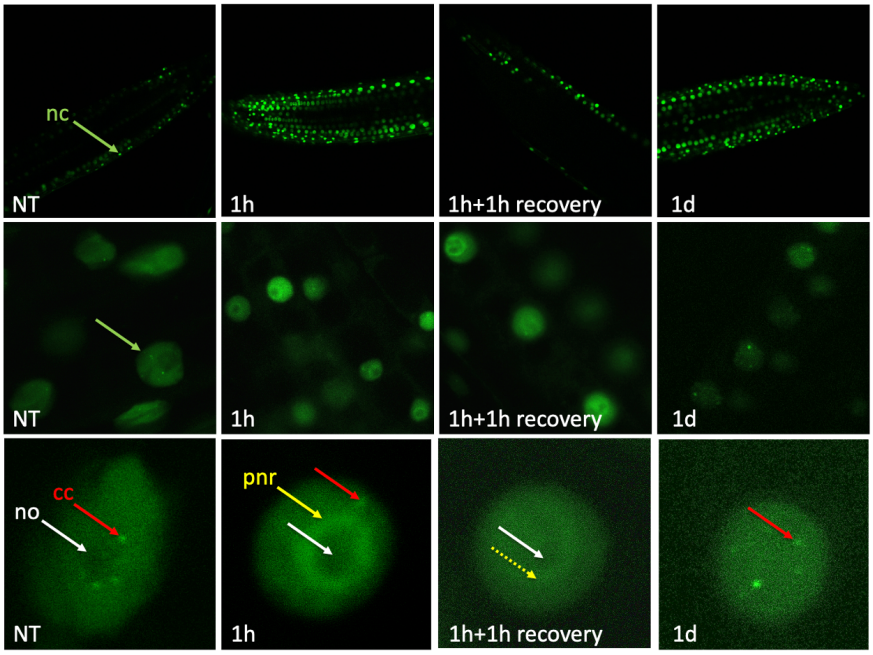


Figure 1: TFIIS localization and protein structure dynamics during HSR. YFP-TFIIS-expressing stable transgenic *A. thaliana* plant roots were visualized by confocal microscopy under UV light exposure (NT, non-treated; 1h, one hour heat treatment at 37˚C, 1h+1h recovery, heat treatment followed by 1h recovery at 21˚C; 1d, one day treatment at 37˚C; nc, nucleus; no, nucleolus; cc, chromocenter; pnr, perinuclear ring).

To understand it's dynamics during heat stress conditions, we analysed TFIIS mRNA level changes in wild-type (wt) *A. thaliana* Col-0 ecotype plants (Fig 2A-C). We measured TFIIS mRNA levels at one-hour (1h), 4h, one day (1d) of 37°C heat stress and 1d stress followed by two days recovery (1d+rec) treatments. A rapid and dramatic (6-10 x) transient accumulation of *TFIIS* mRNA was observed during early heat treatments (1-4h). Later the *TFIIS* mRNA levels declined, at 1d it was only half of that was measured at 4h. We also monitored the levels of the unspliced transcript form (*uTFIIS*) as a proxy for transcription activity. The changes in *uTFIIS* level showed a very similar trend to the spliced form (Fig 2C). Thus, we concluded that heat intensifies the transcription of TFIIS gene. In accordance with this we have found three HSE *cis* motifs within the TFIIS locus (Fig 2A). Additionally, when we re-analysed a previously published ChIPseq data[22] we have found that HsfA1a binds to TFIIS gene locus. These data suggest that in plants HsfA1 *trans* factors are involved in the transcriptional activation of TFIIS. Indeed, heat did not induce *TFIIS* mRNA accumulation in the quadruple *hsfa1a; a1b;a1d;a1e* (*QK)* mutant, in which all four members of the HsfA1 TF family are absent (Fig 2D).

To prove that heat-triggered transcriptional induction of *TFIIS* is manifested in increased protein amounts, we analysed the level of TFIIS protein in independent transgenic complementation lines, in which TFIIS protein was expressed from the own promoter: TFIIS protein accumulated significantly in response to heat (Fig 2E).
In the TFIIS T-DNA mutant (*tfIIs-1,* SALK_056755) the locus may still be transcriptionally

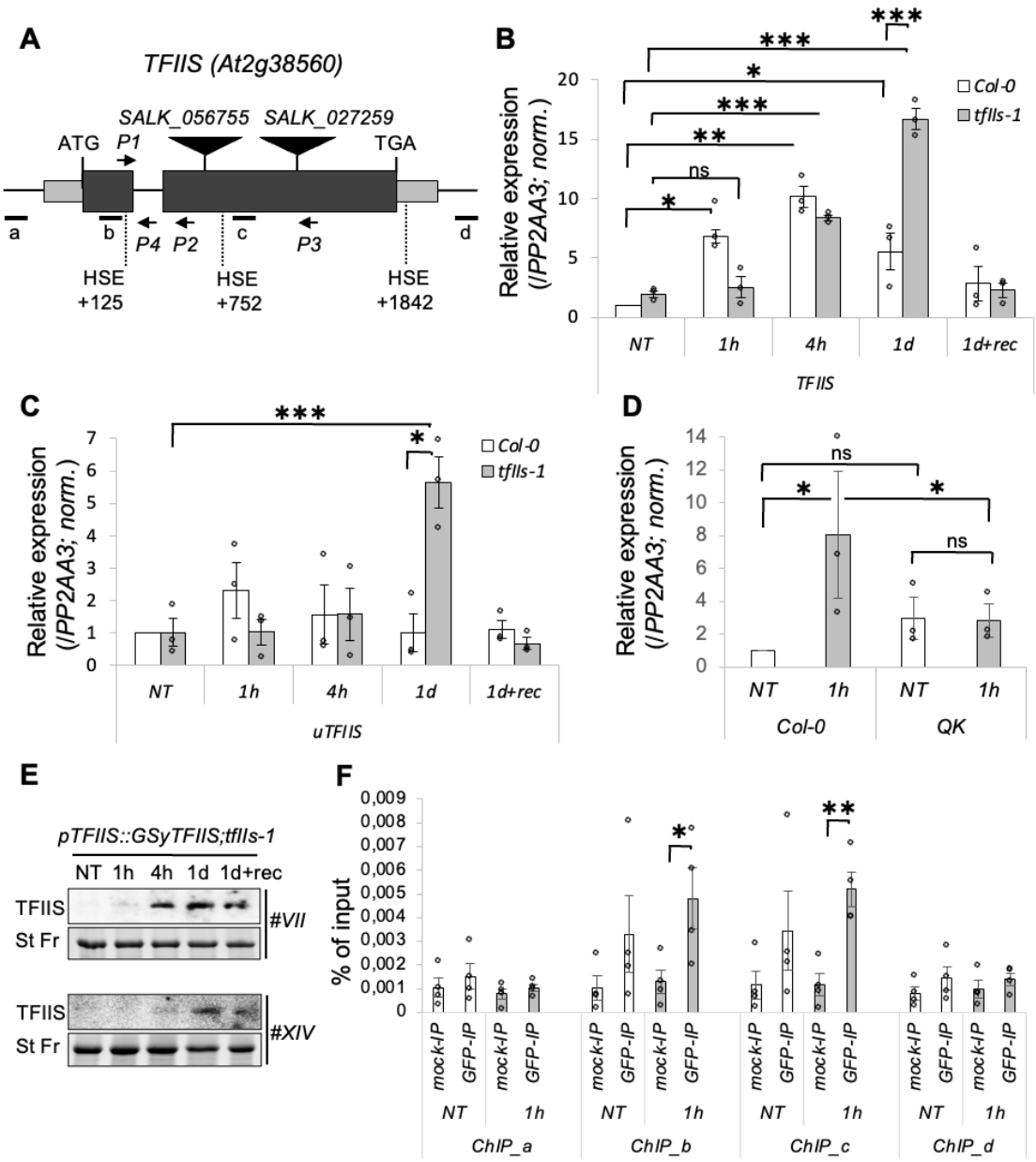


Figure 2: TFIIS is induced by heat. (A) Schematic representation of TFIIS gene locus; exons are black boxes, UTR regions are grey boxes, T-DNA insertions are shown above; heat shock cis elements (HSE), genotyping or qRT-PCR (P1, P2, P3, P4) primers are shown below; (B) qRT-PCR analysis of TFIIS spliced and (C) unspliced mRNA, values were normalized to NT Col-0 plants; (D) Relative expression of TFIIS spliced transcripts in *hsfa1* quadruple knock-out (QK) mutant; (E) western blott analysis of GSy-TFIIS protein in two independent *pTFIIS::GSy-TFIIS;tfIIs-1* lines; stain free as loading control (F) TFIIS-ChIP-qPCR at TFIIS locus, locations of amplicons are shown in panel A (a, b, c, d). Standard errors based on at least three bio reps; p values based on two-tailed Student's t-test (*p<0.05, **p<0.01, ***p<0.001).

active, generating a chimera *tfIIs-tdna* transcript. To analyse the transcriptional activity of the locus in the absence of a functional TFIIS protein during HSR we measured the level of *tfIIs-tdna* transcript in the *tfIIs-1* and Col-0 plants (using primers upstream to the insertion point, Fig 2A). Remarkably, at 1h heat treatment the *tfIIs-tdna* RNA level was not increased in the *tfIIs-1* mutants while at 4h both the *tfIIs-tdna* and *TFIIS* mRNA transcripts accumulated to similar levels (Fig 2B). Under persistent heat (1d) *TFIIS* mRNA was efficiently suppressed, but the *tfIIs-tdna* RNA in the mutant did not decrease, but instead was further elevated. Similar

dynamics were observed in case of the unspliced (*uTFIIS*) transcript forms (Fig 2C). The observations that in the mutant the HS-mediated transcriptional activation is slow and not repressed at persistent heat suggest that TFIIS protein itself could play a complex auto-regulatory roles at the TFIIS locus.

To confirm TFIIS direct autoregulatory roles at TFIIS locus, we performed a chromatin immunoprecipitation (ChIP) experiment: TFIIS protein associated with the gene body but not exogenic regions in heat-regulated manner (Fig 2A, F).

Accumulation of TFIIS during HSR suggests a functional requirement during temperature adaptation. To underpin this assumption, we tested the conservation of TFIIS' heat regulation. First, putative TFIIS homologs were identified in *Chlamydomonas reinhardtii* unicellular algae, dicot *Brassica napus* and monocot *Hordeum vulgare* genomes based on their domain organization, predicted structures and presence of catalytic core sites (Fig 3). Notably, HSE *cis*

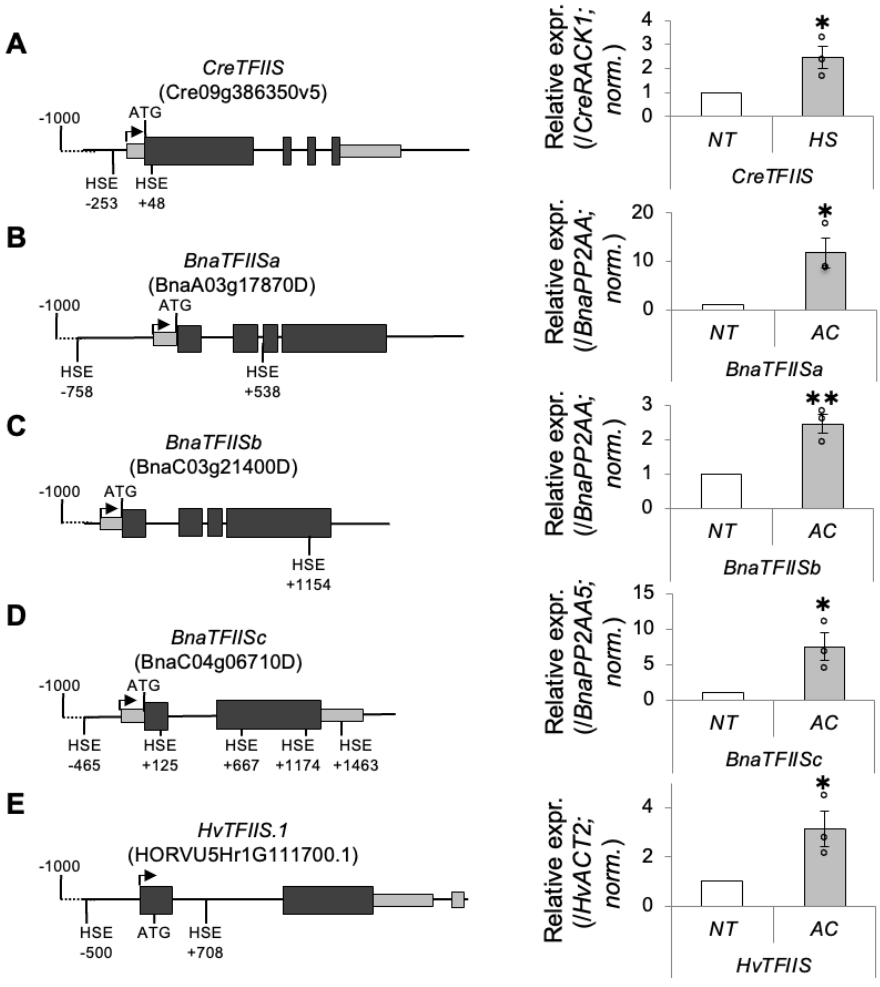


Figure 3: Conservation of TFIIS heat-regulation. (A-F left panels) schematic representation of TFIIS homologous genes in *Chlamydomonas reinhardtii* (A), *Brassica napus* (B, C, D) and *Hordeum vulgare* (E); exons as black boxes, introns as lines, UTRs as grey boxes, ATG start codons are shown above; heat shock *cis* elements (HSE) are shown below; qRT-PCR of TFIIS gene homologous are shown (A-E, right panels). 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).

elements were detected within all these loci, suggesting transcriptional control through HsfA *trans* factors activities during heat exposure. Indeed, we analysed mRNA changes during ambient and heat stress conditions and found that the green algae *CreTFIIS*, the three homologs in rapeseed *BnaTFIISa, b, c* and the barley *HvTFIIS* mRNAs were all expressed and

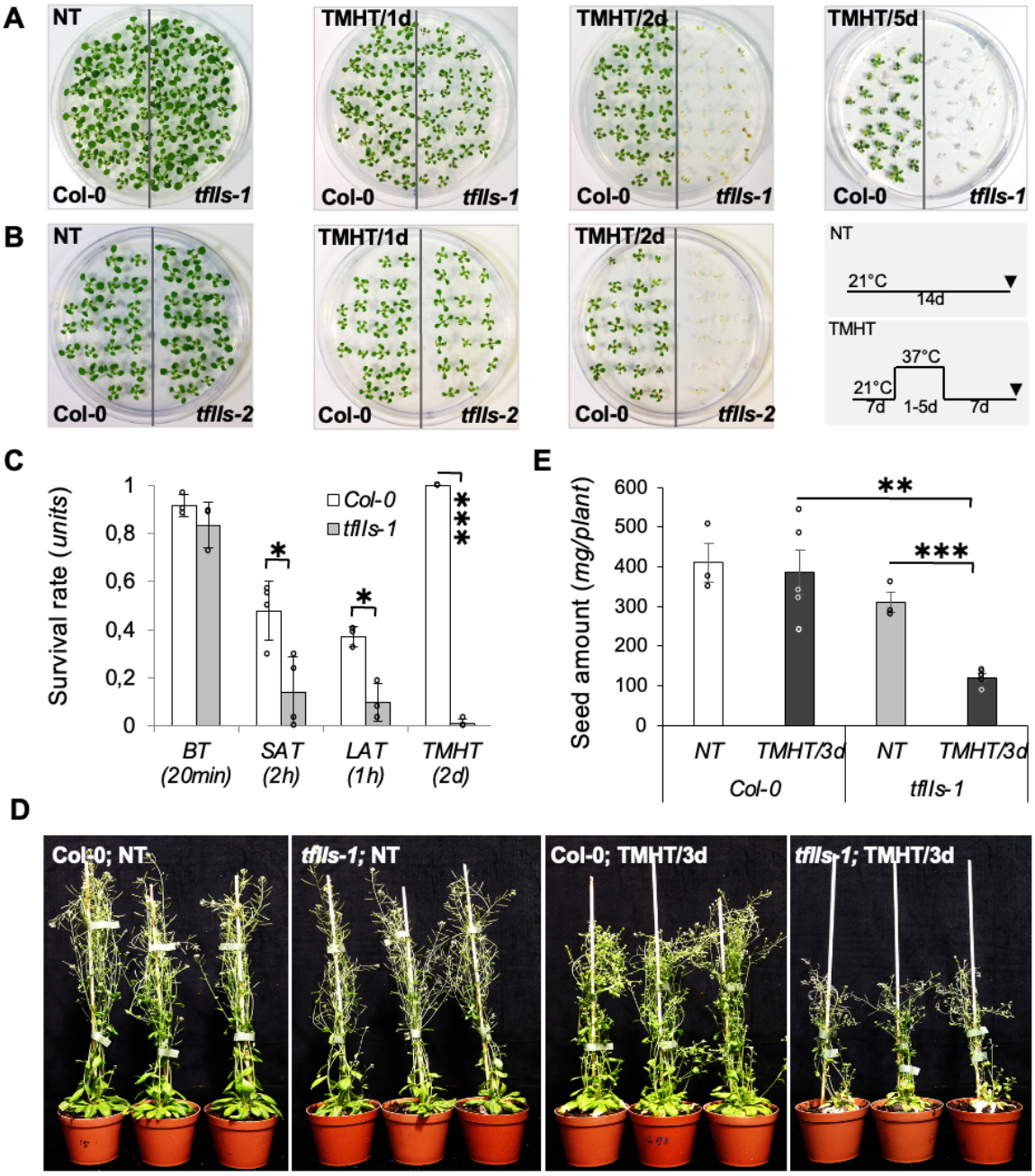


Figure 4: TFIIS is essential for heat stress adaptation throughout the life cycle of *A. thaliana*. (A-B) TMHT heat-treatment of (A) *tfIIs-1* or (B) *tfIIs-2* mutant plants compared to Col-0; (C) Survival rate of Col-0 and *tfIIs-1* plant to basal thermotolerance (BT), short acquired thermotolerance (SAT), long acquired thermotolerance (LAT) and TMHT regimes; (D Flowering Col-0 and *tfIIs-1* mutant plants and (E) seed amounts collected from these following TMHT treatment (TMHT/3d). 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).

significantly induced in response to heat (Fig 3). The widely conserved regulation of TFIIS in these distant plant species separated by cca 500 Mya evolution suggests functional relevance.

To prove the biological relevance of TFIIS requirement for heat adaptation, we analysed the

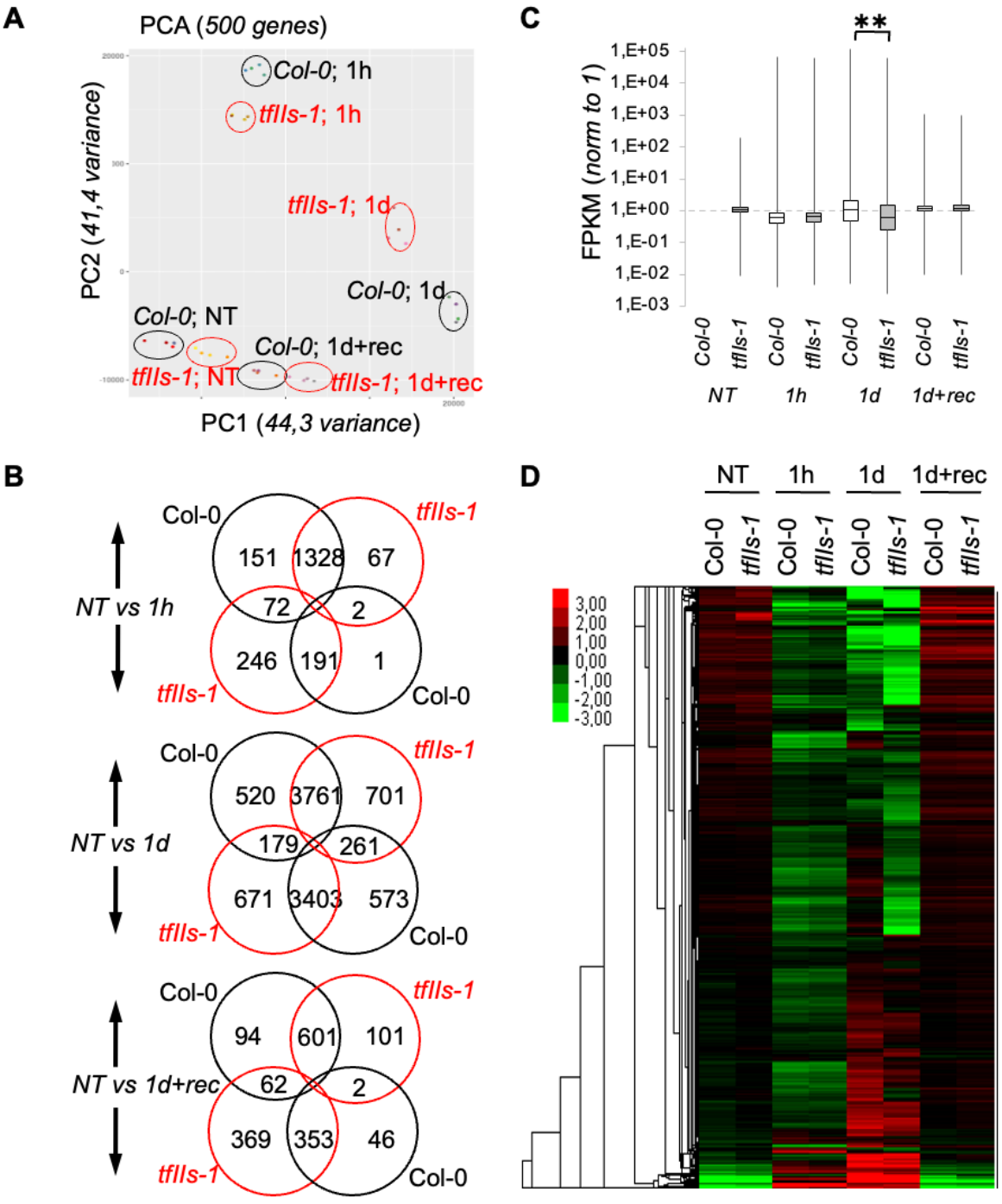


Figure 5: Transcriptome analysis of heat-induced changes in Col-0 and *tfIIs-1* plants. (A) PCA of transcriptome samples of Col-0 or *tfIIs-1* plants; (B) Differentially up- or down- regulated transcript numbers (min. 2-fold change, P<0.01), non-treated (NT) compared to 1 hour (1h), 1 day (1d) heat stress or 1d heat followed by 2 days recovery (1d+rec) treatments; (C) Normalized FPKM changes and (D) heat map analysis of significantly changed transcripts without fold-change restrictions in Col-0 and *tfIIs-1* plants; p values based on two-tailed Student's t-test (**p<0.01).

heat sensitivity of the previously characterized *tfIIs-1* (SALK_056755) and *tfIIs-2* (SALK_027259) T-DNA lines [20,21] (Fig 4). For this, we employed various heat treatments including BT, SAT, LAT and TMHT programs. Significant differences in survival rates were found between *tfIIs* mutants and Col-0 plants in response to SAT, LAT and TMHT regimes (Fig 4A-C). The most striking difference was observed in response to TMHT. Complementation assays were conducted to prove that the lack of TFIIS protein is responsible for the heat-sensitive phenotype of *tfIIs-1*, using complementation lines *pTFIIS::GSyTFIIS;tfIIs-1* and *p35S::MycTFIIS;tfIIs-1* (*not shown*). Relevantly, the heat-sensitive phenotype of *tfIIs-1* was reverted by both constructs in several independent lines.

Next, we investigated the heat sensitivity of *tfIIs-1* mutant during flowering (Fig 4D, E). Col-0 control plants were mildly affected by 3 days of TMHT treatment: leaf yellowing, petal wilting, and pollination defects were observed one-week post-stress. After a couple of weeks, however, the plants resumed their growth and fertilization, producing normal amount of seeds. The *tfIIs-1* plants were more seriously affected by heat: almost all rosette leaves and siliques got yellow and died; the central growing floral stem died back; re-growth was resumed very late from secondary, auxiliary buds; *tfIIs-1* plants remained small and produced shorter siliques with few seeds. These results show that TFIIS is needed for heat adaptation throughout the whole life cycle of plants to support both survival and reproduction success.

As TFIIS is a transcription elongation factor, we reasoned that it regulates thermal adaptation at the transcriptional level. To explore genome-wide transcriptional differences between Col-0 and *tfIIs-1* plants, we performed an RNA transcriptome analysis (Fig 5). Although many up/down-regulated transcript differences between the Col-0 and the *tfIIs-1* mutant are likely the indirect consequences of the TFIIS absence (especially at 1d), a clear separation of transcriptome profile emerged upon TMHT treatment, with Col-0 transcriptome leaning to reshape the RNA metabolism, efficiently down-regulate photosystem-linked transcripts and hormonal pathways but switching on detoxification. On the contrary, *tfIIs-1* continues to produce photosynthesis-linked transcripts or hormone-responsive transcripts but has decreased levels of transcripts required for oxidative stress response, and structural components like epidermis development, cytoskeleton, and cell wall. In sum, Col-0 plants switch efficiently to a stress management transcriptome program, while *tfIIs-1* plants lag in this shift (Fig 5B-D, and *data not shown*).

We have further analysed the transcriptome differences in response to heat between the two genotypes by including all significantly changing transcripts ($P<0.01$) without fold-change restrictions (Fig 5D). During early (1h) heat treatment, a marked but similar downregulation of overall transcriptome was observed in both genotypes. Upon persistent heat (1d) however, this trend was changed and a clear separation of the two genotypes was observed: in the Col-0 transcriptome significantly more transcripts were expressed at higher level compared to *tfIIs-1* mutant (Fig 5C, D).

In conclusion, the absence of TFIIS directly or indirectly impacts many transcripts (n=14553) significantly, although mildly. These data suggests that TFIIS activity is not required for regulation of selected/specific cascades of heat adaptation pathways but aids a prompt

transcriptome shift to stress response program.

To confirm the main findings of the transcriptome analysis, we validated changes observed in accumulation of HSFs, HSPs, alternative splicing dynamics, photosynthetic-related transcripts etc. (*not shown*).

To show the direct need of TFIIS for transcriptional regulation at these specific loci, we performed chromatin immunoprecipitation (ChIP) using GSy-TFIIS complementation line (Fig

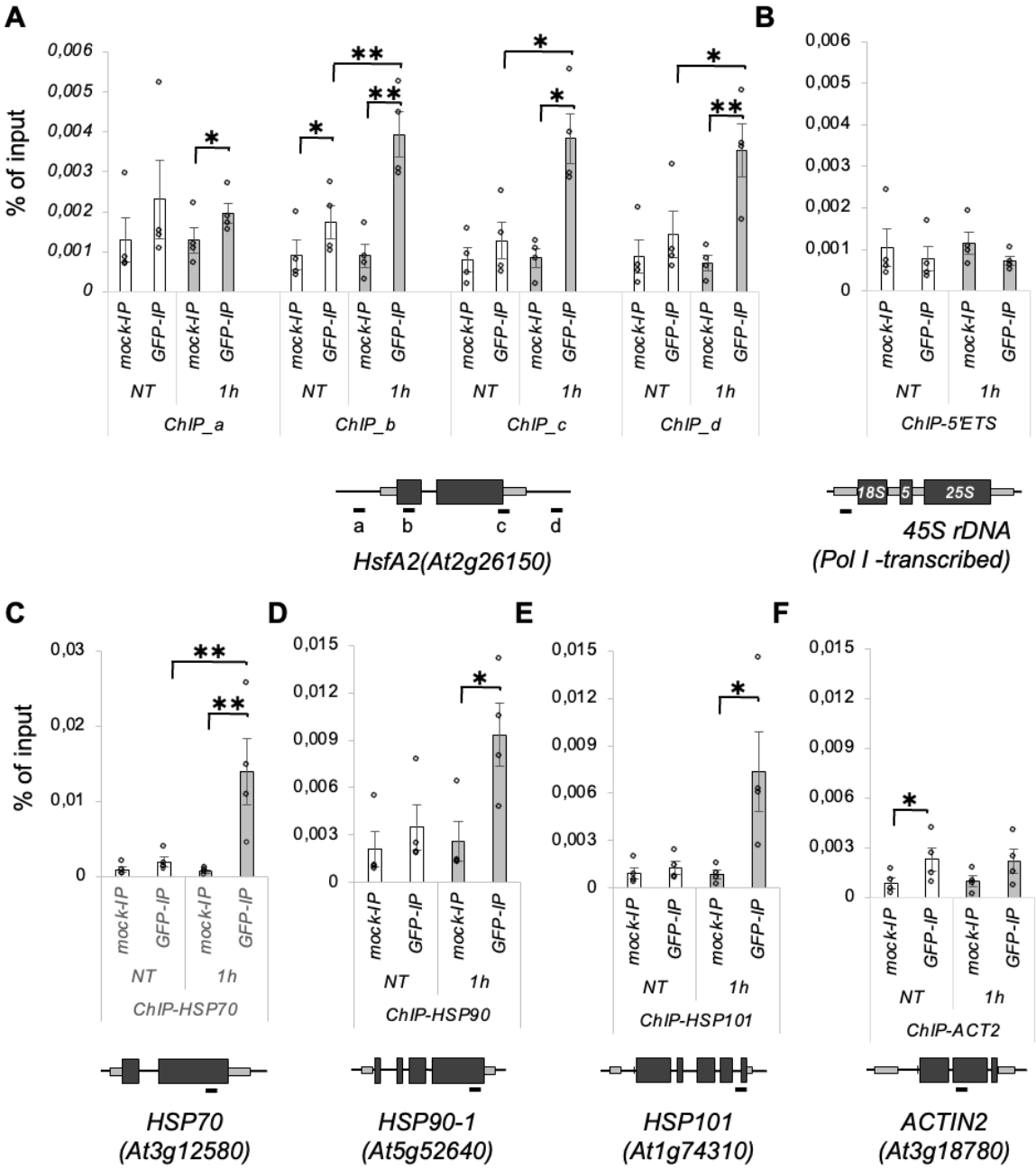


Figure 6: TFIIS is associated with Pol II-transcribed gene loci in a heat-dependent manner. Chromatin immunoprecipitation – qPCR analysis of GSy-TFIIS binding to (A) HsfA2, (B) 45S ribosomal locus, (C) HSP70, (D) HSP90, (E) HSP101 and (F) ACT2 gene loci. 45S rDNA locus is transcribed by Pol I, therefore was used as a negative control. Location of qPCR amplicons are shown as horizontal segments below the schematic of each locus (not to scale); 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).

6.). We have shown that TFIIS binds to the gene body of HS-induced genes but is not present upstream or downstream of gene bodies, nor at RNAPI-transcribed ribosomal DNA loci. Additionally, TFIIS binding at HS loci was heat-specifically induced, while at the same time depleted from housekeeping loci (Fig 6).

To further show the importance of TFIIS in overall transcriptome program switch, we assayed HSP protein levels: the ATP-dependent chaperones (HSP70, HSP90 and HSP101) were significantly under-accumulated in the early heat treatment period, while the ATP-independent holdase sHSP-CI proteins were over-accumulated in the late phase of HSR (*data not shown*). These finding suggested that in the absence of TFIIS the transcriptome program switch delay leads to a proteotoxic stress, probably through protein misfolding and aggregation, that could be the basis of lethality. Indeed, we have observed significant differences in sumoylation and

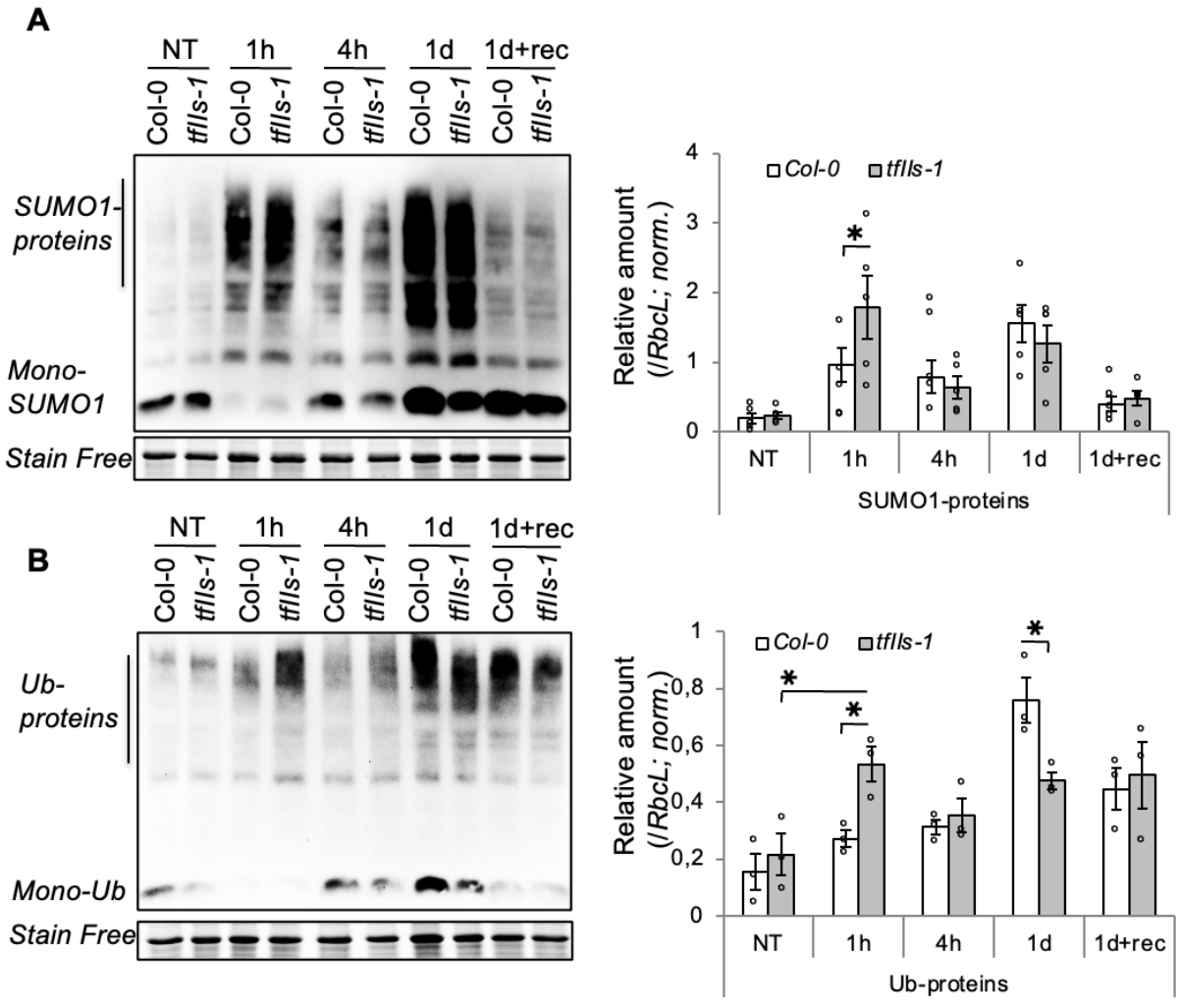


Figure 7: Post-translational modification of proteome is altered in the absence of TFIIS. (A-B) Sumoylation (A) and ubiquitination (B) post-translational modifications were measured by western blot analysis in Col-0 and *tfIIs-1* plants, stain free images of Rubisco large subunit (RbcL) are shown as loading controls (left), treatments conditions as in Figure 4. Quantifications are shown on the right; 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).

ubiquitination of proteomes in TFIIS mutant compared to wt during heat treatment (Fig 7). Importantly, have detected accumulation of protein aggregates at significantly higher level in the *tfIIs-1* mutant compared to wt, both at non-treated and heat-treated conditions (Fig 8).

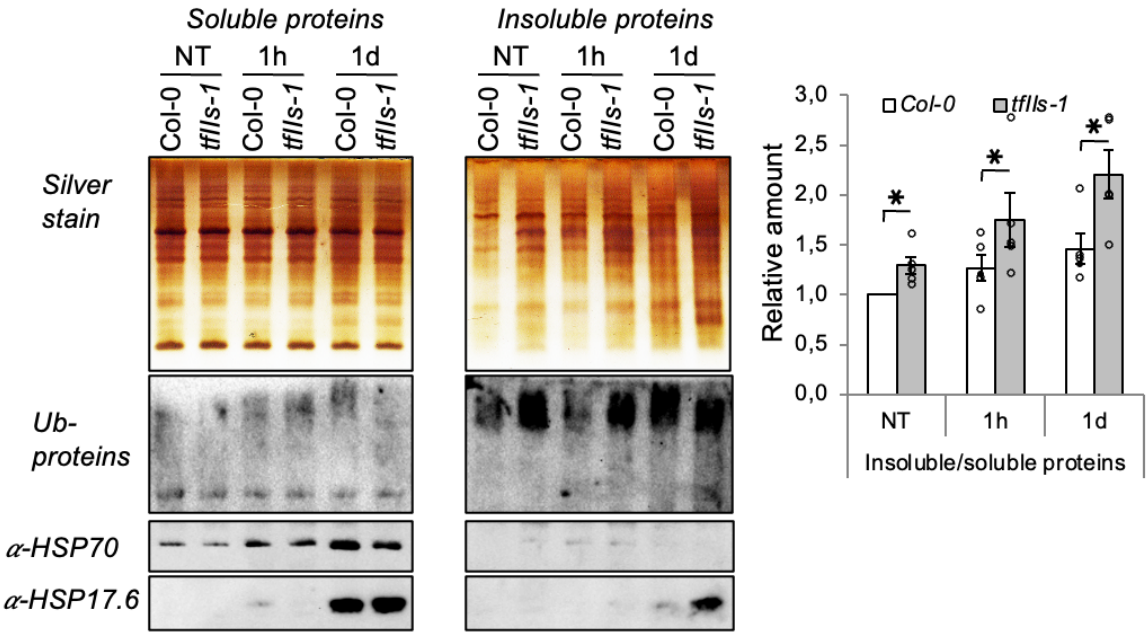
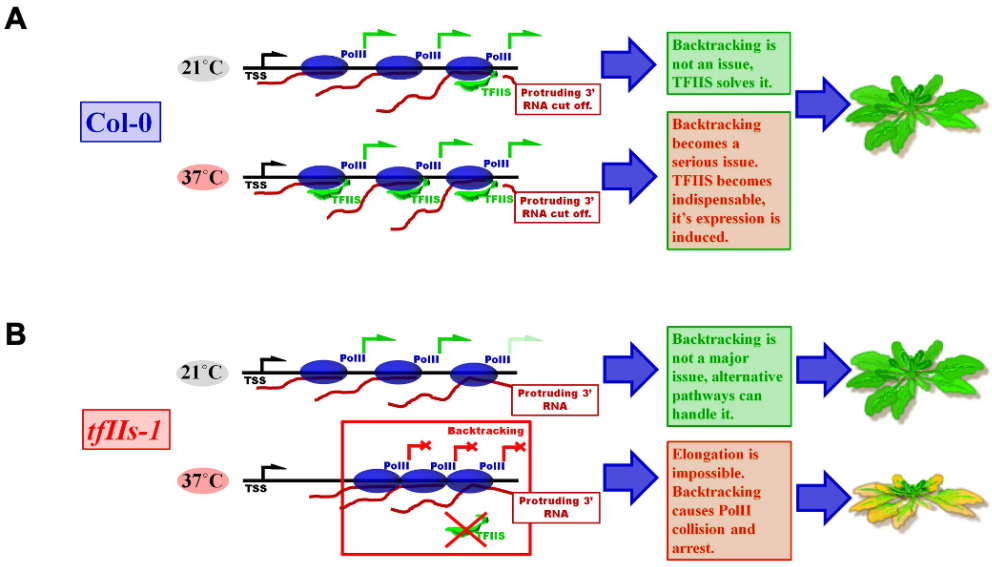


Figure 8: Insoluble protein aggregates accumulate to higher level in *tfIIs-1* mutant plant in response to heat. Silver-staining gel images of soluble and insoluble protein fractions from Col-0 and *tfIIs-1* plants are shown (top left); sister gels were analyzed for ubiquitin-conjugates, HSP70 and sHSP-CI contents (bottom left); treatments conditions as in Figure 4. Quantifications of silver-stained soluble and insoluble protein amount ratios are shown (right); bars represent standard errors based on at least three biological replicates; p values based on two-tailed Student's t-test (*$p<0.05$).

Based on these results, we have proposed a working model for TFIIS elongation factor activity. At ambient conditions in wt plants TFIIS interacts with RNAPII to enable efficient transcription, the arrested RNAPII complexes are efficiently resolved. During heat stress conditions however, the HsfA1s trans factors transcriptionally induce TFIIS to increase its accumulation that is needed for an efficient HSR transcriptome program through *de novo* RNAPII transcription. The absence of TFIIS can be tolerated during non-stress conditions, probably because the arrested RNAPII events are surpassed by RNAPII intrinsic activity and potentially resolved through alternative routes, therefore the plant development remains largely unaffected. During heat stress the absence of TFIIS cannot be compensated due to probably multiple causes, such as increased amounts of arrest/backtracking events, the decreased intrinsic nuclease activity of RNAPII or overloaded alternative mechanisms directed to resolve RNAPII arrested/backtracking events. These events lead to delayed and improper transcriptional output and indirectly causes lethality (Fig 9).



Supplementary figure 9: Working model for TFIIS roles during heat stress adaptation. (A) In Col-0 plants TFIIS interacts with RNAPII to enable efficient transcription; arrested complexes are efficiently resolved; During heat stress conditions HsfA1s *trans* factors transcriptionally induce TFIIS to increase efficiency of RNAPII arrest resolution and transcriptional output: plants are heat tolerant. (B) In the absence of TFIIS factor (in *tfIIs* mutants) arrested RNAPII events are surpassed by RNAPII intrinsic activity and potentially resolved through alternative routes; plant development is unaffected. During heat stress the absence of TFIIS cannot be compensated due to either (i) increased amounts of arrest/backtracking events, (ii) the decreased intrinsic nuclease activity of RNAPII or (iii) overloaded alternative mechanisms directed to resolve RNAPII arrested/backtracking events; this leads to delayed and improper transcriptional output and indirectly causes lethality.

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To show the relevance of our findings in crop plants, we attempted to create *B. napus* and *H. vulgare* TFIIS mutant lines. In rapeseed there are three loci encoding for TFIIS homologues (BnaTFIISa, BnaTFIISb and BnaTFIISc), while in barley one homologue (HvTFIIS)[1]. We designed CRISPR guide RNAs for targeting and creating mutations within these genes. We have failed to obtain triple-mutant barley plants (due to technical issues in the lab), however we successfully generated multiple independent homozygous mutants of barley CRISPR-mutagenized TFIIS lines. From these we have selected two lines for further studies (named as *hvtfIIs-cr1* and *hvtfIIs-cr2*, respectively) (Fig 10A). So far, we have shown that absence of TFIIS does not affect development at ambient (non-stress) conditions, however it is needed for survival under heat stress (Fig 10B)

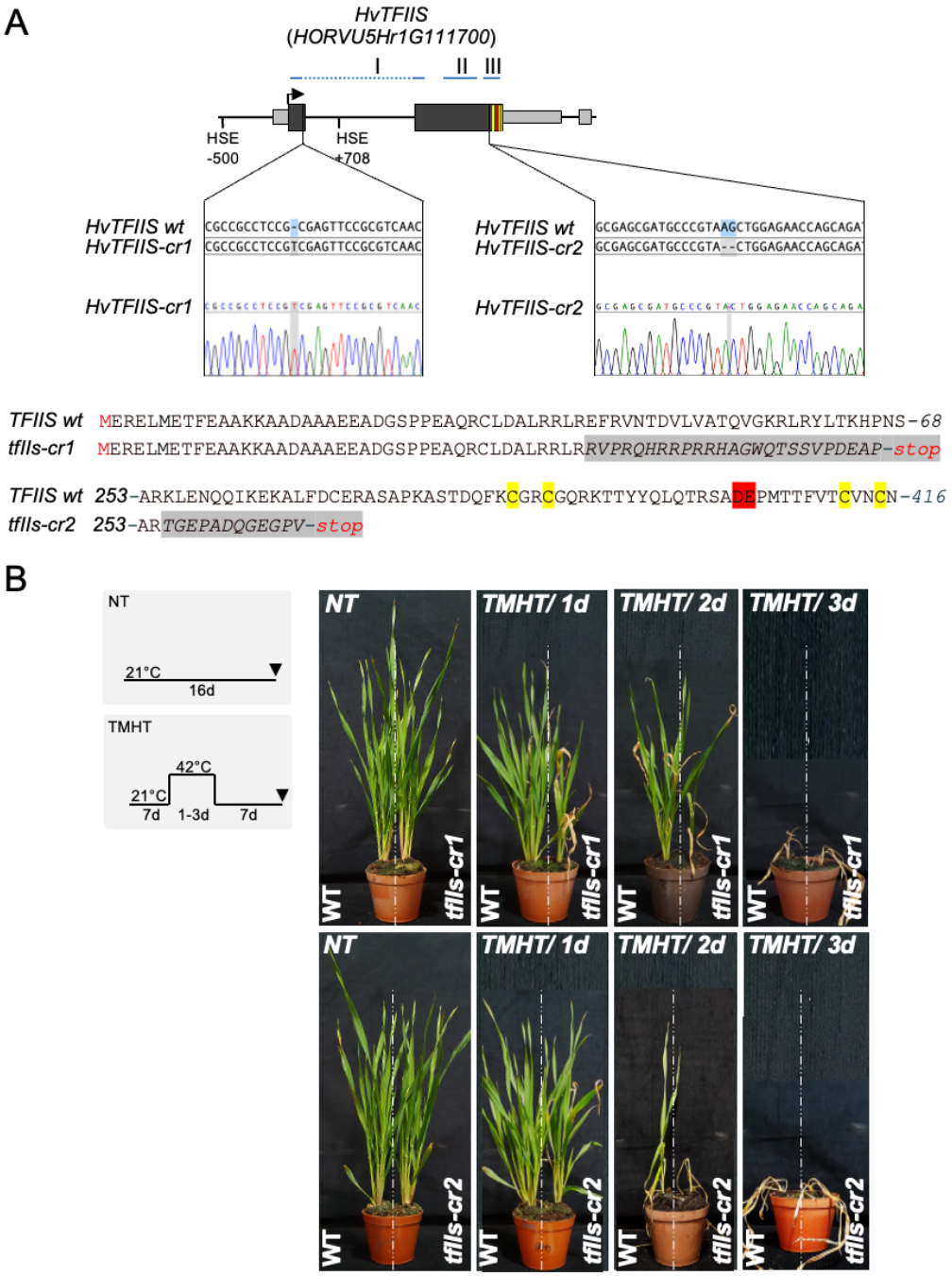

Figure 10: TFIIS elongation factor is needed for heat stress adaptation in *H. vulgare*. (A) TFIIS mutants of barley were created by CRISPR mutagenesis using two independent guide RNAs; Insertion or deletion mutation within the TFIIS gene and protein amino-acid changes with premature stop is shown at bottom. (B) Non-treated (NT) and thermotolerance to moderately high temperature (TMHT) regimes are shown on left; wild-type (WT) or *tfIIs* mutants are shown on left and right side of picture, respectively.

Next, we are planning to perform a thorough analysis of barley mutants, focusing on common and aspects of HSR (by comparing it to the dicot model *A. thaliana* system). Our manuscript is in preparation (*Ahmad et al., unpublished*).

2. Transcriptional regulation study of barley RNA silencing pathway components during heat adaptation.

Connected to our transcriptional regulation during heat stress studies in barley system, we have built an in-house cooperation with *Plant Development group* and *Epigenetics group*. In this work we have studied the transcriptional regulation of RNA silencing *trans* factors under HS conditions.

Most of the knowledge in the RNA silencing field was based on *A. thaliana* and *N. benthamiana* systems, although studies on other dicot or monocot species' RNA silencing and sRNAs have been also done before[23-26]. Despite its economic importance, there was only scattered information on RNA silencing machinery and its temperature regulation in monocot crop barley[27,28]. The key components of RNA silencing are the Dicer-like proteins (DCLs), Argonautes (AGOs) and RNA-dependent RNA polymerases (RDRs). First, we have identified five DCL (HvDCL), eleven AGO (HvAGO) and seven RDR (HvRDR) genes in the barley genome using bioinformatic tools. Genomic localization, phylogenetic analysis, domain organization and catalytic motifs were also defined. Then, we have experimentally analysed the

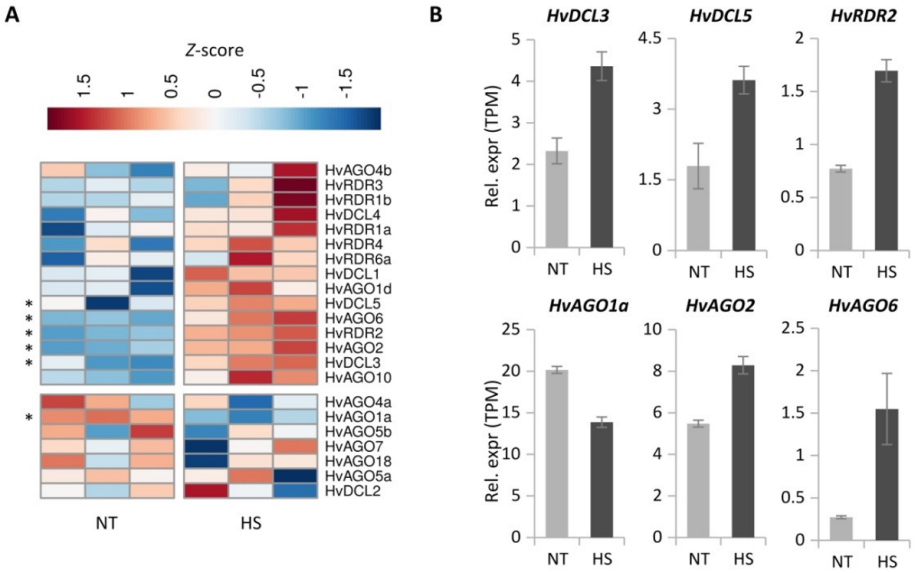

Figure 11: RNA slinencing *trans* factors are regulated transcriptionally during heat stress. RNA-seq analysis of the data published by Pacak et al. [2016]. (A) Heat map representation of the expression pattern of silencing-related genes in heat-shocked (HS) and not treated (NT) barle plants (data for the three biological replicates are shown separately). Colours represent Z-scores, which show how many standard deviations the given value is above or below the mean of all values in a row. Genes that are significantly differentially expressed between the NT and HS samples are marked with asterisks. (B) Bar chart representation of the expression values of the significantly differentially expressed RNA silencing-related genes. Expression values are normalised transcript per million (TPM) units. Error bars represent standard errors.

transcriptional changes of DCLs, AGOs and RDRs in response to moderate, persistent, and gradient heat stress treatments (Fig 11-12).

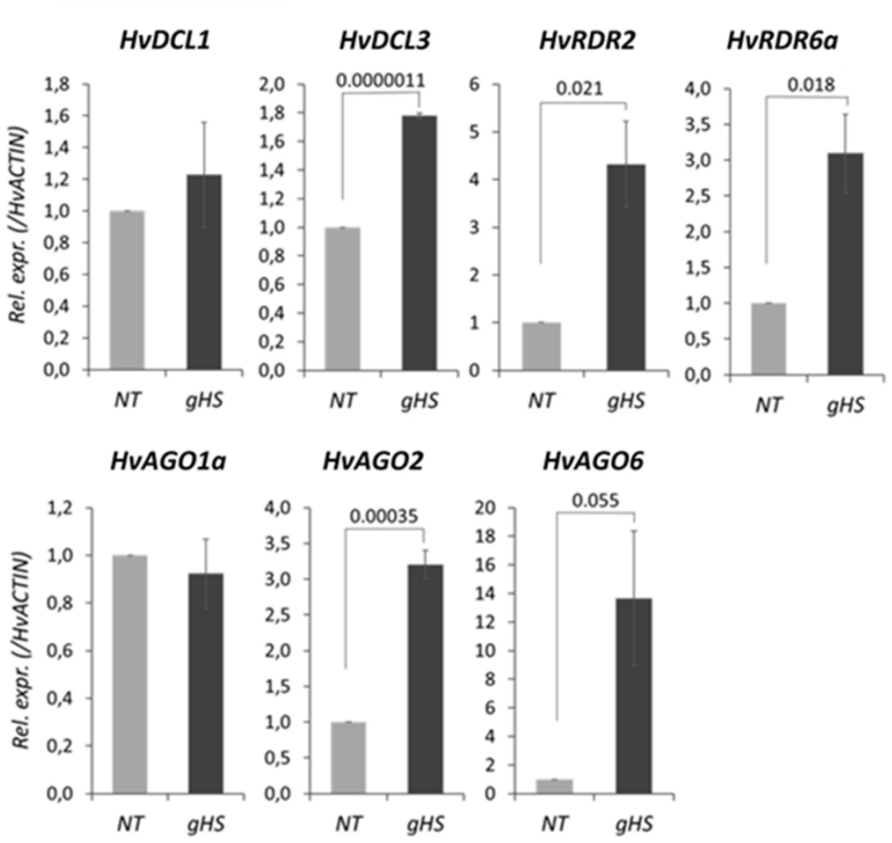

Figure 12: RNA slinencing *trans* factors are regulated transcriptionally during heat stress. RT-qPCR analysis of selected RNA silencing factor transcripts in gradient heat-shock (gHS) -treated and non-treated (NT) barley leaves. p-values for significant changes are shown above each pair.

Literature data (RNA-seq[29]) and the semi-quantitative and RT-qPCR measurements have shown transcriptional accumulation of factors enrolled primarily in siRNA-based silencing, including heterochromatic-sRNA, phased-sRNA and RDR6-dependent sRNA pathways. Importantly, our gradient heat treatment mimicked natural situations, e.g., temperature changes during a summer day, therefore our data may be relevant also in field conditions. As DCL3, AGO2, AGO6, RDR2 and RDR6 factors were all involved in TGS or double-stranded DNA break repair RNA silencing could have chromatin regulatory and protective roles in barley during heat stress acclimation[2].

Publications: Hamar É, Szaker HM, Kis A, Dalmadi Á, Miloro F, Szittya G, Taller J, Gyula P, Csorba T*, Havelda Z*. (2020) Genome-Wide Identification of RNA Silencing-Related Genes and Their Expressional Analysis in Response to Heat Stress in Barley (Hordeum vulgare L.). Biomolecules. 2020 Jun 18;10(6):E929. doi: 10.3390/biom10060929. *corresponding author*

3. Analysis of Homeobox protein NDX a transcriptional and epigenetic regulator of chromatin structure.

The homeodomain (HD) domain containing protein Nodulin homeobox (NDX) was reported in previous works as a transcriptional regulator of euchromatin localized genes[30,31]; amongst its targets, FLC is an integrator of flowering time, while ABI4 and ABI5 are key regulators of abscisic acid (ABA) hormonal signalling pathway. NDX was shown to transcriptionally regulate these loci through stabilization of an RNA:DNA hybrid structure (so-called R-loop) at these loci. To understand the genome-wide effects of NDX on R-loop dynamics we conducted an R-loop immunoprecipitation followed by deep sequencing. Besides we have performed a chromatin immunoprecipitation using NDX protein as bait. Combining these data, we planned to study the colocalizations of R-loops and NDX protein.

Surprisingly, we have found that NDX is primarily a heterochromatic regulator[4]. We gathered multiple lines of evidence to support our findings (Fig 12). ChIPseq measurements revealed that NDX is associated with heterochromatin. Microscopy in living cells confirmed that NDX is a chromatin-bound factor with slow nuclear dynamics incorporated into heterochromatin. NDX preferentially associated with pericentromeric het-siRNA loci involved in non-CG methylation and caused significant sRNA level changes. CHH/CHG hypo- methylation of pericentromeric regions in *ndx* mutant significantly overlaps with NDX binding sites. Finally, the loss of NDX function results in extensive 3D chromatin structural changes at pericentromeric regions[4].

Based on these findings, we proposed a working model of NDX actions. Inactivation of NDX results in chromatin decompaction at highly condensed pericentromeric regions. The relaxed heterochromatin structure leads to het-siRNA accumulation and DNA methylation changes. Subsequently, chromatin regulatory genes and transposons are derepressed or repressed, that can indirectly lead to higher order 3D chromatin changes. Genetic analysis of the above and other factors is expected to lead to a better understanding of heterochromatin homeostasis as the driver of genome organization and stability and its response to developmental signals and environmental stimuli[4].

Publication: Karányi Z, Mosolygó-L Á, Feró O, Horváth A, Boros-Oláh B, Nagy É, Hetey S, Holb I, Szaker HM, Miskei M, Csorba T, Székvölgyi L*. NODULIN HOMEOBOX is required for heterochromatin homeostasis in Arabidopsis. (2022) Nat Commun. 2022 Aug 27;13(1):5058. doi: 10.1038/s41467-022-32709-y. *corresponding author*

Interestingly, our RNA transcriptome analysis from *ndx* mutant plants revealed flowering time and organ morphology changes. Besides, we have detected developmental alterations caused by NDX absence that are reverted by high ambient temperature. The molecular events at the core of these changes are being analysed (*Szaker et al., unpublished*).

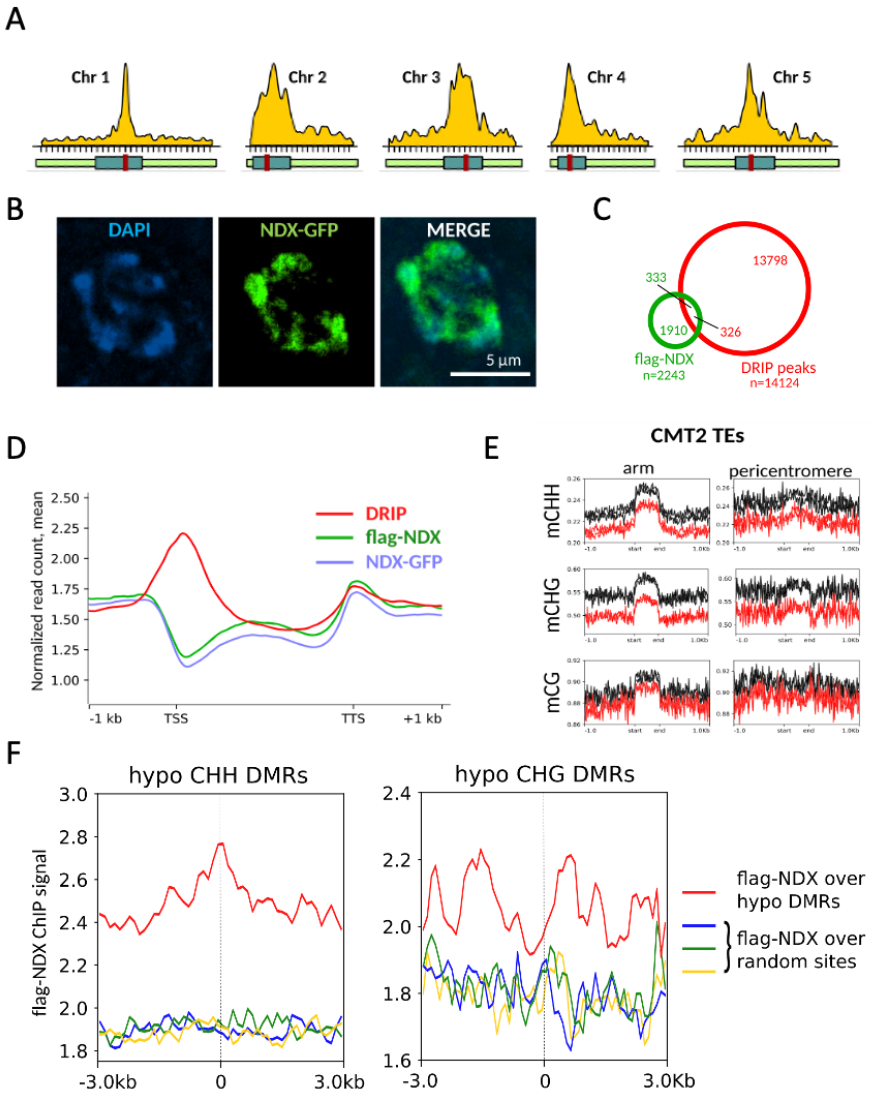

Figure 13: NODULIN HOMEOBOX (NDX) is a heterochromatic regulator. (A) Genome-wide mapping of chromosomal binding sites of NDX; (B) Microscopic localization of NDX: A minor fraction of cells with typical chromocenter structure (DAPI foci) shows strong NDX-GFP enrichment at chromocenters; (C) NDX binding sites and R-loops' genomic distribution is antagonistic; (D) Metagene profile of flag-NDX, NDX-GFP and DRIP signal intensities over protein coding loci. TSS: transcription start site. TTS: transcription termination site. (E) Metaplots of DNA methylation levels in CHH/CHG/CG contexts in Col-0 (black) and ndx1–4 (red) plants in CMT2 TE loci. (F) Anchor plot of flag- NDX ChIP signal over hypo CHH and CHG DMRs (red line) and random sites (blue, green, yellow lines). The NDX signal is enriched in the hypo-CHH and hypo-CHG DMRs.

4. The study of miR824/AGL16 module in regulation of flowering time during elevated temperature conditions.

A vast amount of data has been generated on protein coding genes' transcriptional regulation during heat stress, but much less is known about non-coding RNA (ncRNAs). We have analysed the non-coding transcription under HS by employing a gradient heat stress response through a comprehensive RNA transcriptome analysis. From ncRNAs that changed in a HS-dependent manner we selected miR824 and its target AGAMOUS-Like 16 (AGL16) for further in-depth study.

We have shown that miR824 accumulates gradually in response to heat due to the combination of transient transcriptional induction and posttranscriptional stabilization. miR824 induction requires heat shock *cis*-elements and HsfA1 family and HsfA2 transcription *trans* factors. Parallel to miR824 induction, its target AGL16 is decreased, implying cause-consequence relationship. However, AGL16 repression during heat stress is more complex, it comprises of a miRNA-independent and a miR824-dependent component. AGL16 downregulation in response to heat leads to a mild de-repression of FT, a positive regulator of flowering time. Notably, heat stress regulation of miR824/AGL16 is conserved within *Brassicaceae*, including *B. napus*. In conclusion, miR824/AGL16 module integrates high temperature cues to fine-tune FT levels and consequently alter flowering time transition. Stress-induced miR824, therefore, can act as a "post-transcriptional memory factor" to extend the acute impact of environmental fluctuations in the post-stress period.

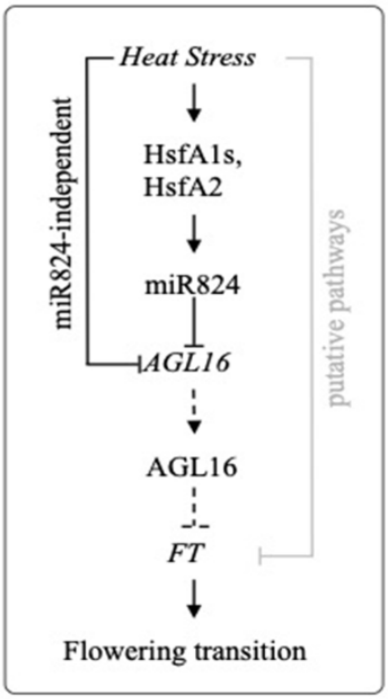

Figure 14: Working model of miR824/AGL16 module during heat stress regulation. Heat stress induces transcription of miR824 through HSFA1a family and HSFA2 transcription factors. *AGL16* is depleted through a miR824-dependent and a miR824-independent pathway. Stable downregulation of *AGL16* leads to derepression of *FT*, a central integrator of flowering transition (dotted lines depict downregulated steps during and following HS). *FT* level may be also altered by other putative heat stress (HS)-regulated factors (gray line).

Publication: Szaker HM, Darkó É, Medzihradszky A, Janda T, Liu HC, Charng YY, Csorba T, (2019) miR824/AGAMOUS-LIKE16 Module Integrates Recurring Environmental Heat Stress Changes to Fine-Tune Poststress Development, Front Plant Sci. 2019 Nov 25;10:1454. doi: 10.3389/fpls.2019.01454. eCollection 2019. *corresponding author*

5. Additional works related to transcriptional regulation of temperature adaptation.

Based on our works on transcriptional regulation of heat stress response we have been invited to revise the role of RNA silencing and its regulation during temperature adaptation. In this review, we summarized the current understanding of small RNA-mediated modulation of high-temperature stress regulatory pathways including basal stress responses, acclimation, and thermo-memory. We gather evidence that suggests that small RNA network changes, involving multiple up-regulated and down-regulated small RNAs, balance the trade-off between growth/development and stress responses, to ensure successful adaptation. Finally, we highlighted characteristics of small RNA-based temperature stress regulation in crops and explored the perspectives of using small RNAs in breeding programs to increase resilience and stress tolerance, which may be crucial for agriculture in the future.

Publication: Szaker H.M., Gyula P., Szittya G., Csorba T. (2020) Regulation of High-Temperature Stress Response by Small RNAs. In: Miguel C., Dalmay T., Chaves I. (eds) Plant microRNAs. Concepts and Strategies in Plant Sciences. Springer Cham, DOI: 10.1007/978-3-030-35772-6, *corresponding author*

Besides, we were invited to summarize the role of a long non-coding RNA called APOLO. APOLO integrates endogenous hormonal and exogenous temperature signals and, in combination with an intricate network of epigenetic and transcriptional factors, controls multiple aspects of root development. APOLO acts as both positive and negative regulator to orchestrate gene expression in *cis* and in *trans*.

Publication: Csorba T (2021) APOLO lncRNA, a self-calibrating switch of root development. Mol Plant. 2021 Jun 7;14(6):867-869. doi: 10.1016/j.molp.2021.05.015. *corresponding author*

V. Conclusions

I summary the present grant has funded our attendance to ***5 scientific conferences,*** *the publication of* ***5 scientific papers*** *and* ***1 book chapter.*** Additionally, the work contributed to the completion of a ***PhD program (Szaker HM, ELTE).***
On long terms these findings will contribute to the development of heat-tolerant plant lines that may have an immense significance in the light of global warming and climate change. Furthermore, these findings significantly extended the knowledge on heat stress transcriptional regulation functions in general, which may be relevant in all eukaryotic organisms.

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