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FINAL REPORT

The role of long non-coding RNAs during heat-stress response in plants

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Abbreviations:

AGO: Argonaute protein
A. thaliana: Arabidopsis thaliana
B. napus: Brassica napus
DCL: DICER-LIKE
H. vulgare: Hordeum vulgare
HSE: Heat Shock element
HSF: Heat Stress Factor
lncRNA: long non-coding RNA
miRNA: microRNA
mRNA: messenger RNA
NAT-ncRNA: natural antisense non-coding RNA
ncRNA: non-coding RNA
RDR: RNA-Dependent RNA Polymerase
RISC complex: RNA Induced Silencing complex
RITSC: RNA Induced Transcriptional Silencing complex
siRNA: short interfering RNA
sncRNA: short non-coding RNA
ta-siRNA: trans-acting siRNA
TAS ncRNA: non-coding RNA transcript, precursor of trans-acting siRNAs

Summary

Plants as sessile organisms are exposed to persistently changing environment. High temperature stress (heat stress, HS) is considered to be one of the major abiotic stresses affecting the distribution and productivity of agriculturally important plants worldwide. Plants cannot avoid the exposure to these factors but need to adapt morphologically and physiologically.

Cells exposed to high temperatures activate heat shock responses (HSR), which consists of a series of dramatic biochemical and physiological changes that results in a reprioritization of cell physiology to support survival. During HSR about 5% of plant transcriptome is up-regulated two-fold or more. While protein-coding transcriptional changes and its downstream impact has been extensively studied, the role of non-coding RNAs (ncRNA) lags behind. In the present work we have studied multiple aspects of non-coding RNAs' role during heat stress adaptation and post-stress development.

To identify non-coding RNAs (ncRNAs) involved in HSR, we heat-stressed *Arabidopsis thaliana* (Col-0) seedlings and performed strand-specific RNA transcriptome analysis. For in-depth studies we have selected different classes on ncRNAs, such as a pri-miRNA, a TAS ncRNA, and an angiosperm-specific conserved long intergenic ncRNA family.

(i) Translation-dependent mRNA quality control systems protect the protein homeostasis of eukaryotic cells by eliminating aberrant transcripts and stimulating the decay of their protein products. Although these systems were intensively studied in animals, little was known about the RNA quality control in plants. We characterize the mechanism of nonstop decay (NSD) system and have shown that plant NSD efficiently degrades nonstop mRNAs. We demonstrate that in Pelota, Hbs1 and SKI2 proteins are *trans* factors of NSD. We have further shown that NSD and RNA silencing systems cooperate and that NSD is required for the

elimination of RISC 5′ cleavage product ***when the cleavage occurs on protein-coding transcripts but not non-coding transcripts***[1] (e.g. long non-coding TAS transcripts). (Szadeczky-Kardoss I.*, Csorba T* et al., 2018, NAR.)

(ii) 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 due to the combination of transcriptional induction and posttranscriptional stabilization. miR824 induction is dependent on HSE *cis*-elements and HSFA1 and HSFA2 transcription factors. Parallel to miR824 induction, target *AGL16* mRNA level is decreased. AGL16 repression during HS comprises of a miRNA-independent, and a miR824-dependent pathway. AGL16 downregulation in response to heat leads to a mild derepression of Flowering locus T (FT), the central positive regulator of flowering time transition. Due to the enhanced post-transcriptional stability of miR824, stable repression of AGL16 is achieved following heat stress that may serve to ***fine-tune FT levels and alter flowering time***. Stress-induced miR824, therefore acts as a "***post-transcriptional memory factor" to extend the acute impact of environmental fluctuations in the post-stress period***. Finally, we present evidence showing that miR824/AGL16 module regulation is conserved within *Brassicaceae* crop species[2]. (Szaker et al., 2019, Frontiers in Pl Sci, Szaker et al., 2020, Springer Nature.)

(iii) We have studied the role of a long non-coding RNA family during heat stress adaptation and development. This lncRNA family comprises of two members in *A. thaliana* (MASTER-1 and -2) but have 11 members in *B napus*. First we confirmed HS-induction of these by northern blot and qrtPCR techniques. Then have shown that the homologs and HS regulation of MASTER lncRNAs is conserved in angiosperm species (including *B. napus* and monocot crop *H. vulgare*). To prove their roles during HS, we tested *Arabidopsis* single mutants, double mutant and p35S overexpressor lines in diverse heat-stress assays but could not find any clear phenotype apart of a mild alteration of flowering time. Next we generated KO mutants by CRISPR mutagenesis. 2 independent CRISPR constructs were designed and tested. Multiple independent CRISPR lines resulted in ***sterile fenotype, suggesting involvement of MASTER lncRNAs in fertilization process***. Fenotype could be reverted by cross-pollination using wild-type pollen suggesting male sterility. In support of these, we have shown strong promoter activity of MASTER-1 and -2 in anthers and pollen grains. Preparation of a new manuscript based on the results is in progress ("*Szaker and Csorba; MASTER lncRNA family regulates fertilization in angiosperms in a temperature-dependent manner*", *in prep*). The results and figures shown in this part of the work are presented in details and they outline the high quality and importance of data, which are anticipated to be published in a top academic peer reviewed journal with high impact factor, and it is expected to be highly cited in future.

In summary, the findings of this research proposal have been presented on international and national conferences comprising ***13 conferences, 4 scientific publications with IF and 1 review***).

Content:

1. Introduction

Plants are sessile organisms which are exposed to persistently changing environments[3]. High temperature stress (heat stress, HS) is considered to be one of the major abiotic stresses affecting the distribution and productivity of agriculturally important plants worldwide[4]. When the temperature rises 5°C above the optimal, it is already experienced as HS. As a consequence of greenhouse effect future episodes of high temperature are expected to occur more frequently increasing the vulnerability of crop plants[5].
Cells exposed to high temperatures activate heat shock responses (HSR), which consist of a series of dramatic biochemical and physiological changes that results in a reprioritization of cell physiology to support survival. During HSR about 5% of plant transcripome is upregulated two-fold or more[6,7]. The heat stress transcription factors (HSFs) are central regulators of downstream expression of heat-stress-responsive components (e.g. heat-shock protein, HSP, antioxidant enzymes etc.)[8]. Accumulation of HSPs is assumed to have a central role in acquired thermotolerance[9]. Besides, HSR also includes a general repression of the basal transcription and translation, changes in transcripts of genes associated with control the level of sugars, metabolites, hormons, and unsaturated fatty acids[10,11]. Increase in temperature results also in epigenetic changes like histone occupacy or histone isoform replacements[12,13]. The existence of environmentally-induced epigenetic modifications and heritable adaptive response in plants following heat-stress also exists, since improvements in the heat-specific fitness was observed in offsprings of heat-stressed parental lines[14-16].
In the model plant *Arabidopsis thaliana* less than 50% of its genome is capable of coding proteins[17]. Non-coding transcripts (ncRNAs) are widespread in eukaryotes with evidences showing that up to 30% of transcriptional units in diverse eukaryotes have ncRNA transcription[18]. ncRNAs are diverse: some are structural (tRNA, rRNA, snRNA, snoRNA) others are regulatory ncRNAs. Based on their lenghts, regulatory RNAs can be short ncRNAs (sRNA), or long ncRNAs (lncRNA). In the recent years it has become evident that the regulatory ncRNAs contribute enormously to the complexity of the higher organisms. ncRNAs were implicated in many biological processes including development, signal transduction, or stress responses[19-22]. Non-protein coding genes may be important players involved in the regulation of HS-responses[15]. There are many mechanistic ways lncRNAs can use to regulate cellular processes. They may have an effect epigenetically, transcriptionally or post-transcriptionally[22]. Many plant lncRNAs were identified but not functionally characterized therefore the understanding of their roles is still largely elusive[21,23-26].
Our hypothesis was that lncRNAs are important regulators of HSR. In the present programme we studied ***lncRNAs' homeostasis*** (transcription and decay), the ***specific role and conservation of selected ncRNAs during thermal adaptation***. *We will briefly revise results, which have been published, and will give a detailed description of the findings, which are in progress of publication.*

2. Results

To identify strand-specifically ncRNAs during HSR, we heat-stressed *A. thaliana* (Col-0) seedlings. To understand the HSR that occurs naturally, we employed gradual and repeated heat stress (37°C gradual priming) followed by lethal stress (45°C). Treatments were as follows: non-treated (NT), gradually-primed at 21°-to-37°C in course of 4 hours (acclimation done on days 4, 5 and 6, ACC), recovery 1 day following the priming treatment (REC) and 30 minutes heat-shock (ACC+HS). Data quality control by PCA analysis confirmed that the treatments worked well and the biological repeats are very similar to each other. The

transcriptome during the priming (ACC, 21°-to-37°C) is surprisingly different from the transcriptome at HS (45°C), both being significantly different from control and recovery samples. Besides, it also shows that one-day recovery is a period long enough for the transcriptome to almost completely regain the transcriptome pattern following the heat treatment.
To understand the genome-wide changes, the reads were first mapped to the Ensemble TAIR10 transcript set for gene expression analysis and mapped to NONCODE A. thaliana ncRNA transcript set for ncRNA expression analysis using Bowtie2 version 2.1.0. The gene expression level was estimated using RSEM v1.2.15. TMM (trimmed mean of M-values) was used to normalize the gene expression. For ncRNA expression analysis, differentially expressed genes were identified using the edgeR program.
ncRNA transcripts showing altered expression with $p < 0.05$ and more than 2 fold changes were considered differentially expressed (see Table 1.).

ncRNAs detected in total	**2572**		
Conditions	***ACC***	***REC***	***ACC+HS***
Total changes (vs CTL)	315 (12,2%)	49 (1,9%)	166 (6,4%)
Positive changes	150 (5,8%)	29 (1,1%)	105 (4,0%)
Negative changes	165 (6,5%)	20 (0,8%)	61 (2,4%)

Table 1: Global ncRNA changes in response to diverse heat treatments

De novo assembly and ncRNA filtering/search was also done. We have found 62, 2, 28 novel (longer than 200 nt) ncRNAs that were significantly changed during ACC, REC and ACC+HS, respectively. These were mostly transcripts having very low expression levels. The ncRNAs that were significantly changes (vs NT) were categorized as follows:

(i) ACC: 2 snRNAs, 11 miRNA precursor (pre-miR), 2 trans-acting RNA precursor (TAS), 62 natural antisense RNA (NAT-ncRNA), 18 intergenic ncRNAs, 108 pseudogene, 67 transposable element transcripts and 46 unknown transcripts;
(ii) REC: 1 pre-miRNA, 1 TAS, 4 NAT-ncRNA, 10 intergenic ncRNAs, 15 pseudogene, 17 transposable element transcripts and 1 unknown transcripts;
(iii) ACC+HS: 1 rRNA, 1 snRNA, 4 pre-miRNA, 5 TAS, 27 NAT-ncRNA, 4 intergenic ncRNAs, 58 pseudogene, 64 transposable element transcripts and 2 unknown transcripts.

Next, we selected 10 ncRNAs for heat stress sensitivity/tolerance phenotypic studies: pri-miR824 (AT4G24415), Gene with Unstable Transcript 15 (GUT15, At2G18440), Cytokinin Repressed 20 (CR20, AT4G36648), natural antisense of NRT1 (NAT-NRT1, AT5G01175), natural antisense of TET2 (NAT-TET2, AT2G19582), natural antisense of ASY1 (NAT-ASY1), natural antisense of pTAC18 (NAT-pTAC18, AT2G32179), natural antisense of TCP3 (NAT-TCP3, AT1G53233), natural antisense of PHD (NAT-PHD, AT1G33415), natural antisense of PrKin (NAT-PrKin, AT5G26146). T-DNA mutants have been ordered and genotyped to find homozygous mutants. We unambiguously validated six of these lines to be homozygous (the other seed stocks did not contained mutant seeds). The homozygous mutant lines have been tested in different heat stress treatment programs (based on literature): direct heat stress (HS), short acclimation treatment (SAT), long acclimation treatment (LAT), gradient acclimation and heat stress (ACC+HS) or thermo-tolerance to moderately high

temperatures (TMHT)). *hot1-3* mutant (HSP101), the quadruple mutant *hsfa1a/b/d/e* (Heat Shock Factor A1 family) and hsfa2 mutants were used as positive control in the different assays. We ***could not find any obvious difference in seedling survival following the different heat stress regimes*** in these mutants. In spite of these, we focused our study on selected ncRNA such as ***TAS ncRNAs***, ***pri-miR824***, ***GUT15*** and ***CR20 lncRNA*** family. The reason for selection was that TAS transcripts were involved in heat stress response previously, miR824 was shown to regulate stomatal development and finally in case of GUT15 and CR20 lncRNA family we have found extensive conservation amoungst angiosperms suggesting important functions (see later).

2.1. ncRNAs' homeostasis during to heat stress

TAS genes encode ncRNA transcripts (about 1kb long) that are the precursor molecules of trans-acting small interfering RNAs (ta-siRNAs). Following RNAPII-mediated transcription TAS transcripts are sliced by miRNA-loaded RISC complex, then either the 5'- or 3'-miRISC cleavage product (e.g. 5' cleavage product in case of TAS1 family) converted to dsRNA by the activity of RDR6 complex. The dsRNA then is maturated by DCL4 to give rise to ta-siRNAs[27,28]. We have found in our RNAseq that TAS family transcripts significantly decrease in response to HS, implying that their absence is required during HSR (Fig. 1).

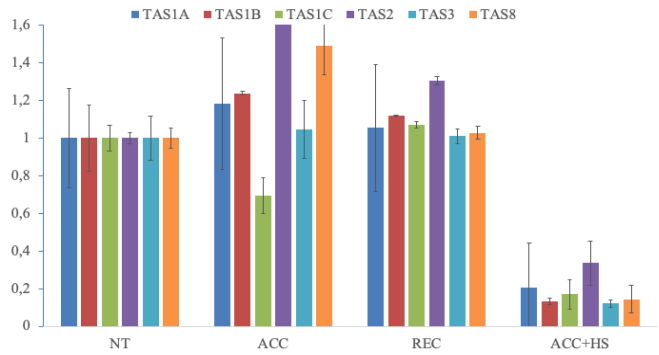

Figure 1: TAS ncRNA changes in response to heat stress conditions

Indeed, TAS ncRNAs have been implicated in HS previously as negative regulators[29]. Additionally, recently was shown that downregulation of lncRNA TAS-derived tasiRNA downregulation and induction of HEAT-INDUCED TAS1 TARGET 5 (HTT5) by REF6-HSFA2 regulatory loop are required for transgenerational early flowering[30]. As TAS transcripts roles have been intensively studied and recently described, we focused our attention on their processing and/or stability regulation.

To find out what cellular components are involved in TAS lncRNA and its fragment processing or destabilization, we analyzed it in plants which were either Virus-Induced Gene Silenced (VIGS) or mutants for *xrn4* (the main cytosolic 5'-3' exonuclease), *ski2* (co-factor of exosome, the main cytoplasmic 3'-5' exonuclease) or *pelota* (described in mammal, or DOM34 in yeast) and *hbs1* (RNA quality control components of non-stop decay (NSD) pathway (described in yeast and mammals) genes. To do this, first, we have shown that NSD operates in plants, Pelota and Hbs1 components are conserved trans factors of NSD pathway, since these were required for elimination of transcripts without stop codons (Fig 2A, B). Additionally, we have shown that NSD cooperates with RNA silencing to eliminate RISC

cleavage products. Importantly, NSD is responsible for the degradation of RISC cleavage product only when the substrates are targeted within the coding region (Fig 2B). In case of TAS lncRNAs, which were shown to be bound by ribosomes but not translated, stabilization of RISC fragments could be found in neither *xrn4-, ski2-, pel-* or *hbs1*-silenced/or single mutant plants. To show that these findings are not restricted to sensor constructs or selected RNAs but occur genome-widely, we employed RNA transcriptome sequencing from wild-type or mutant plants, respectively. Based on these, ***we excluded NSD being involved in TAS ncRNA destabilization***. Instead TAS ncRNAs are protected following miRISC cleavage and probably directly converted to dsRNA by the RDR complex to generate ta-siRNAs (Fig. 2C).

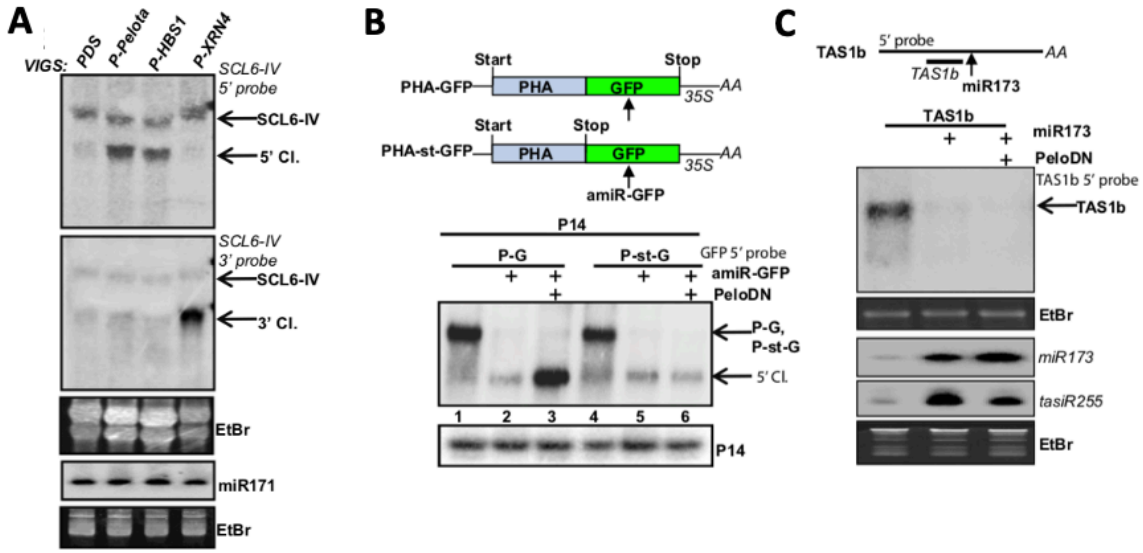

Figure 2: NSD pathways drive RISC 5'cleavage product degradation when cleavage occurs within protein-coding transcripts but not lncRNAs. (A) SCL6-IV 5'-RISC cleavage product accumulates in the absence of Pelota and HBS1, while 3' RISC-cleavage product accumulates in absence of XRN4; (B) 5' RISC-cleavage product accumulates only when a downstream stop codon is present; (C) ncRNA TAS1c 5'RISC-cleavage product give rise to tasiRNA independently of NSD pathway (NSD was inactivated by a dominant negative form of *Pelota* trans factor (PeloDN)).

These findings have been published[1]: (Szadeczky-Kardoss*, Csorba* et al., 2018, NAR, *shared first authors).

2.2. Short ncRNAs' role during thermal adaptation

miR824 is a *Brassicaceae*-specific miRNA, its unique target is AGAMOUS-Like16 (AGL16), a MADS-box transcription factor. We have found in our RNAseq data that *pri-miR824* strongly accumulated, while AGL16 target was significantly decreased by the ACC heat treatment. These changes have been confirmed by qrtPCR and/or northern blot (Fig. 3). Based on literature miR824/AGL16 module was reported to be involved in stomata complexity regulation[31]. As the cooling effect of water evaporation may have a moderating impact on heat stress damage, we reasoned that miR824 potentially may contribute to heat tolerance of plants through stomatal density regulation.

To test whether *pri-miR824* accumulation during HS is caused by transcriptional induction, we generated a GUS reporter stable transgenic lines, where GUS expression was driven by the miR824 promoter ($p824_{wt}$*::GUS*). GUS expression was vein-specific and overlapped with AGL16 target expression localization (Fig 6). We studied *in silico* the promoter of *pri-miR824*; heat stress responsive elements (one HSE and further 2 corrupted HSE) were

detected upstream of start site. To validate these elements as *bona fide* functional motifs, we introduced point mutation within the HSE (*p824$_{hse1}$::GUS*) and assayed GUS RNA levels following HS (at both 37° and 45°C). 37°C strongly, while 45°C mildly induced GUS expression. HS-induced activation of *p824$_{hse1}$:GUS* reporter transgene RNA was significantly diminished (4-fold decrease compared to *p824$_{wt}$::GUS*)(*data not shown*). We have also generated the GUS reporter by using the triple HSE mutant promoter (*p824$_{123}$::GUS*), however this behaved similarly to *p824$_{hse1}$:GUS*, suggesting that HSE-like elements do not confer additional heat-responsiveness (*data not shown*).

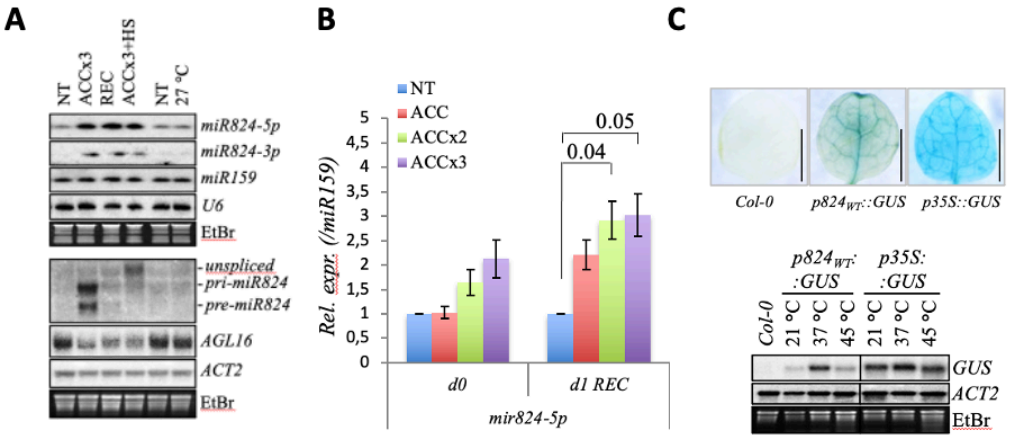

Figure 3: *pri-miR824* is induced while *AGL16* target is downregulated by heat. (A) miR824 is transcriptionally induced and accumulates during repeated heat stress treatments; miR824 target *AGL16* mRNA levels are decrease during heat treatment; (B) quantification of miR824 mature form during repeated heat treatments; (C) miR824 promoter shows vein-specific and heat-responsive activity. Bars represent the mean ± standard deviation of at least 3 independent measurements. Number denotes two-tailed Student's t-test p values.

To find out which *trans* factors are required for miR824 transcriptional induction, we tested *pri-miR824* heat-responsiveness in several *hsf* mutants: we have found that HS-induction was compromised in *hsfa1a, hsfa1b, hsfa1d, hsfa1e* triple or quadruple mutants and in *hsfa2* mutants (Fig 4A), but not in *hsfa3, hsfa6a, hsfa6b, hsfa7a* or *hsfa7b* mutants (*data not shown*).

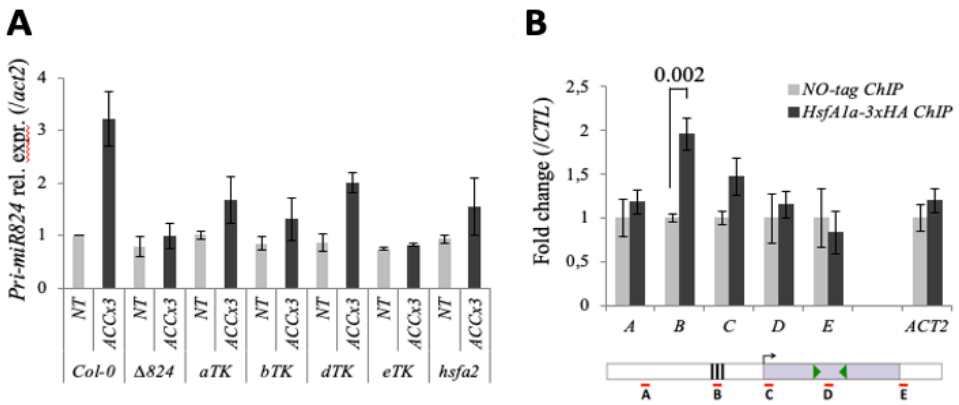

Figure 4: HS-induction of pri-miR824 requires HSE *cis* elements and HsfA1 and A2 trans factors. (A) *pri-miR824* induction is incomplete in HsfA1 triple mutants *aTK, bTK, dTK, eTK* and *hsfa2* mutants; (B) HsfA1d directly bind promoter region of miR824 gene, ChIP amplicons are depicted below, the three back lines denote HSE elements within promoter region of the gene, number denotes two-tailed Student's t-test p value.

To ask whether that HSF *trans* factors are directly required for *pri-miR824* HS-induction, we did chromatin immunoprecipitation (ChIP) using *phsfa1d::3xHA-HSFA1d* (in *hsfa1a/b/d/e* quadruple mutant background) stable transformant line. Direct interaction of HSFA1d with miR824 promoter in the HSE element containing promoter region, but not upstream or downstream regions of miR824 gene was observed (Fig. 4B).

Besides HSE elements a drought responsive element (DRE) was also found in the promoter of miR824 gene. This prompted us to assess miR824 induction during drought and combined (HS + drought) stress. We have found that miR824 is not induced under drought conditions but combined stress (HS + drought) has a synergistic effect (much stronger induction of *pri-miR824* compared to HS only)(*data not shown*).

AGL16 mRNA level decrease parallel to miR824 induction during HS suggested cause-effect relationship between the two phenomena. Interestingly however, the level of *AGL16* decreased immediately at 45°C (in the very early stage of heat stress), when miR824 induction was not complete, suggesting miR824-independent behaviour. To understand miR824 roles on AGL16 down-regulation during HS, we monitored *AGL16* mRNA levels during a time-course in wt (Col-0) and miR824 mutant (*Δ824*) or mimicry line (*MIM824*). In *Δ824* line the miR824 is not induced, while in *MIM824* is induced but remains inactivated by the mimicry construct[32,33]. We found that in the presence of active miR824 (in Col-0) HS-induced down-regulation of *AGL16* mRNA lasted for at least 3-4 days, while in its absence (*Δ824* and *MIM824* lines) was transient. In conclusion therefore, *AGL16* downregulation during mild heat exposure is caused by at least two pathways, a miR824-dependent and a miR824-independent one (Fig. 5).

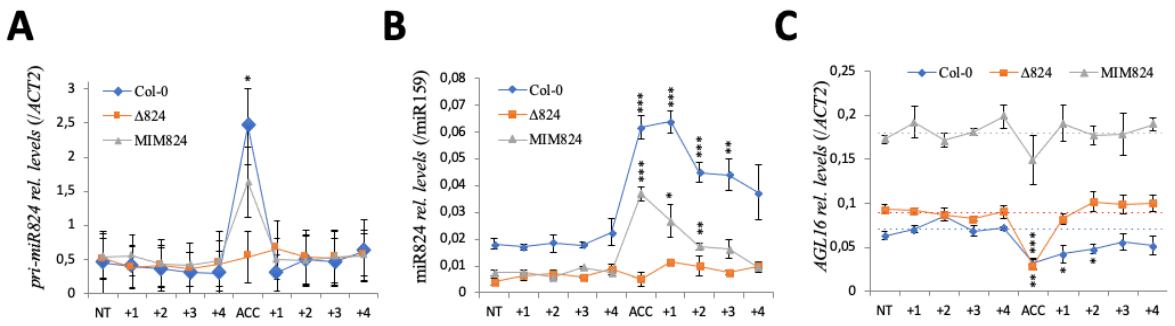

Figure 5: AGL16 downregulation is caused by a miR824-independent and a miR824-dependent pathway. (A) pri-miR824 is transienty induced by heat treatment; (B) half-life of mature miR824 is about 3-4 days; (C) AGL16 is transiently downregulated by heat miR824-independently, and stabily repressed following heat for 3-4 days by miR824 pthway. Bars represent the mean ± standard deviation of at least 3 independent measurements. Stars represent significant changes based on two-tailed Student's t-test p values (*p<0.05, **p<0.01, ***p<0.001).

To understand the causes of miR824-independent AGL16 mRNA down-regulation (transcriptional block versus elevated decay) we assayed the level of *AGL16* unspliced form (*uAGL16*), as a proxy for transcriptional changes: a mild downregulation has been found between non-treated and heat treated samples. Additionally, we also generated an AGL16 promoter driven GUS construct (*pAGL16:GUS*) and have shown that heat inhibits AGL16 promoter activity.

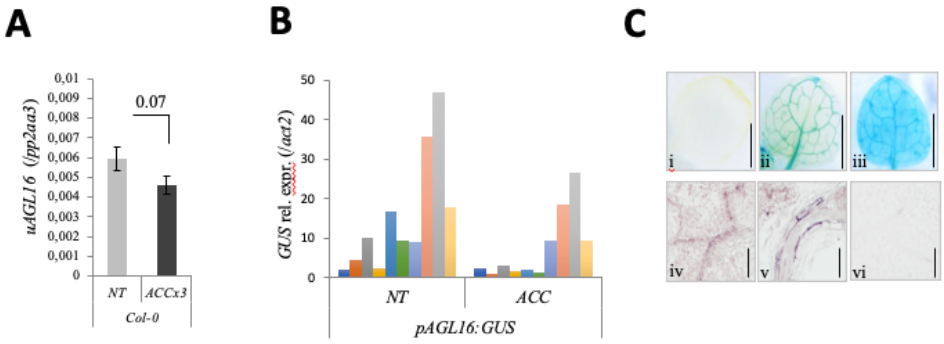

Figure 6: AGL16 is transcriptionally repressed during heat stress. (A) unspliced AGL16 RNA is mildly decreased by heat; AGL16 promoter activity is downregulated by heat in multiple independent transgenic lines; (C) AGL16 promoter activity is vein-specific; i. non-transformed line, ii. *pAGL16::GUS* –transformed line, iii. *p35S::GUS*-transformed control line, iv-v. *AGL16* mRNA *in situ* hybridization, vi. *in situ* negative control). Bars represent the mean ± standard deviation of at least 3 independent measurements. Number denotes two-tailed Student's t-test p value.

Next we wanted to understand the biological relevance of miR824 induction and AGL16 downregulation. miR824 was reported to be involved in stomata complexity regulation through down-regulation of AGL16[31]. AGL16 overexpressor lines develop higher order stomata complexes while miR824 overexpressor line have lower amount of these stomata. Stomata are important cellular components of HS response: they regulate evaporation and therefore contribute to the cooling of the leaf surface.

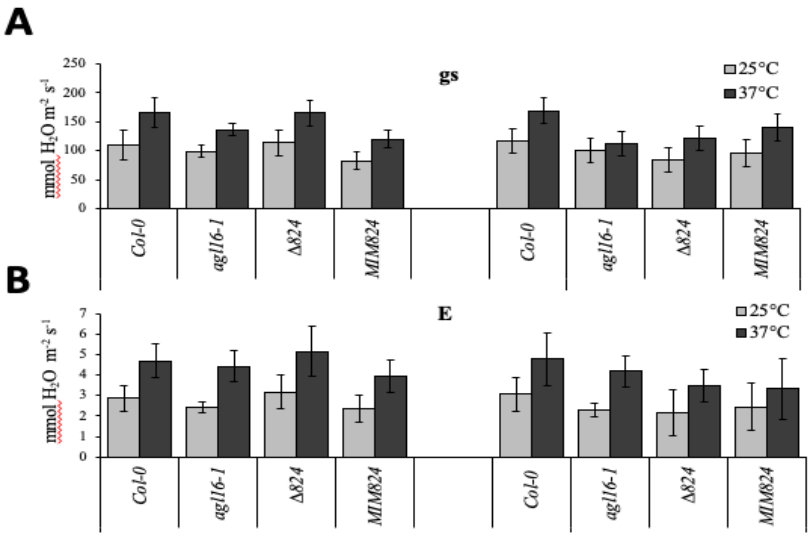

Figure 7: miR824/AGL16 module does affect heat stress response through stomata complexity changes. (A) stomatal conductance (*gs*) and (C) transpiration rate (*E*) were determined at 25 °C and 37 °C in the non-treated (NT) and acclimated (ACCx3) mutants compared to wild-type Col-0. Bars represent the mean ± standard deviation of 6 independent measurements originated from different plants in each genotype and treatment.

To find out whether miR824/AGL16 module is important for HS response regulation through stomata complexity regulation, we assayed evaporation and gas exchange in *agl16-1*, and miR824-defective *Δ824* and *MIM824* lines. Measurements were done on both untreated and heat-acclimated plants in control (25°C) and HS (45°C) conditions. Significant differences were found between non-treated and acclimated conditions but not between wild-type and mutant genotypes. These findings ***excluded miR824/AGL16 module as regulators of HS adaptation through stomata complexity regulation*** (Fig 7).

The other role of miR824 – AGL16 module is flowering time acceleration[33]. AGL16 forms a multiprotein complex with Flowering Locus C (FLC) and Short Vegetative Phase (SVP). Alteration of *AGL16* mRNA levels by manipulation of miR824 abundance influences flowering time. The effect of AGL16 on flowering time is fully dependent on the presence of Flowering Locus T (FT). To assay the effects of HS on flowering time mediated by miR824 /AGL16 module, we crossed our mutant and overexpressor lines into FRIGIDA (FRI) background. FRI is a strong activator of Flowering Locus C (FLC, the cofactor of AGL16); in the presence of FRI and active FLC flowering is strongly delayed. The difference caused by AGL16 inactivation (*agl16-1*) on flowering in this background is much higher (Col-*FRI* vs Col-*FRI/agl16-1* about 30%) than in Col-0 background (Col-0 vs *agl16-1* about 14%)[33]. The double homozygous plants were assayed for *FT* changes under NT and heat treated conditions: as ambient temperature elevated FT levels and accelerate flowering transition, instead of ACC treatment we employed direct 37°C treatments. Under non-treated conditions *FT* levels are significantly higher in Col-*FRI;agl16-1* plants and slightly lower in Col-*FRI;MIM824* plants. Heat treatment resulted in similar *FT* mRNA levels in Col-*FRI* and Col-*FRI;agl16-1* but significantly different in Col-*FRI;MIM824* line. Flowering time changes correlated with *FT* changes. These findings suggest that ***heat-induced miR824/AGL16 module changes compensate for the negative impact of HS and accelerate flowering*** through derepression of FT (Fig 8).

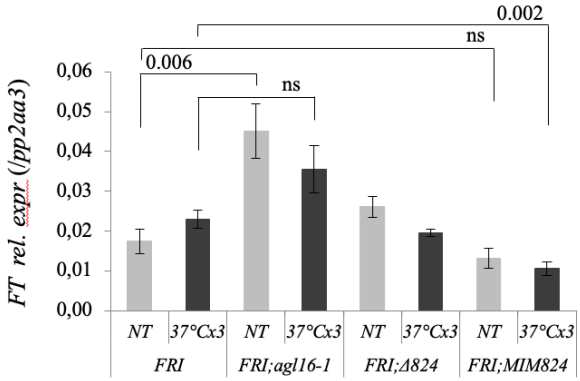

Figure 8: FT mRNA changes in wild-type and miR824/AGL16 modul mutants in response to heat. Bars represent the mean ± standard deviation of at least three independent measurements; numbers denote two-tailed Student's t-test p values (ns, non significant).

To assess the conservation of miR824/AGL16 module HS-mediated regulation we analyzed it in the *Brassica napus* spring var. RV31 and winter var. Darmor, *Raphanus sativus L., B. oleracea var. botrytis, B. oleracea var. capitata* and *B. rapa var rapa* (*data not shown*). *miR824* induction occured in all these agronomically important crops, suggesting that the module is conserved and may have regulatory functions during HS response (Fig 9).

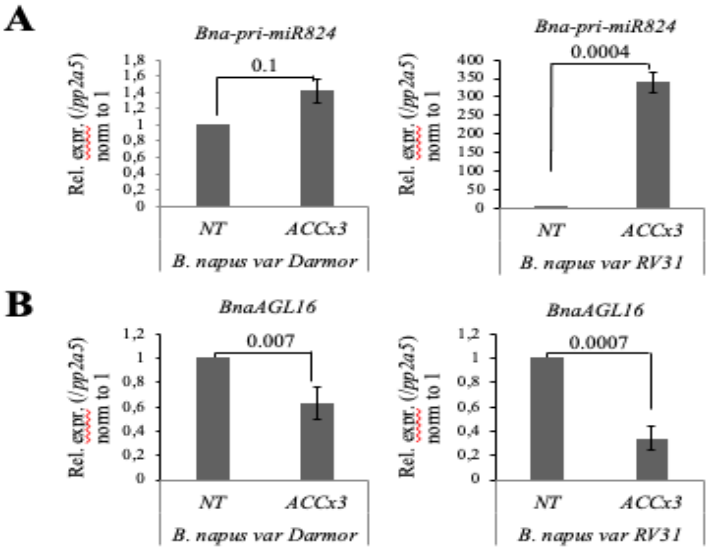

Figure 9: Changes of miR824/AGL16 module components in *B. napus* in response to heat . (A) *B. napus pri-miR824* is significantly induced, while (B) *BnaAGL16* is repressed in both spring and winter varieties. Bars represent the mean ± standard deviation of three independent measurements; numbers denote two-tailed Student's t-test p values.

Based on these findings we proposed a working model (Fig 10). Beyond the ***detailed molecular characterization of miR824/AGL16 module regulation*** our study have reached also two general conclusions: (i) we have shown that ***repeated stresses may lead to gradual accumulation of stress-responsive miRNAs*** due to their extended half-life, and (ii) stress-induced miRNAs (as shown for miR824, miR398a in our study or for miR156, miR831 earlier[15]) have an extended effect on their target mRNAs post-stress and as such should be regarded as "***post-transcriptional memory factors***" that modulate development post-stress. ***These data have been published***[2].

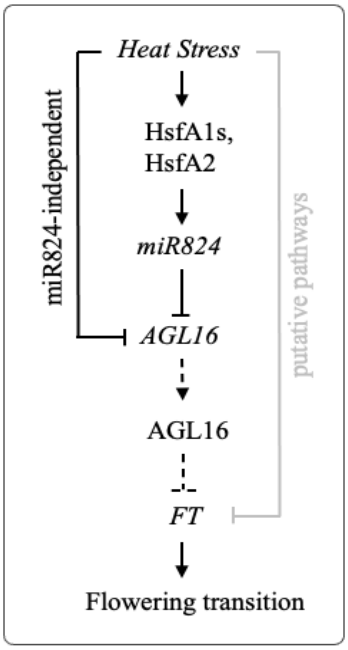

Figure 10: Proposed working model of miR824/AGL16 module heat stress regulation. Heat stress induces transcription of miR824 through HSFA1a family and HSFA2 transcription factors. *AGL16* is depleted through a miR824-dependent and a miR824-independent pathway. Stable downregulation of *AGL16* leads to de-repression of *FT*, a central integrator of flowering transition (dotted lines depict down-regulated steps during and following HS). *FT* level may be also altered by other putative HS-regulated factors (grey line).

Based on our work on stress-related short ncRNAs, we have been asked to summarize the literature on their roles during thermal adaptation. In this review, we revised the current understanding of small ncRNA-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, in order to ensure successful adaptation (Fig 11).

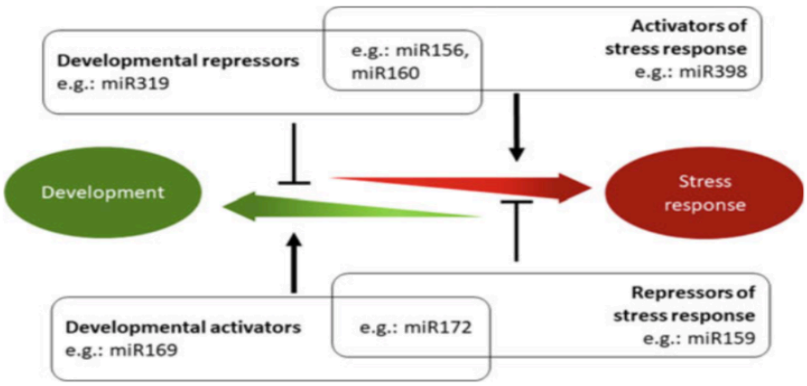

Figure 11: Small RNAs act in a complex network to regulate the balance between developmental pathways and stress responses.

Finally, we highlighted specific characteristics of small RNA-based temperature stress regulation in crop plants, and explored the perspectives of the use of small RNAs in breeding to improve stress tolerance, which may be relevant for agriculture in the near future. ***The review paper has been published (Szaker et al., 2020; DOI: 10.1007/978-3-030-35772-6).***

2.3. Long ncRNAs' role in thermal adaptation

Tobacco Gene with Unstably Transcript 15 (GUT15)[34] and cucumber Cytokinin Repressed 20 (CR20)[35,36] are intergenic ncRNAs, members of the same ncRNA family. In spite of being ***identified about 25 years ago, the physiologcal role of these lncRNAs is still unknown***. The fact that these lncRNAs are conserved, possess a structured secondary stem-loop, and are significantly induced in our HS screen prompted us to try to understand the biological relevance of these genes/transcripts.

As the name of these genes does not depicts their relatedness, and does not point to a real physiologcal role, we renamed them as *Male Sterile-1* (*MASTER-1,* At2G18440) and *MASTER-2* (AT4G36648)(see later). We will relate to these as such from now on.

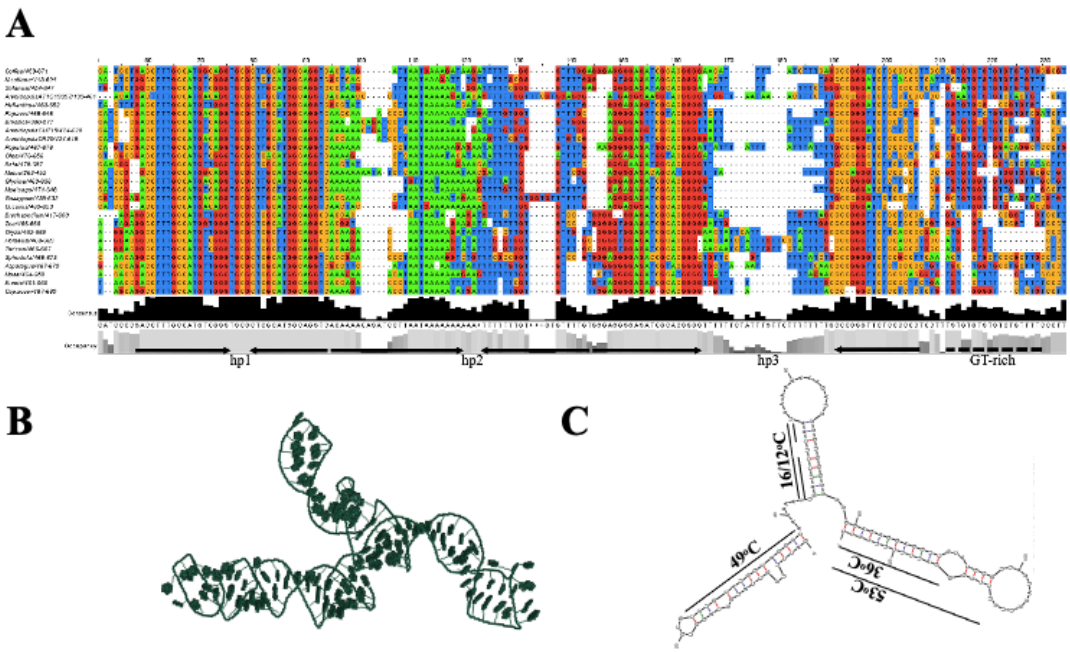

Figure 12: Conservation of the secondary structured domain within MASTER lncRNA family in angiosperm species. (A) Conservation in selected species, positions of the hairpins are depicted below, (B) 3D predicted folding of the triple hairpin, (C) 2D predicted structure, predicted physiological melting temperatures are shown on each branch region.

First we thoroughly analysed conservation of MASTER genes throughout plant kingdom: MASTER lncRNA genes have been found in angiosperm (detected by us in all angiosperms studied with available genome sequence data), but absent in gymnosperms or other evolutionary distant lineages (Fig 12A). MASTER genes contain a central triple stem-loop (instead of two stem-loops, as suggested earlier) and an additional immediate downstream GU-rich region which is partly also conserved. The central stem-loop has been predicted to have a low melting temperature (Arabidopsis consensus MASTER transcripts' AU-rich stem-loop Tm≈28°C), making it suitable to function as a temperature sensor (Fig 12B, C).

Next, we confirmed that *MASTER-1* and *MASTER-2* are induced under HS by northern blot and qrtPCR. We have also analysed their induction on different temperatures, including cold or elevated ambient heat: we have found that both are HS-induced with an optimum at 32°-37°C. We also assayed MASTER lncRNAs' levels during different environmental conditions: MASTER-1 and -2 were neither responsive to dark, cold, salt, drought or sucrose. Heat ***induction occurs also in the dicot B. napus or the monocot H. vulgare crops*** (Fig 13).

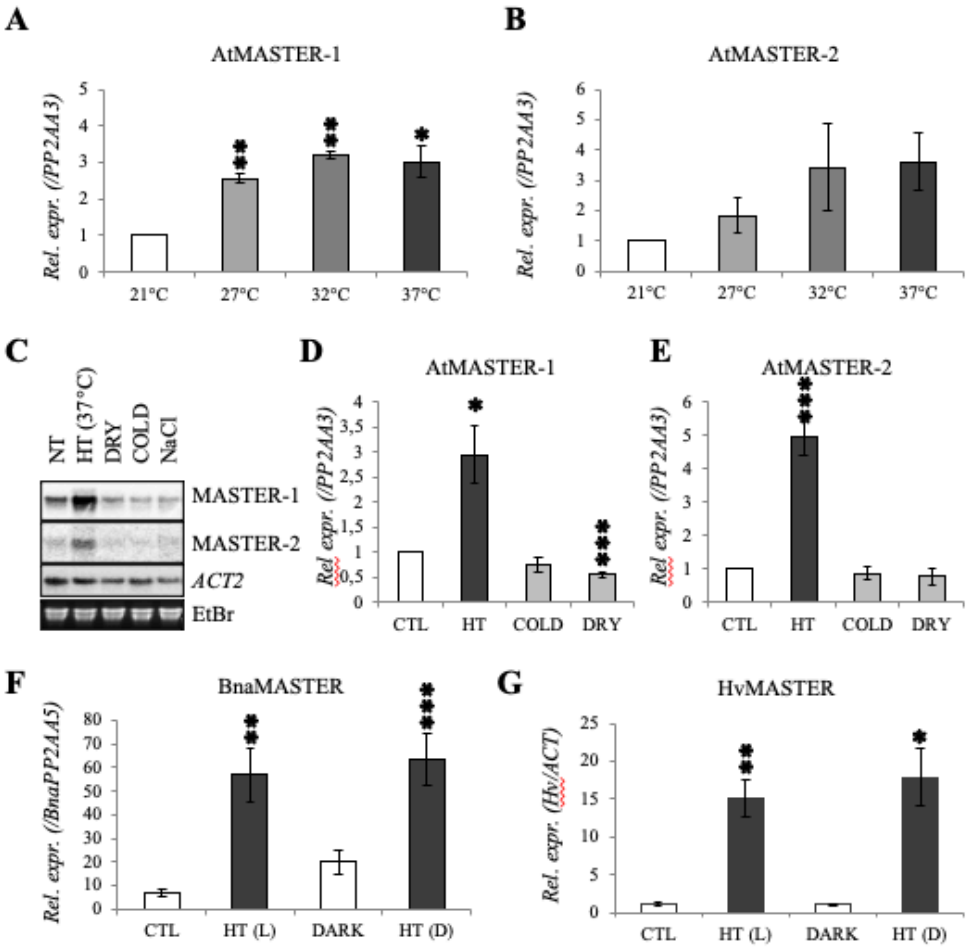

Figure 13: MASTER lncRNAs are induced by heat. (A, B) Arabidopsis MASTER-1 and -2 are induced by elevated ambient heat and moderate heat; (C, D, E) MASTER-1 and -2 are not responsive to cold, drought or salt stress; (F) heat induction of MASTER lncRNA in *B. napus* and (G) *H. vulgare*. Bars represent the mean ± standard deviation of at least 3 independent measurements. Stars represent significant changes based on two-tailed Student's t-test p values (*p<0.05, **p<0.01, ***p<0.001).

Heat-induced accumulation was observed in case of unspliced forms, suggesting transcriptional induction (Fig 14). The heat-inducibility of *pMASTER-1::GUS* reporter construct further confirmed this hypothesis (*data not shown*). As HSE *cis* elements have been found in the promoter and gene body of both genes (Fig 14A, B), we assayed their induction in the quadruple mutant *QK* (*hsfa1a;hsfa1b;hsfa1d;hsfa1e*)[37], lacking HsfA1 factors, the central regulators of HSR (Fig 14C-F). Both basal transcription and heat-induced activation of *MASTER-1* and *2* was elevated, suggesting that HsfA1s *trans* factors negatively (probably indirectly) affect their transcription.

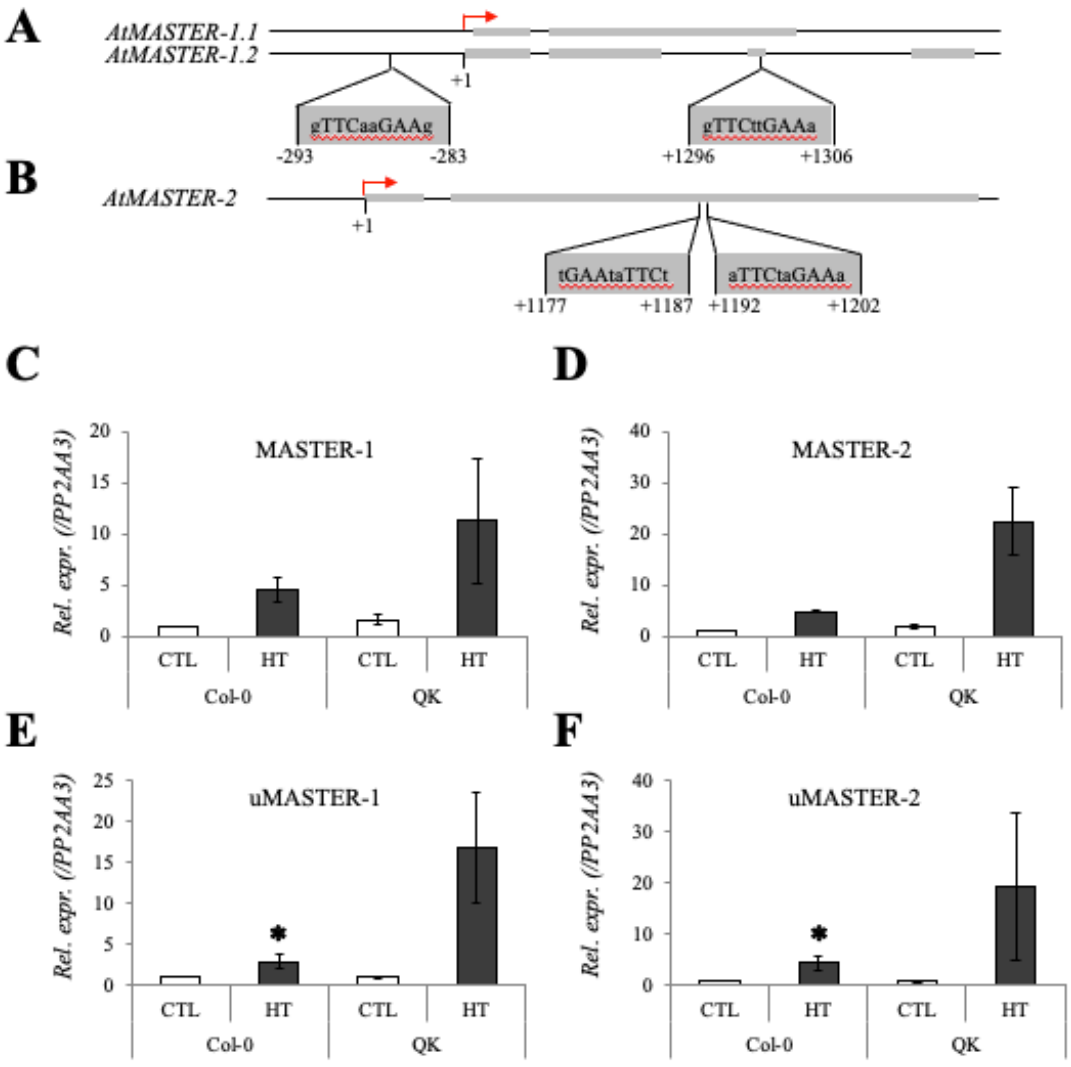

Figure 14: MASTER lncRNAs are heat-induced at transcriptional level. (A,B) HSE cis elements are present in promoter and gene body of MASTER-1 and -2 gene loci; (C,D) HsfA1 family trans factors negatively regulate transcrition of MASTER-1 and -2. Bars represent the mean ± standard deviation of 2 independent measurements. Stars represent significant changes based on two-tailed Student's t-test p values (*p<0.05, **p<0.01, ***p<0.001).

Accumulation of *MASTER-1* (3,2-fold induction) was observed in the *p35S::MASTER-1* line as well. Of note, *p35S* promoter is only mildly heat-inducible (1,2-fold induction). These data therefore indicate that post-transcriptional stabilization contributes to *MASTER-1/2* lncRNAs' accumulation (Fig 15).

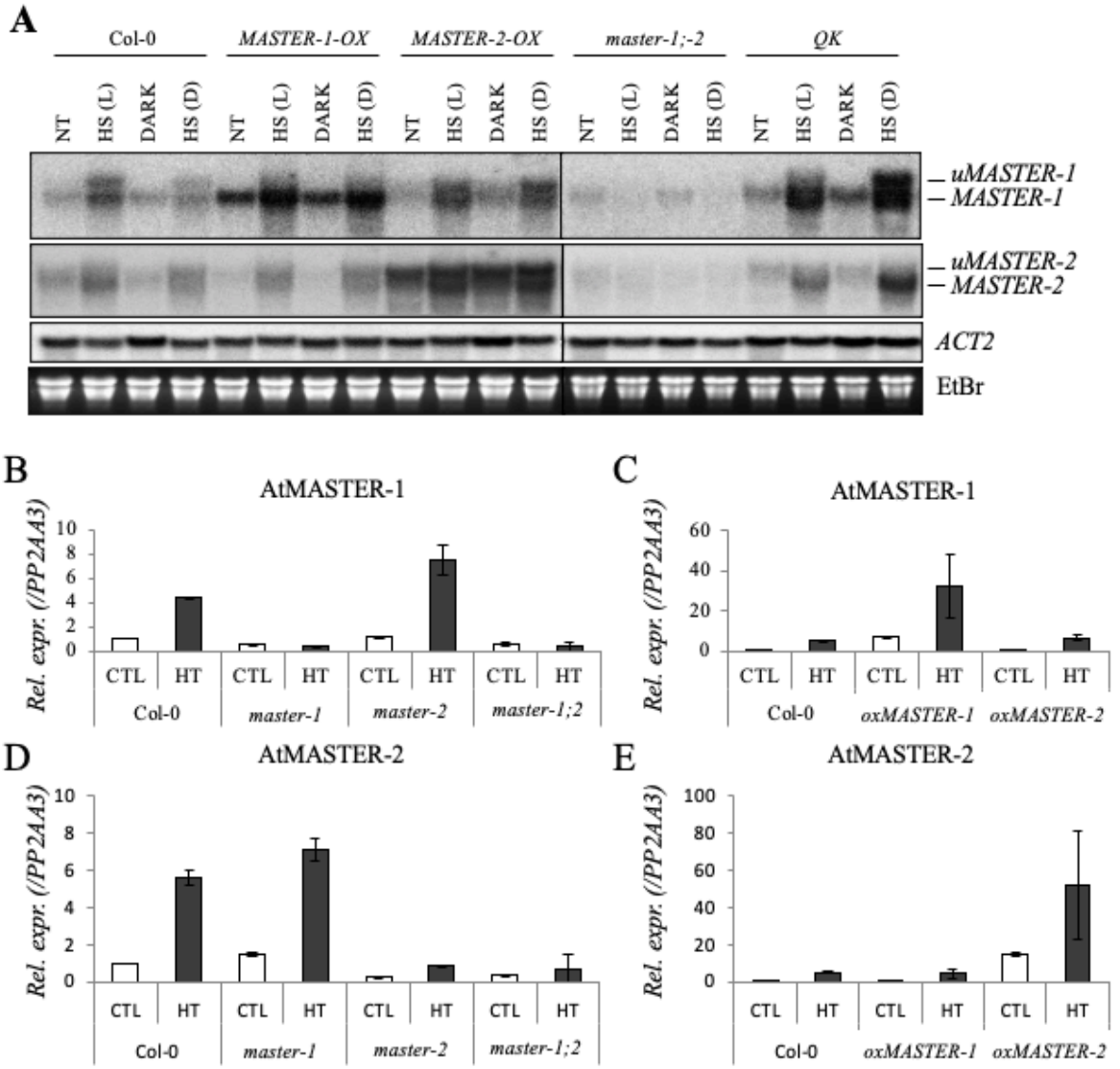

Figure 15: Heat-induction of MASTER-1 and -2 in single and double mutants or MASTER-1 and -2 overexpressor lines. (A) Northern blot analysis, (B-E) qrtPCR assays. Bars represent the mean ± standard deviation of 3 independent measurements.

To study the roles of MASTER lncRNAs we have obtained homozygous *master-1* and *master-2* T-DNA mutants and did crosses to generate double mutant *master-1;2* line (Fig 15). We tested the mutant and overexpressor lines in diverse HS treatment assays, such as basal thermo-tolerance, short and long acquired thermo-tolerance, hypocotyl elongation, thermo-tolerance to moderately high temperatures) but ***could not find any heat sensitivity or tolerance phenotypes***, arguing against a direct role in heat adaptation.

Development and flowering time were also analyzed during long day, short day, continuous light: ***flowering transition was slightly accelerated*** by overexpression of both *MASTER-1* and *-2* under long day in 30°C (Fig 16).

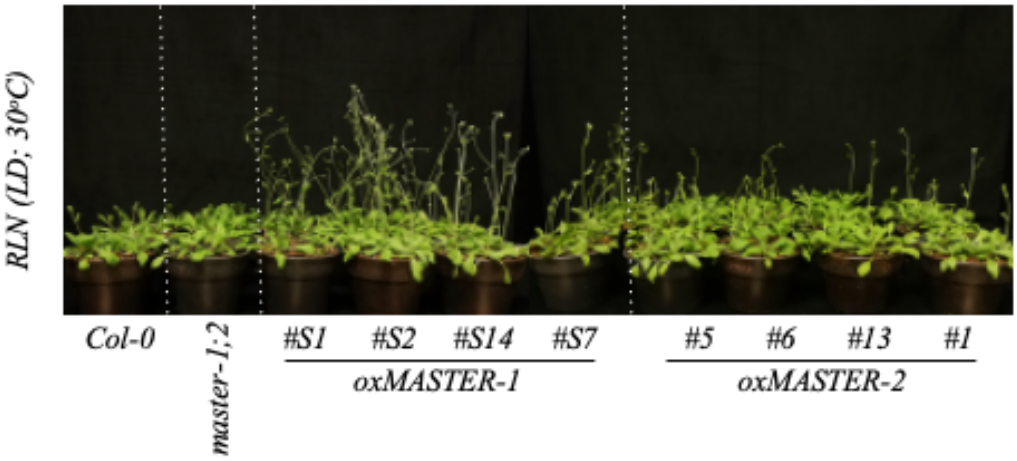

Figure 16: Overexpression of MASTER-1 or -2 from p35S promoter accelerates flowering transition in response to elevated temperatures elevated (independent lines are shown for each).

A possible explanation for our failure to find strong phenotype and by this prove biological significance of these factors, may be the leaky transcription of MASTER lncRNAs in single or double mutant lines (Fig 15). To completely erase MASTER lncRNAs' we did CRISPR mutagenesis, targeting specifically the stem-loop region of the two genes simultaneously. 6 different CRISPR constructs have been tested, two of which were successful. We analyzed homozygous and heterozygous T0 plants. 3 transgenics out of 20 available T0 plants were ***completely sterile displaying strong secondary floral phenotypes as well*** (including underdeveloped anthers, unfused carpels and delayed flowering termination). Then we tested the phenotype of T1 progenies of 12 (from the remaining 17) T0 plants: in 8 line all progenies were fertile, while 4 lines had sterile progenies with the ration of 17:6, 18,5, 15:5 and 20:15 (fertile:sterile), respectively. To link the phenotype with the genotype we sequenced the MASTER-1 and -2 genes amplified from 14 sterile and 10 fertile (from the 20:15 segregating ratio line) and 6 sterile and 6 fertile (from the 15:5 segregating ratio line) plants. Unfortunately, ***phenotype could not be directly linked to genotype*** (mutations have been found in both sterile and fertile plants). Of note, in accordance with the literature 90% of mutations within the genes were 1bp insertions or deletions. Therefore, we reasoned that the phenotype may not be fully penetrant, caused by this minor alteration of the lncRNAs' structure (opposingly to a protein coding gene where a framshift would occur). At this point we could not exclude the possibility of an off-target effect causing the sterile phenotype. As *in silico* off-targets were not predicted by CRISPOR or other similar online tools, we searched the *A. thaliana* genome using BLAST search, and have found 4 potential target genes that may have been affected. We sequenced these genes amplified from 2 sterile and fertile plants, respectively, but could not find any alterations within any of these. Besides, we could not find mutation within the MASTER-1 gene at the MASTER-2-CRISPR target site, or in the opposite direction (in spite of the fact, that the difference between MASTER-1 and -2 target sites was a single nucleotide only). These data suggests that, the CRISPR guides used by us are highly specific to the unique MASTER genes designed against (*data not shown*).

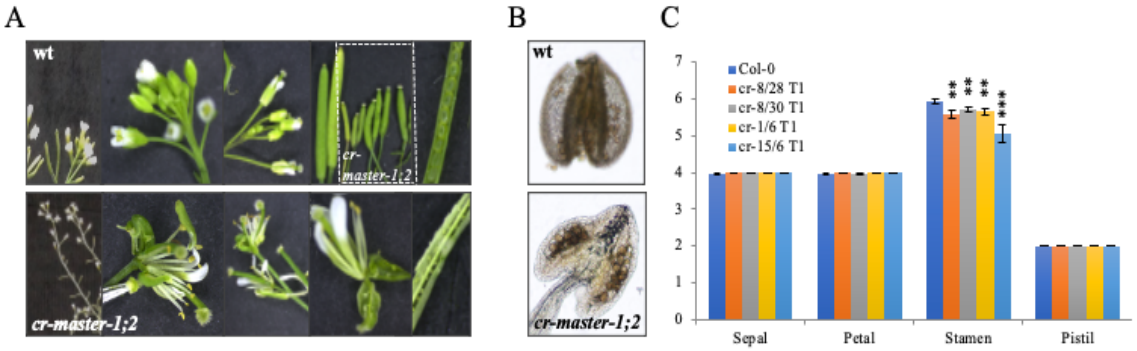

Figure 17: CRISPR mutants of MASTER-1 and -2 cause sterility, floral developmental alterations, and delayed floral termination. (A) Floral phanotypes of wild type plants (wt, top line) or mutant plants (*cr-master-1;2*, bottom line and dashed box), (B) anther phenotype, (C) CRISPR *master-1;2* mutants develop fewer stamens compared to wild type plants (multiple independent lines are shown).

To demonstrate that the phenotype is indeed caused by the mutations within the MASTER-1 and -2 genes, we designed an alternative CRISPR guide construct, targeting the MASTER-1 and -2 genes 6 nucleotides downstream from the previously used target site and have re-done the mutagenesis. This generation of CRISPR plant again lead to sterile plants (10 out of 52), reinforcing the hypothesis, that MASTER gene knock-out mutations cause sterility, but potentially minor mutations (e.g. 1bp indels) are potentially not fully penetrant (as it would be in case of a protein coding genes due to frame-shift).

To circumvent these uncertainties, we decided to check causality by alternative means:

(i) try to regenerate plants from root cultures of sterile plant lines and complement these using *pMASTER-1::MASTER-1* construct (or *p35S::GUS*, as negative control);

(ii) transform Col-0 wild-type or *master-1;2* T-DNA double mutant plants (which do not have sterile phenotype) with *pMASTER-1::MASTER-1*$_{mut}$ constructs (these constructs were cloned form the sterile plant, and have 1 nucleotide A or U insertion within the conserved region of the gene); we hope the mutant form of MASTER-1 may behave as *dominant negative.*

(iii) Cross-pollinate the plant having a big (18bp) deletion in MASTER-1 with another one having 19bp deletion in the MASTER-2 gene (these plants are already available), and obtain homozygous double mutant crispr plants; we hope that the big deletions within MASTER genes will cause *a fully penetrant phenotype*, and all double homozygous plant will be sterile, while the phenotype will be rescued by a wild-type or even a 1 nucleotide indel containing copy of either MASTER-1 or -2 genes.

These experiments are under way, being finalized hopefully in a couple of months.

In support to our model, *pMASTER-1::GUS* and *pMASTER-2::GUS* reporter constructs show ***strong promoter activity in anthers, pollen grains, stigma and replum***, with expression observed also in shoot apical meristem, young leaves and root (Fig 18).

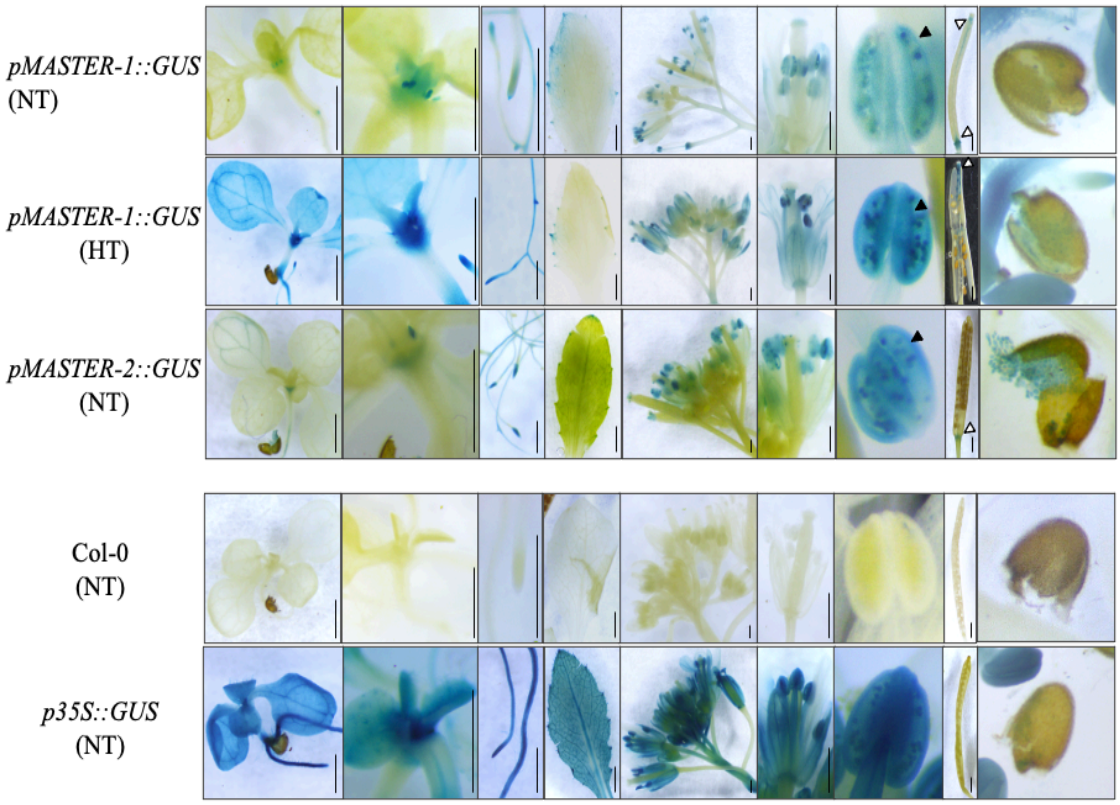

Figure 18: Tissue-specific activity of MASTER lncRNAs' promoter. The different lines are indicated on the left (NT, non-treated; HT, heat treatment).

These finding are in line with the observed phenotype and suggest a central role of MASTER-1 and -2 in temperature-sensitive flowering induction and fertilization processes.

To start to unravel molecular mechanism of this lncRNA family we analyzed the subcellular localization of MASTER-1 and -2 and found it to be enriched in the nuclear fraction (a low level was found also in the cytoplasm)(Fig 19). To double-check the subcellular localization, we are attempted to do *in vivo* RNA staining, using Mango labelling technology, however, these technologies were unsuccessful due to unknown reason (probably the staining material could not penetrate cell walls).

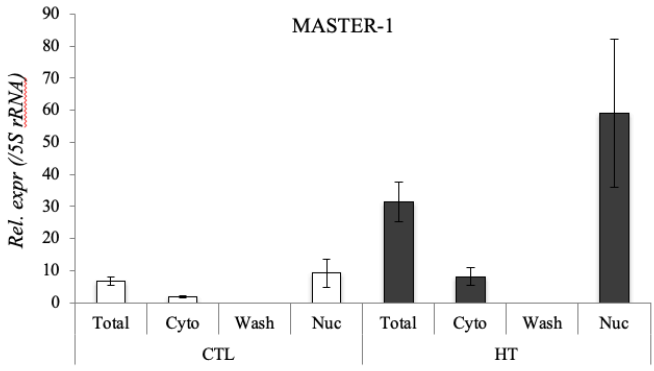

Figure 19: MASTER-1 lncRNA sub-cellular localization (CTL, control; HT, heat treated; Cyto, cytoplasmic fraction; Nuc, nuclear fraction).

The nuclear fraction of MASTER lncRNAs may have genomic/gene regulatory roles, alongside protein partners. We hypothesized that the conserved RNA secondary structure may serve as scaffold for protein binding or complex assembly. To unravel potential protein partners, we did RNA-IP using a tiled biotin-DNA oligo set (similarly as in ChIRP). MASTER-1 but not *ACTIN2* RNA was robustly enriched (30-fold) in the biotin-IP but not in the mock-IP. We ***have tried to identify the putative protein partners*** by Mass Spectrometry analysis (in collaboration with Laboratory of Proteomics, BRC, Szeged), but ***failed so far***. Besides we tried to do electromobility shift assays using *in vitro* synthesized MASTER1, and prove the existance of a molecular partner, again without success. Next we will do Chromatin Isolation by RNA Purification (ChIRP) in combination with DNAseq, in order to reveal potential genomic loci targeted by MASTER-1 and -2 transcripts and reveal direct target genes, potentially involved in fertilization. These expriments *have been put to hold*, until the genotype-phenotype causality is verified.

Hopefully we will be able to finalize these experiment soon, and thoroughly characterize a novel regulatory lncRNA acting in angiosperms, in a very important process, and which were elusive since 25 years. Manuscript in preparation: *Szaker HM and Csorba T: MASTER lncRNA family regulates fertilization in angiosperms in a temperature-dependent manner.*

3. Cooperation related to the project

Previously we have revised the role of short RNAs in temperature sensing and role in keeping the required balance between developmental processes and stress responses. Most of the knowledge in the RNA silencing field is based on *A. thaliana* and *N. benthamiana* systems, although studies on other dicot or monocot species' RNA silencing and short ncRNAs have been also done[38-41]. Despite its economic importance, there was only scattered information on RNA silencing machinery and its temperature regulation in monocot crop barley[42,43]. The key components of RNA silencing are the Dicer-like proteins (DCLs), Argonautes (AGOs) and RNA-dependent RNA polymerases (RDRs). In this study, we identified five DCL (HvDCL), eleven AGO (HvAGO) and seven RDR (HvRDR) genes in the barley genome. Genomic localization, phylogenetic analysis, domain organization and functional/catalytic motif identification were also performed. To understand the regulation of RNA silencing, we experimentally analysed the transcriptional changes in response to moderate, persistent or gradient heat stress treatments: transcriptional accumulation of siRNA- but not miRNA-based silencing factor was consistently detected. These results suggest that ***RNA silencing is dynamically regulated and may be involved in the coordination of development and environmental adaptation in barley*** (and probably other monocot crop relatives, like wheat). Our work ***provides background information about barley RNA silencing components*** and will be a foundation for the selection of candidate factors and in-depth functional/mechanistic analyses[44]. This work has been done in cooperation with Epigenetic group and Plant Developmental group at ABC.

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 authorship

Besides we have been involved in another cooperation on transcriptome changes during biotic stresses[45].

Pesti R., Kontra L., Paul K., Vass I., Csorba T., Havelda Z., Várallyay É. (2019) Differential gene expression and physiological changes during acute or persistent plant virus interactions may contribute to viral symptom differences, PLoS One. 2019 May 3;14(5):e0216618. doi: 10.1371/journal.pone.0216618. eCollection 2019.

4. Conclusions

In animal systems lncRNAs were specifically involved in regulation of different aspects of temperature response. In spite of many lncRNAs have been identified, only a few lncRNAs are functionally characterized and directly linked to HSR in plants. In the present programme we have done a deep transcriptome analysis, and based on this selected short and long ncRNAs for in-depth study. We ***unravelled the roles of selected ncRNAs*** in HSR in *A. thaliana* plants and extended our findings at some extent to the dicot *Brassica napus* and monocot *Hordeum vulgare* crops. We presented our finding at ***13 scientific conferences, published 4 scientific papers and wrote 1 review paper.*** Additionally, a ***PhD program has been completed during the project (Szaker HM, ELTE).*** We hope that our findings will contribute to the development of heat-tolerant plants in long term that may have an immense significance in the light of global warming and climate change. Furthermore, we believe that our findings can extend significantly the knowledge on lncRNA functions in general, which may be relevant in all eukaryotes.

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