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I. Zusammenfassung

In Eukaryonten ist die DNA um Histone gewunden und bildet mit diesen eine kompakte Struktur, das Chromatin. Diese Struktur beeinflusst die Zugänglichkeit der DNA und damit auch die transkriptionelle Regulation der Gene. In *Arabidopsis thaliana*, dem pflanzlichen Modellorganismus der Genetik, wird das Ausmaß der Chromatinkondensation durch physiologische Prozesse, unterschiedliche Entwicklungsstadien, sowie von Umweltfaktoren verändert. Epigenetische Faktoren scheinen an der Verarbeitung von Umweltsignalen beteiligt zu sein und Einfluss auf die Struktur des Chromatins zu haben. Die beteiligten Mechanismen sind aber noch nicht ausreichend untersucht.

Eine bekannte Auswirkung von Hitzestress bei Pflanzen ist die Dekondensation von Chromatin und eine damit einhergehende Aktivierung von solchen Genen, welche unter normalen Bedingungen durch epigenetische Kontrolle stillgelegt sind. DNA Methylierung und postranslationale Histonmodifikationen, die sonst mit epigenetischen Veränderungen verbunden sind, bleiben unter Hitzeeinwirkung weitestgehend unverändert. Allerdings geht die Bindung der DNA an die Histone an manchen Stellen des Genoms verloren. In dieser Arbeit habe ich die Auswirkung von anderen abiotischen Stressfaktoren auf die Chromatinorganisation untersucht. Getestet wurden ein limitiertes Nährstoffangebot, oxidativer Stress, sowie hohe und niedrige Lichtintensitäten. Eine von mir neu etablierte Methode erlaubte es, die zytologisch sichtbare Heterochromatindekondensation, welche während des Hitzestresses auftritt, zu quantifizieren. Dadurch war es mir möglich, die auftretende Dekondensation mit der Aktivierung von Genen in Beziehung zu setzen. Zu diesem Zweck wurden unterschiedliche Ökotypen untersucht und verglichen. Transkriptionelle Aktivierung und Dekondensation zeigten eine beträchtliche Variationsbreite zwischen den Ökotypen in der Reaktion auf Hitze. Aktivierung und Dekondensation sind beides transiente Effekte, welche nach Ende der Hitzeeinwirkung weitgehend in den ursprünglichen Zustand zurückkehren. Das Vorhandensein von Licht während der Hitzeeinwirkung beeinflusste das Ausmaß der Aktivierung. Andere getestete Stressarten hatten keinen oder kaum Einfluss auf die transkriptionelle Aktivierung. Hitze scheint deshalb eine spezifische Wirkung auf der Ebene des Chromatins auszulösen.

II. Abstract

In eukaryotes, DNA is wrapped around histones to form a higher order structure called chromatin. This structure is crucial for transcriptional regulation of genes by modifying the accessibility of the DNA. In the model plant *Arabidopsis thaliana*, the condensation level of chromatin can be modified by physiological, developmental, and also environmental factors. Epigenetic factors at the chromatin level are thought to be involved in processing environmental stimuli. but the mechanisms are yet not well understood.

Heat stress has been shown to disrupt condensed chromatin and to activate genes, which are transcriptionally silent at moderate temperatures. This effect occurs without changes in DNA methylation and only minor changes in histone modifications, but with reduction of nucleosome occupancy. Thereby, heat causes prominent effects on epigenetic regulation. In this work, I have tested other stresses with regard to interference with epigenetic regulation, including limiting nutrient factors, oxidative stress, high and low light intensities. I have further developed a protocol to quantify the cytologically visible heterochromatin decondensation during heat stress. I have applied this to investigate the correlation between decondensation and transcriptional activation of epigenetically controlled genes and to compare the degree of decondensation upon heat stress between different ecotypes of *Arabidopsis*. Natural variation in response to heat exists between the ecotypes, at the level of transcription and decondensation. The effects are transient, since silencing is re-established and heterochromatin condensation returns largely to pre-stress levels. Nevertheless, I found that the effect of heat is modulated by the amount of light that the plants receive during the heat exposure. Diverse other stress types showed only minor or no response, demonstrating the significant and specific effect of heat.

1 Introduction

1.1 Epigenetics and environment

Unfavourable environmental conditions disrupt the homeostasis of organisms and lead to stress. Accurate and adequate stress response is crucial for the survival of an organism. Part of the stress response is exerted by quick and transient changes of signal transduction, transcriptional activity, regulation of RNA stability, translation efficiency, post-translational modifications, or regulation of enzyme activity, but there is growing evidence that epigenetic gene regulation at the level of chromatin configuration is involved in stress responses. Changes in epigenetic modifications in response to environment were shown in many organisms, reviewed by (Feil and Fraga 2012). Epigenetic modifications on DNA or chromatin lead to changes in gene expression without altering the nucleotide sequence. Epigenetic mechanisms influencing the transcriptional activity of a gene include histone modifications, DNA methylation and small RNAs. These potentially reversible modifications are mitotically heritable, and sometimes also meiotically, resulting in a non-Mendelian segregation.

Remodelling of chromatin structure provides a flexible but at the same time relatively stable way to adapt transcription to cope with environmental stresses. These alterations can occur at specific regions or genome-wide. Though most of the modifications return to previous levels after stress exposure, occasional imperfect restoration or even maintenance of stress-induced changes may provide an evolutionary advantage to the organism and its population (Zhu *et al*. 2012). For instance, in *Caenorhabditis elegans,* mutationally induced deficiencies in chromatin modifiers prolonged the lifespan of individuals even in wild type descendants up to the third generation (Greer *et al*. 2011). In addition, silencing factors administered with the food of *C. elegans* caused long-term epigenetic memory (Ashe *et al*. 2012). Here, nuclear RNAi factors and chromatin regulators were essential for the lasting effect. Interestingly, once the modification was established, the trigger was no longer needed.

Recent studies provide evidence that also stress-induced changes on the chromatin level can be trans-generationally heritable. It was shown in *Drosophila* that

heat shock and osmotic stress disrupt a transcriptionally silenced chromatin state called heterochromatin (Seong *et al*. 2011). This effect of stress lasted multiple generations and was inherited in a non-Mendelian fashion. The change of chromatin structure seemed to be crucial for the stress-induced epigenetic inheritance, independent of RNAi mechanisms.

In contrast to animals, plants as sessile autotrophs cannot escape from local acute stresses. Therefore, rapid and adequate response to environmental changes may be of even more fundamental importance to them. Quick adaptation of the epigenetic status could be a key component of their flexibility. Epigenetic mechanisms were shown to be involved in response to different acute abiotic stresses in plants, such as extreme temperatures, light conditions, water and nutrient availability. However, so far no profound evidence has been found that acquired stress-induced epigenetic changes fulfil all criteria to claim heritable effects in plants (Pecinka and Mittelsten Scheid 2012).

In plants, the most extensively studied effect among abiotic stresses involving large-scale chromatin reorganisation is induced by heat (Pecinka *et al*. 2010). It is important to understand the impact of heat on plants, because temperature plays a major role in determining crop yield, and temperature increase due to climate changes is a factor with global relevance. Although *Arabidopsis thaliana* is not a crop plant, it has many advantages as a model organism to study heat-induced chromatin changes, since numerous different ecotypes collected from all over the world might respond differently to heat.

1.2 Chromatin organisation

In eukaryotes, DNA is wrapped around histone proteins forming a higher order structure called chromatin. A single unit of the chromatin, called nucleosome, consists of 147bp of DNA wrapped around a histone octamer. The octamer consists of two H2A-H2B dimers and a tetramer of H3-H4. Histone H1 mediates higher chromatin condensation by associating with linker DNA between nucleosomes. Diverse protein complexes act as histone chaperons that mediate nucleosome assembly/disassembly during replication, repair or epigenetic regulation. Exchanging canonical histones with variants, adding posttranslational modifications (PTMs),

movement of the histones relative to the DNA, or removing them from the DNA can alter nucleosome properties and accessibility of the DNA for other proteins as shown in Figure 1 (Zhu *et al*. 2012). The amino terminal tails of the histones are likely to be modified posttranslationally, such as lysine and arginine methylation, lysine acetylation, serine and threonine phosphorylation, lysine ubiquitination. These modifications can influence the interaction between DNA and histones by changing the electrostatic constitution. Furthermore, they serve as recognition sites for binding factors that can exert additional changes of chromatin structure or gene activity. The accessibility of the DNA and the presence of transcription factors decide about the transcriptional activation of a gene, together with its regulatory regions. A transcriptionally active state is referred to as euchromatin, which is mostly concomitant with activating epigenetic marks, such as histone acetylation, histone 3 methylation at lysine 4 (H3K4me1, 2 or 3) and little or no DNA methylation. Euchromatin is usually decondensed to ensure accessibility for transcription factors and polymerases and comprises of gene-rich sequences. In contrast, heterochromatin contains regions of a transcriptional silent state. Heterochromatin is associated with dense DNA methylation at cytosine residues (mC) and deacetylated histone H3 but dimethylation of lysine 9 of H3 (H3K9me2) (Vaillant and Paszkowski 2007; Roudier *et al*. 2009). Short interference (si) RNAs are also associated with heterochromatin formation (Kanno and Habu 2011). Heterochromatin is organised in cytologically visible structures called chromocenters, which contain mainly repetitive DNA, like telomeric and centromeric regions and part of the ribosomal repeats.

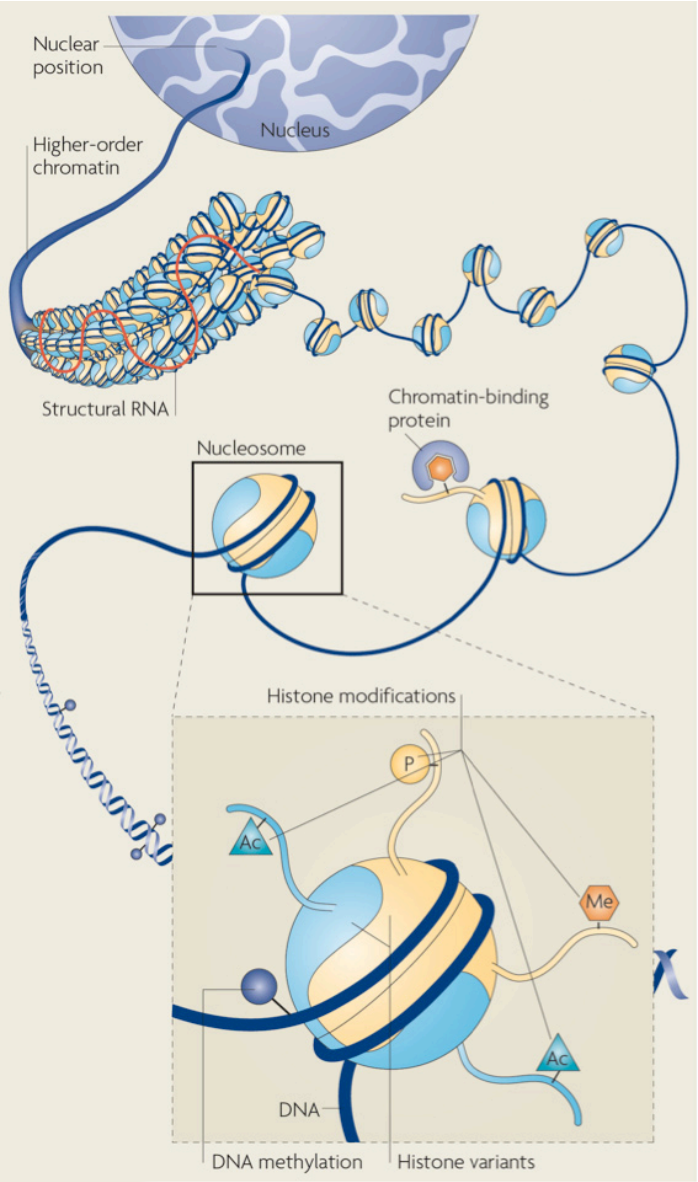

Figure 1. Epigenetic marks influence the organisation of chromatin.
DNA methylation, histone modification marks, histone variants, chromatin binding proteins and chromatin remodeller determine the accessibility of the DNA (Probst et al. 2009).

1.3 Stress response of chromatin in *Arabidopsis thaliana*

Stress-induced histone tail modifications can involve a stress-specific response or prepare for faster or stronger transcription reviewed in (Gutzat and Mittelsten Scheid 2012). For instance, drought stress induces increased acetylation marks at the regions of drought stress-responsive genes (Kim *et al*. 2008). A transient up-regulation of H3 phosphoacetylation and histone H4 acetylation were

shown under salinity and cold stress (Sokol *et al*. 2007). Genome-wide histone H3K4 methylation was observed in response to dehydration (van Dijk *et al*. 2010). Histone deacetylation by histone deacetylase HDA6 was shown to be required in freezing tolerance (To *et al*. 2011).

Beside the genes encoding canonical histones, which are highly conserved and mostly expressed during S phase of the cell cycle, genes for histone variants are expressed throughout the cell cycle. Similar to histone modifications, histone variants are found at specific regions or loci of the genome. By incorporation into nucleosomes they can change structural or transcriptional features of the region. The most studied histone variants are H3 and H2A subtypes (Zhu *et al*. 2012). For example, H3 variant CenH3 is located at centromeric regions and important for chromosome segregation. H3.3 is incorporated predominantly within promoters and regions that are transcriptionally active (Deal and Henikoff 2011). Although suggested, an involvement of the H3 variant H3.3 in stress response has not been proven. H2A variants were shown to be involved in DNA repair pathways and stress response. Incorporation of H2A.Z into the region close to the transcription start site of genes poises heat gene expression in an inducible manner (March-Diaz and Reyes 2009). Recently, it was demonstrated, that lack of H2A.Z incorporation at ambient temperatures mimics the change of gene expression under increased temperatures (Kumar and Wigge 2010). In response to higher temperatures, H2A.Z gets lost from regions of heat response genes, which become up- or down-regulated. A similar reaction occurred in response to phosphate starvation, where H2A.Z got evicted from promoter regions of phosphate starvation response (PSR) genes, followed by their transcriptional activation (Smith *et al*. 2010). H2A.Z and H3.3 are also often found in double-variant nucleosomes at transcriptionally active loci and are less stable than in nucleosomes with single variants (Zhu *et al*. 2012). Additionally, H1 linker variants were shown to be expressed under drought stress in *Arabidopsis* (Zhu *et al*. 2012).
Large-scale chromatin reorganisation occurs not only under stress, but also under certain developmental and physiological conditions. Loss of chromocenter organisation corresponds with floral transition, seedling differentiation, ageing of rosette leaves, dedifferentiation of protoplast formation, and pathogen infection (Mathieu *et al*. 2003; Tessadori *et al*. 2007; Tessadori *et al*. 2007; van Zanten *et al*. 2012). Also in other plants, loosening of chromocenters and hypomethylation was observed in response to biotic stress, for instance in tomato upon infection by

Pseudomonas syringae (Pavet *et al*. 2006). Furthermore, natural variation in chromatin organisation found in 21 different *Arabidopsis* ecotypes correlates with latitude of origin and is dependent on light intensity. Photoreceptor PHYTOCHROME-B (PHYB) and histone modifier HDA6 were shown to control light-dependent chromatin status. Sequence polymorphism in PHY-B gene and HDA6 promoter was found to be responsible for lower chromatin compaction in the Cape Verde Islands (Cvi-0) ecotype in comparison to others (van Zanten *et al*. 2010) (Tessadori *et al*. 2009). Nevertheless, a significant loss of chromocenter organisation occurs also in response to prolonged heat stress in Columbia-0 ecotype (Pecinka *et al*. 2010).

1.4 Heat stress interference with epigenetic regulation

Together with the general loss of chromocenter organisation upon prolonged heat stress in *Arabidopsis thaliana,* epigenetic regulation was disrupted resulting in manifold gene expression changes and transcription of otherwise silent transposable elements(Pecinka *et al*. 2010; Tittel-Elmer *et al*. 2010). Interestingly, this was concomitant with only minor changes in histone modification and no change in DNA methylation, but with loss of nucleosome occupancy and release of transcriptional gene silencing (TGS) as shown in Figure 2. (Pecinka *et al*. 2010). Loss of chromocenter organisation was shown for heterochromatic repeats, e.g. the 180bp repeats highly represented in centromeres. The chromocenters of differentiated cells of ecotype Columbia became dispersed significantly after extended heat stress and did not recover for the subsequent 7 days. In contrast, meristematic tissue seemed to be protected from that effect (Pecinka *et al*. 2010).

The loss of nucleosome occupancy occurred on multiple loci and was not restricted to regions of transcriptional activation. Reloading of nucleosomes required the CHROMATIN ASSEMBLY FACTOR 1 (CAF-1) complex (Pecinka *et al*. 2010). In wild type plants, the nucleosome dissociation was transient, but mutant plants lacking subunits of CAF-1, *fas1* and *fas2*, showed no reloading even after 7 days of recovery at ambient temperature, and a lasting transcriptional activation of TGS targets.

Several repeats known to be under control of TGS showed induced expression under long heat stress conditions. TGS is a mechanism controlling

repression of repetitive elements and heterochromatinization. Reactivation of transposable elements (TE) causes a threat to genome integrity via reintegrating of new copies (reviewed in Mirouze and Paszkowski 2011). Other genes found to be expressed under extended heat include the multicopy transgenic reporter gene GUS in line L5 (Morel *et al*. 2000), TRANSCRIPTIONALLY SILENT INFORMATION (TSI, an endogenous family of ATHILA related retrotransposons) and COPIA78 (an LTR retrotransposon family with 8 copies in the Columbia ecotype). Interestingly, transcript levels of all TEs returned to pre-stress levels upon recovery, with the exception of COPIA 78 showing delayed recovery. Delayed silencing after heat exposure was further observed for TSI in *fas1* and *fas2* mutants. This again indicates that nucleosome loading rather than histone modification is important in heat response.

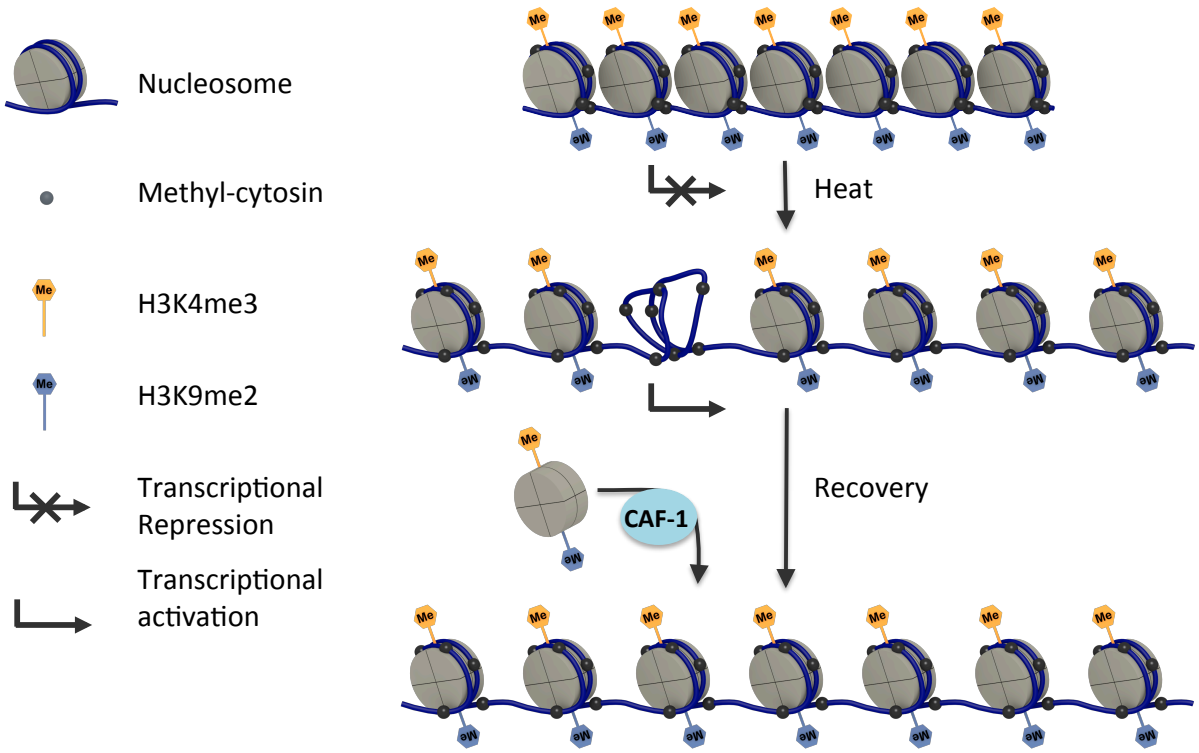

Figure 2. Summary of heat stress effects in *Arabidopsis thaliana*.
Release of TGS is concomitant with chromatin decondensation, loss of nucleosome occupancy as a result of lasting heat stress (Pecinka *et al*. 2010). DNA methylation and histone modifications are barely effected. CAF-1 is necessary for nucleosome reloading.

In summary, a complex interplay of diverse factors including histone variants, modifications, chaperons and nucleosome loading is likely to be involved in the response to stress, as exemplified by extended heat exposure. Different other stresses were also investigated for release of transcriptional gene silencing. Some transcriptional activation and histone dissociation was shown for UV-B radiation (Lang-Mladek *et al*. 2010; Pecinka *et al*. 2010). Stresses showing no response in this regard include UV-C irradiation (3000 J/m³), salinity, freezing at -4°C for 24 h in dark and at -20°C, drought (Lang-Mladek *et al*. 2010; Pecinka *et al*. 2010; Tittel-Elmer *et al*. 2010), however, several factors relevant for plants were not tested.

1.5 Aim of this work

In this work I describe experiments to investigate the possible interference of stress factors like phosphate starvation, high and low light intensity and oxidative stress with epigenetic regulation, using the transcriptional activation of previously characterized TGS targets as indicators. Furthermore, I analyse diverse factors influencing the heat stress response, including the light regime and light duration during heat exposure. I also established an automatic way to quantify heterochromatin decondensation, which allows correlating it with transcriptional activation in different ecotypes.

2 Materials and Methods

2.1 Plant material

Arabidopsis thaliana line L5 containing a single multicopy insert of P35S:GUS in Columbia-0 (Col-0) background (Morel *et al*. 2000), line L5 ddm1 - L5 crossed with *ddm1-5* mutant in Zh background (Mittelsten Scheid *et al*. 1998). The following wild type ecotypes were used: Columbia (Col-0), Cape Verde Islands (Cvi-0), Landsberg erecta (Ler-1) and Marturba (Mt-0).

2.2 Growth conditions and stress treatments

Prior to high light, low light, heat and oxidative stress treatments, plants were grown in vitro on germination medium (GM) for 21 days under long day (16 hours light /8 hours dark) conditions at 22°C subjected to a light intensity of 30 µmol m^{-2} s^{-1}. For phosphate starvation stress treatments, plants were grown for 4 weeks on GM medium containing 0, 6, 60 and 600 µmol PO_4^{-2}, whereby 600µl is the concentration in the GM medium. For high light (94 µmol m^{-2} s^{-1}) and low light (4 µmol m^{-2} s^{-1}) treatment, plants were put to a Percival growth incubator for 4 days. Paraquat, dissolved in liquid GM in a concentration of 2 µM, was applied to seedlings for 30 h. Heat stress treatments were performed in Percival growth incubators as described in the Results section.

2.3 Seed sterilization

Seeds corresponding to a volume of approximately 50 µl were aliquotted into a 2 ml Eppendorf tube and openly placed in a plastic box together with a beaker with 100 ml 10% sodium hypochlorite solution. Additionally, a cut Falcon tube containing 10 ml concentrated HCl was inserted into the beaker. The lid of the box was closed and the box gently shaken, so that the content of the Falcon tube was poured into the hypochlorite solution. Mixing of the two solutions lead to the generation of chlorine gas, which sterilised the surface of the seeds during an incubation period of 15 to 20

minutes. Sterilised seeds were stored at room temperature or used directly after sterilisation.

2.4 Germination medium (GM)

Stock solutions were prepared and afterwards filter-sterilized. One litre of MS macro contained 19 g KNO, 16.5 g NH_4NO_3, 4.4 g $CaCl_2$ x H_2O, 3.7 g $MgSO_4$ x 7 H_2O, 1.7 g KH_2PO_4. For 100 ml of B5 micro, 1 g $MnSO_4$ x H_2O, 300 mg H_3BO_3, 200 mg $ZnSO_4$ x 7 H_2O, 75 mg KJ, 25 mg Na_2MoO_4 x 2 H_2O, 2.5 mg $CuSO_4$ x 5 H_2O and 2.5 mg $CoCl_2$ x 6 H_2O were mixed. MS vitamin consisted of 5 g m-inositol, 100 mg glycine, 50 mg thiamine, 25 mg pyridoxine and 25 mg nicotinic acid. Five gram ammonium iron citrate were dissolved in 500 ml. Fourteen gram MES were dissolved in 100 ml H_2O and adjusted to pH 6.0. For the final GM, two solutions were prepared using the stock solutions. Solution 1 consisted of 50 ml MS macro, 1 ml B5 micro, 5 ml ferric citrate, and 10 g sucrose in a volume of 100 ml H_20. Solution was adjusted to pH 5.6 and filter-sterilized. Solution 2 was prepared with 4 g Merck agar and 2.5 ml MES. It was filled up with H_2O to 450 ml and autoclaved. Fifty millilitre of solution 1 and 450 ml of solution 2 were mixed for the final GM.

2.5 GUS histochemical staining

GUS solution consisting of sodium phosphate buffer (pH=7, 100 mM), EDTA (10 mM), Triton X-100 (0.1% (w/v)), chloramphenicol (100 µg/ml), potassium ferrocyanide (2 mM), potassium ferricyanide (2 mM) and X-glucuronide (0.5 mg/ml in dimethylformamide) was mixed and the volume adjusted to 500 ml with water. The solution was filter-sterilised and kept at 4°C in the dark. For staining, seedlings were transferred to 15 ml Falcon tubes and covered with GUS solution. For infiltration, the Falcon tubes were exposed to vacuum in a desiccator. A nylon mesh kept seedling in the solution during infiltration. The seedlings were incubated in GUS solution over night at 37°C. After 24 hours incubation, the GUS solution was exchanged by 70% EtOH and seedlings incubated for another 24 h at 37°C. A repetition of clearing via ethanol was performed if necessary. Pictures were acquired with Leica Mz Apo stereomicroscope.

2.6 RNA extraction

About ≤ 100 mg tissue (4 to 5 seedlings) was harvested and immediately frozen in liquid nitrogen. The tissue was stored at -80°C till I proceeded.
RNA extraction was performed using Qiagen RNeasy Plant Mini Kit (Catalog no. 74904) according to the kit protocol. To remove residual DNA, the on-column DNAse digest for RNA was performed (Fermentas, #EN0251). RNA was eluted in 30 µl and concentration measured with the Nanodrop photometer. For storage, RNA was frozen at -80°C.

2.7 cDNA synthesis for qRT-PCR

Adapted from Nicole Lettner

Previous to reverse transcription, DNAse (Fermentas, #EN0251) digest was performed with an incubation period of 30 min at 37°C. Additionally, Ribo Lock^{TM} RNase Inhibitor (Fermentas, #E00381) was added.

Reaction Mix for DNAse treatment:

2.5 µg RNA + H_2O	18.75 µl
+ 10 x buffer with $MgCl_2$	2.5 µl
+ RNAse Inhibitor	1.25 µl
+ DNAse	2.5 µl
Total volume	25 µl

The reaction was stopped by adding 2.5 µl EDTA (25 mM) to each tube. Samples were heated to 65°C for 10 minutes and afterwards cooled on ice.

cDNA Transcription mix:

RNA after DNAse treatment	27.5 µl
+ H_2O	2.5 µl
+ RNAse Inhibitor	1.5 µl
+ Random Hexamer Primers (Fermentas, #S0142)	2.5 µl
+ 5 x buffer	10 µl
+ dNTPs 10 mM each (Fermentas, #R0192)	5 µl
Total volume	49 µl

For reverse transcription, the cDNA transcription mix was added to the samples. To test for genomic DNA contamination, 10 µl of each reaction mix were transferred to a new reaction tube as a control. No reverse transcriptase was added to these controls.

To the remaining 39 µl, 1 µl of the RevertAid^{TM} H Minus M-MuLV Reverse Transcriptase (Fermentas, #EP0451) was added. Both reactions were incubated as follows: 10 minutes at 25°C, 90 minutes at 42°C, 10 minutes at 70°C. To test for contaminations of genomic DNA I used UBC28q-F and R Primers (At1g64230) in a standard PCR. The reactions conditions for the test PCR for reverse transcription comprised of a denaturing step at 94°C for 30 s, an annealing temperature at 60°C and an extension time at 72 °C for 1 min s in a repetition of 30 cycles. I always used water for a negative control. For the control samples without reverse transcriptase, I used an extension time of 1 min and ran 40 cycles. To check for purity of the DNA, the samples were loaded on a 1% agarose gel in TAE buffer. Expected band sizes are 124 bp for cDNA and 920 bp if contaminated with genomic DNA, due to the presence of an intron.

2.8 Polymerase chain reaction (PCR)

For standard PCR I used 5 PRIME Taq DNA Polymerase in a reaction mix of 10.8 µl dH_2O, 2 µl 10x buffer, 2 µl dNTPs, 2 µl forward primer, 2 µl reverse primer, 0.2 µl Taq Polymerase. One microliter of template was added. The PCR was performed in a thermocycler for 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min.

2.9 Quantitative real time polymerase chain reaction (qRT-PCR)

qRT-PCR analysis was performed in technical triplicates by using SensiMix ™ SYBR& Fluorescein Kit by BIOLINE and iQ5 equipment (Bio-Rad). Expression values were calculated according to Pfaffl (2001) and normalized to values of the expression of UBC28 (AT1G64230) or EIF4A (AT3G13920), genes for which expression did not change significantly under the respectively applied stress conditions. Data analysis was performed with Bio-Rad iQ5 software (Bio-Rad) and Excel (Microsoft).

2.10 Fluorescence in situ hybridization (FISH)

Preparation of *Arabidopsis* leaf nuclei by Cytospin (MPW technical instruments)

Four to five leaves were harvested and washed in distilled water in a 15 ml tube twice for 5 minutes. The samples were kept on ice throughout the preparation. Fixation was performed in cold 4% formaldehyde (6 ml 37%formaldehyde, 50 ml water) for 15 min under vacuum. Afterwards the leaves were washed three times for 5 min with distilled water. To isolate the nuclei the leaves were cut with a razor blade in 250 µl of chromosome isolation (CI) buffer (15 mM Tris, 2 mM EDTA, 0.5 mM Spermin, 80 mM KCl, 20 mM NaCl, 15 mM Mercaptoethanol, 0.1% Triton X-100, pH 7.5). Additional 250 µl of CI buffer were added. The suspension was filtered through a 32-µm-nylon mesh and collected in flow-sorting tubes. They were kept on ice until centrifugation.
Slides were mounted into the Cytospin devices. Hundred microliter of the suspension and 300 µl CI buffer were mounted to the sample chamber and centrifuged in the Cytospin for 5 minutes at 2500 rpm. The slides were washed shortly in ice cold 1% PBS and finally stored in 50% glycerol /1%PBS at -20°C until use.

Probe preparation

Genomic DNA from *Arabidopsis thaliana* Columbia-0 (kindly provided by Laura Sedman) was used for the preparation of the labelling probe using ALU/ALR primers for the 180bp repeats (see Table X).

Reaction mix for PCR labelling of probe:

DNA	1 µl
10X buffer	5 µl
2 mM dNTPs (-dTTP)	5 µl
1 mM dTTP	7.5 µl
Labelled dUTP (1mM)	2.5 µl
10 mM primer pair	5 + 5 µl
H_20	18 µl
Taq	1µl
Total volume	50 µl

The DNA probe was EtOH precipitated by adding 1/10 of the sample volume NaAc (3M pH=5.2) and 2.5-3.0 times the sample volume 95% EtOH. Samples were kept on ice for 15 min. Subsequently the solution was centrifuged at 13400 rpm for 30 min at room temperature. The supernatant was discarded and the pellet washed with 70% EtOH. After centrifugation for 15 min, the supernatant was discarded and the pellet air-dried. The pellet was dissolved in deionized formamide. The DNA probe was stored at -20°C.

Slide pre-treatment

Slides were washed in 2xSSC and treated with RNase. Therefore, 16.1 µl RNase A (10 mg/ml) diluted in a total volume of 100 µl 2X SSC per slide were added and incubated for 30 min at 37°C in a moist chamber. The slides were washed in 2xSSC for 10-15 min. To remove remnants of cytoplasm, pepsin (ROCHE, cat. no. 108 057) treatment was performed. Hundred µl of pepsin (10 mg/ml in 10 mM HCl) were diluted in 50 ml of 10 mM HCl. HCl was preheated at 38°C and the pepsin dissolved

just before adding. The treatment of slides lasted 3 min at 38.0°C. Furthermore the slides were rinsed twice in 2xSSC for 5 minutes. For fixation the slides were incubated in 4% formaldehyde in 2xSSC for 10 min (50 ml 2xSSC, 6 ml formaldehyde). The slides were washed twice in 2xSSC, each time for 5 min. For dehydration, the slides were transferred subsequently to 70%, 90% and 100% ethanol; each step lasting 1 -2 min. The slides were air-dried.

Hybridization

The labelling probe was diluted in deionized formamide up to a volume of 10 µl depending on the DNA concentration and added to 10 µl 20% Dextran sulphate in 4%SSC. Twenty microliter of mixture containing the labelling probe were applied per slide and the slides were incubated at 80°C for 2 min for denaturing. The slides were further incubated for hybridization in a moist chamber at 37°C overnight.

Detection of biotin-labeled probes

Previously to the treatment, SF50, 2xSSC, 4T and TNT solutions were prepared (see recipes below) and preheated at 42°C in a water bath. For incubation of 100 µl solutions, 24x32 mm coverslips were used. The slides were washed 3 times for 5 min in SF50 at 42°C, twice for 5 min in 2xSSC at 42°C and furthermore for 5 min in 4T buffer at 42°C. They were incubated in blocking buffer (BB, recipe below) at 37°C for 30 min and subsequently rinsed in 4T at 42°C. They were then incubate in Avidin~Texas Red (1:1000, 0.1 µl/100 µl) in BB at 37°C for 30 min. Afterwards they were washed in 4T 2x5 min at 42°C and additionally in TNT for 5 min at 42°C. The slides were incubated in goat-anti-avidin~biotin (1:200, 0.5 µl/100 µl, Vector Laboratories) diluted in incubation buffer (IB, recipe below) at 37°C for 30 min. Again they were washed with TNT three times for 5 min at 42°C. They were incubated a second time in Avidin~Texas Red (1:1000, 0.1 µl/100 µl) in IB at 37°C for 30 min.
Finally the slides were washed in TNT, 3x5 min at 42°C and dehydrated in an ethanol series of 70%, 90% and 100%. Each step lasted 2 min and afterwards the slides were air-dried in darkness. Finally, DAPI staining was performed to mark the area of the whole nucleus by adding 10 µl of DAPI-Vectashield (2 µg/ml). The slides were stored at 4°C.

Buffers used for FISH

Incubation Buffer (IB) consists of 1% BSA (0.1 g BSA), 0.1% Tween-20 (10 µl Tween-20) and 4x SSC (2 ml 20x SSC). The volume was adjusted to 10 ml with sterile H_20 and filter-sterilized. Blocking buffer (BB) was prepared by dissolving 5% BSA (0.5 g BSA), 0.2% Tween-20 (20 µl Tween-20) and 4xSSC (2ml 20xSSC) in a total of 10 ml water. For 1 litre of 4T buffer, 200 ml of 20x SSC with 0.5 ml Tween-20 were mixed and adjusted to 1 litre. For 10x TN (1M Tris-HCl, 1.5M NaCl, pH 7.5), 121 g Tris and 87.4 g NaCl were dissolved in water, 60 ml of 37% HCl added and the volume adjusted to 1 litre. For buffer TNT, 100 ml of 10x TN with 0.5 ml Tween-20 were mixed and the volume adjusted to 1 litre. SF50 consists of 50% formamide/2xSSC at pH 7.0, 150 ml formamide, 30 ml 20xSSC and 120 ml ddH_20 (pH 7.0). For 1 litre 20x SSC (pH 7.0), 175.3 g NaCl and 88.2 g sodium citrate were dissolved in H_20. For 10x PBS, 80 g NaCl, 2 g of KCl, 14.4 g Na_2HPO_4 and 2.4 g KH_2PO_4 were dissolved in 1 litre dH_20 (pH 7.4).

2.11 Microscopy

An inverted wide-field microscope (Zeiss Axiovert 200M) for fluorescence with a motorized xyz-stage, a CCD camera (Photometrics) and HBO self-adjusting 50W was used to take pictures of the nuclei in an automatic way. Objective 63x/1.4 plan-apochromat Oil DIC was used. The MetaMorph analysis software controls the components of the microscope. Using a MetaMorph journal it was possible to automatically acquire at least 200 nuclei per slide/time point.

Making use of the MetaMorph software, the settings for acquisition were adapted to my conditions in the “Acquire” main dialog box. At first, I adjusted the focus on the nuclei on the DAPI channel using the live imaging function. For calibration of the exposure time for DAPI (λ_{max} = 405nm) and Texas Red (λ_{max}= 545nm), the “acquisition multiple wavelength” function was used. By pointing the mouse arrow over the brightest point of the acquired image, the grey scale values (on a 16-bit scale) become visible. The highest grey scale value should not exceed 16000 for the DAPI signal and should be approximately 3500 for the Texas Red signal. To adjust further options, the function “setup-name-screen” was selected. The folder for the subsequently acquired pictures was labelled and its directory selected. Furthermore,

the scan stage was programmed according to area and density of the fixed nuclei. The stage should move to relative positions in a vertical “Zig-Zag” pattern, using the coordinates for x= -100, y= -140. The number of scans following this pattern should be repeated 18 times in column and row. After all adjustments were made, a journal (Figure 3) was selected and the scan started. The journal loads the conditions to acquire an image using the current settings specified in the Acquire main dialog box. It differentiates between objects and other parts of an image based on the image's grey scale.

Pictures were taken for both DAPI and the Texas Red signals and scanned at 324 different positions on the slide. The high number of scans was necessary due to variable density or irregularity of the fixed nuclei.

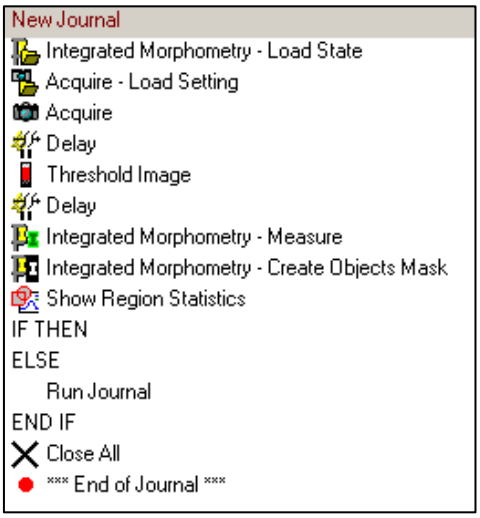

Figure.3: Screen shot shows the journal performed by MetaMorph for automated picture acquisition.

2.12 Image analysis by Definiens

To analyse the large amount of images taken, Definiens software was used. Top-hat filtering was applied to remove background and to focus on the signal of the whole nucleus on the DAPI layer. Detection of nuclei was done by intensity thresholds. Background subtraction was performed on the red channel. For detection of the whole nuclei, the borders were stretched on the red layer to include the red signals. A

filter was applied to exclude objects, which were too big, with a threshold of 1000 px. Another threshold was set so that objects with pixels below a selected percentage of the maximum light intensity (DAPI intensity less than 1000) were removed.
Objects likely to be cut off by the image border were deleted along with objects where no red signal was detected. Serial processing of the identified objects included background subtraction in red to balance intensity differences. Furthermore, an intensity threshold was set for object borders of heterochromatic (red) regions. For statistical analysis, the ratio of the area of the whole nucleus and the area of heterochromatin was calculated using the software Excel (Microsoft) and GraphPad Prism 5.

2.13 Primers used in this study

Table. 1: Primer list

Target	ORF	Primer name	Sequence(5´-3´)	Application
Actin2	AT3G18780	ActinF	TCC CTC AGC ACA TTC CAG CAG AT	qRT-PCR
		ActinR	AAC GAT TCC TGG ACC TGC CTC ATC	
Actin7	At5g09810	Actin7qF	TGGTGATGAAGCTCAGTCCA	qRT-PCR
		Actin7qR	TACATGGCAGGGACATTGAA	
COPIA78	multiple	COPIA78qF2	CGGTGCTCACAAAGAGCAACTATG	qRT-PCR
		COPIA78qR3	ATCCTTGATAGATTAGACAGAGAGCT	
CYC=ROC3	AT2G16600	ROC3F329cyc	GATGGGAAACATGTTGTGTTTG	qRT-PCR
		ROC3R518cyc	AAAGCTACCATTGGATCCTCAA	
EF1-α	AT5G60390	EF1α F	TGAGCACGCTCTTCTTGCTTTCA	qRT-PCR
		EF1α R	GGTGGTGGCATCCATCTTGTTACA	
EIF4A1	AT3G13920	TIF F2	ATC CAA GTT GGT GTG TTC TCC	qRT-PCR
		TIF R2	GAG TGT CTC GAG CTT CCA CTC	
GUS	-	qPCR-GUS-F	TTAACTATGCCGGAATCCATCGC	qRT-PCR
		qPCR-GUS-R	CACCACCTGCCAGTCAACAGACGC	
TSI	-	TSIqF	CTCTACCCTTTGCATTCATGAATCCTT	qRT-PCR
		TSIqR	GATGGGCAAAAGCCCTCGGTTTTAAAATG	
UBC10	AT5G53300	UBC10 F	GACCAAGGTGTTCCATCCCAAC	qRT-PCR
		UBC10_R	GGAAATGGTGAGCGCAGGAC	
UBC28	AT1G64230	UBC28qF	TCCAGAAGGATCCTCCAACTTCCTGCAGT	cDNA
		UBC28qR	ATGGTTACGAGAAAGACACCGCCTGAATA	
		UBC28q_2_newF	AGGCGGTGTCTTTCTCGTAACC	qRT-PCR
		UBC28q_2_newR	TAGGGTGGAACACTTTTGTCCTGAA	
VSP2	AT5G24770	VSP2_qF	GACTTGCCCTAAAGAACGACACC	qRT-PCR
		VSP2_qR	CTCCGGTCCCTAACCACAACC	
180-bp	-	ALR	TGG ACT TTG GCT ACA CCA TG	FISH
		ALU	AGT CTT TGG CTT TGT GTC TT	

3 Results

3.1 TGS reactivation is most prominent upon heat stress

Previous work had provided strong evidence for interference with epigenetic regulation by prolonged heat stress, while other abiotic stresses were not extensively studied (Lang-Mladek *et al*. 2010; Pecinka *et al*. 2010; Tittel-Elmer *et al*. 2010). In a natural environment, stresses rarely appear isolated from each other, and therefore combined effects are possible. Therefore, I tested other stress types, and I have chosen high and low light intensity, phosphate starvation and oxidative stress.
As for the heat experiments, I used the GUS reporter gene system in the line L5 of *Arabidopsis thaliana*, which allows observing release of TGS in a tissue-specific manner. The line contains a multicopy transgene encoding the 35S promoter of the Cauliflower Mosaic Virus attached to the β-glucuronidase (GUS) gene, which is transcriptionally silenced (Morel *et al*. 2000; Probst *et al*. 2004). When the GUS gene is expressed, for instance under stress conditions or in a mutant background, the encoded protein cleaves the substrate X-Gluc, resulting in a blue product. In addition to the visualization, the degree of transcriptional activation was quantified by real-time reverse transcription polymerase chain reaction (qRT-PCR). To extend the analysis to endogenous repeats, I also analysed the expression of TSI and COPIA78 in a quantitative way.

To compare new experiments to standard conditions, I demonstrated the effects of long heat stress once again with the previously established protocol. I grew L5 plants for 21 days under long day (16 hours light and 8 hours dark) conditions at 22°C with a light intensity of 30 µmol m^{-2} s^{-1} during the light period, according the standard settings in the growth chambers. For long heat stress treatments, seedlings were exposed to 37°C for 30 hours (standard heat, SH). As positive control I used the L5 line in the mutant background of *ddm1* (Vongs *et al*. 1993) where the GUS gene is strongly expressed (Figure 4A). For negative controls the plants remained in the growth chamber under standard conditions (mock), where GUS expression was never detectable.

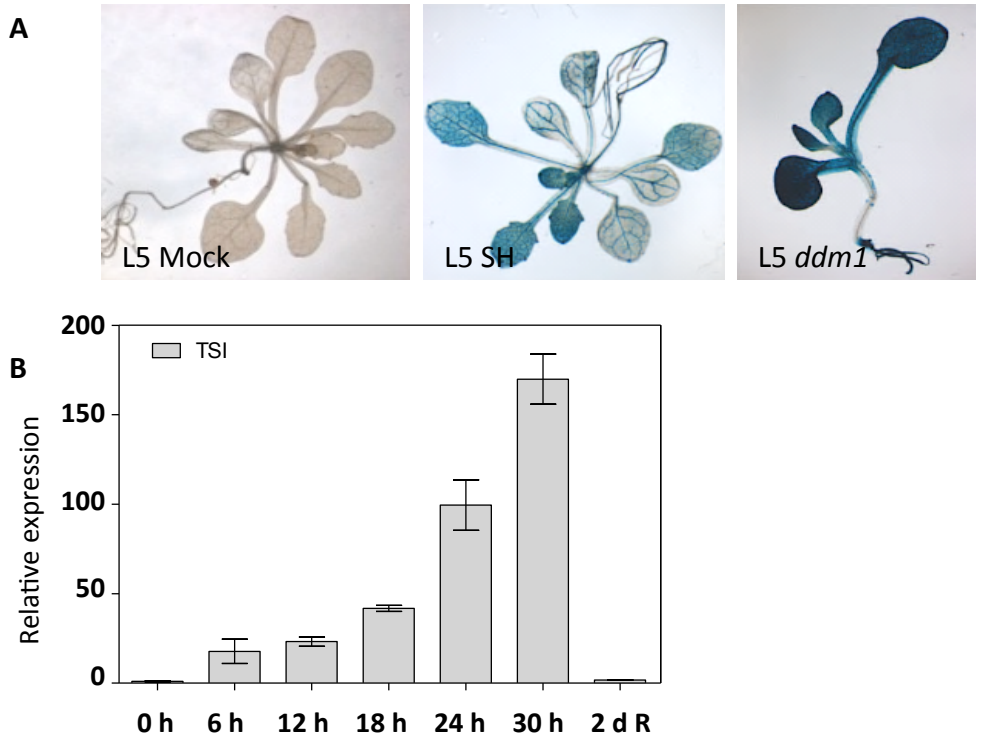

Figure 4 . Prolonged heat stress causes activation of TGS targets.

(A) GUS staining of L5 line plants after mock or standard heat stress (SH) treatment or in *ddm1* mutant background. Line L5 contains a transcriptionally silent p35S::GUS transgene. **(B)** Relative expression of an endogenous TGS target (repeat TSI) in heat-stressed seedlings. Values are normalized to EIF4A1, a gene with equal expression under all conditions tested here. Error bars indicate standard deviation of 3 technical replicates.

However, histochemical staining revealed transcriptional activation of the GUS reporter gene under heat stress conditions, as shown in Figure 4A and in (Pecinka *et al*. 2010). GUS expression is visible in all somatic tissues and is most prominent in the leaf veins, but less pronounced than in *ddm1* mutant plantlets. I also monitored the expression of the TSI retrotransposons sampling every 6 h over the 30 hours of heat stress. This revealed an increase of TSI transcription, which peaks after 30 h. After 2 days of recovery under normal growth conditions, the expression returns to the previously low level. Thus, I confirmed that the long heat stress interferes transiently with epigenetic regulation of several repetitive elements that are usually under control of TGS.

The transcriptional activation of the repeats correlated with a substantial decondensation of the heterochromatin (Pecinka *et al*. 2010), and a similar large-scale chromatin reorganization was shown to occur under low light intensity, also

concomitant with massive changes in transcriptional activity (van Zanten *et al*. 2012). Another common feature of light and temperature stress response is the possible involvement of reactive oxygen species, occurring due to limited photosynthesis efficiency (Rossel *et al*. 2002). Therefore, I decided to include the stress of extreme light intensities into my study of stress-induced release of TGS.

I used 4 µmol m^{-2} s^{-1} for low light stress and 94 µmol m^{-2} s^{-1} for high light conditions. These values were limited by the light range of the available Percival incubators and by the increasing temperature due to higher light intensities. For both conditions and the mock controls, the expression levels of GUS were monitored over a period of 4 days after shifting plants to the new settings. Quantitative analysis revealed no detectable effect of light stresses in the context of TGS release (Table 1). Although I measured a 12 fold up-regulation of expression after 6 hours of high light intensity stress, this up-regulation is likely just a technical variation. Besides, I did not observe GUS staining in any of the plants. (Fig. 2A and B) This lack of response is not due to too mild stress conditions, since qRT-PCR of the Vegetative Storage Protein 2 (VSP2) gene indicated 45-fold up regulation at the end of the stress treatment (Figure 5C) and therefore high light-induced oxidative stress as described by (Rossel *et al*. 2002).

Another frequently occurring natural stress is a limitation of nutrients. To consider the effect of this stress type on TGS targets I chose phosphate starvation. Phosphate is a crucial macronutrient involved in many metabolic reactions, for energy transfer (Yang and Finnegan 2010) and is an important component of nucleic acids. According to literature, the transcriptional response to phosphate starvation can be divided into two segments during on-going stress. An early program lasting from 3 h to 72 h represses biosynthesis of products involved in the use of cytosolic phosphate. The later response starting 7d after stress onset consists of a more specific phosphate deficiency program (Wu *et al*. 2003). I grew line L5 plants on GM media plates containing 0, 6, 60 and 600 µmol PO_4^{-2} (Table 1), in which 600 µmol PO_4^{-2} is the concentration needed for normal growth and therefore used as control. After 30 days of growth I quantified the expression of GUS and COPIA 78 by qRT-PCR (Table 1). Seedlings grown under phosphate depletion showed distinctive and severe growth deficiency but no release of TGS (Figure 5D, and data not shown).

As mentioned previously, Reactive Oxygen Species (ROS) are components accumulating under diverse abiotic environmental stress conditions. To observe the

effects of oxidative stress, separate from others, I applied the viologen Paraquat to seedlings, which produces ROS. Plants were treated with 2 µM Paraquat for 30 hours. No major increase of expression in GUS or COPIA78 was observed under this treatment (Table 1, and data not shown).

In summary, none of the abiotic stresses tested here indicated an alleviation of TGS that was comparable to that observed upon extended heat stress.

Table 1. Test of different stress types for interference with TGS.

Type of stress	Dose	Duration	Fold change	
			GUS	COPIA78
High light intensity	94 µmol/m^2/s^1	6 h	0.86	n.d.
		12 h	1.74	n.d.
		36 h	1.50	n.d.
		60 h	0.69	n.d.
		84 h	0.78	n.d.
		108 h	0.98	n.d.
Low light intensity	4 µmol/m^2/s^1	6 h	11.68	n.d.
		12 h	1.20	n.d.
		36 h	1.34	n.d.
		60 h	1.45	n.d.
		84 h	1.00	n.d.
		108 h	1.00	n.d.
Phosphate starvation	6 µmol	30 d	0.93	2.19
	60 µmol	30 d	1.18	0.70
Oxidative stress	2 µmol PQ	30 h	0.82	2.18

PQ paraquat
n.d. not determined

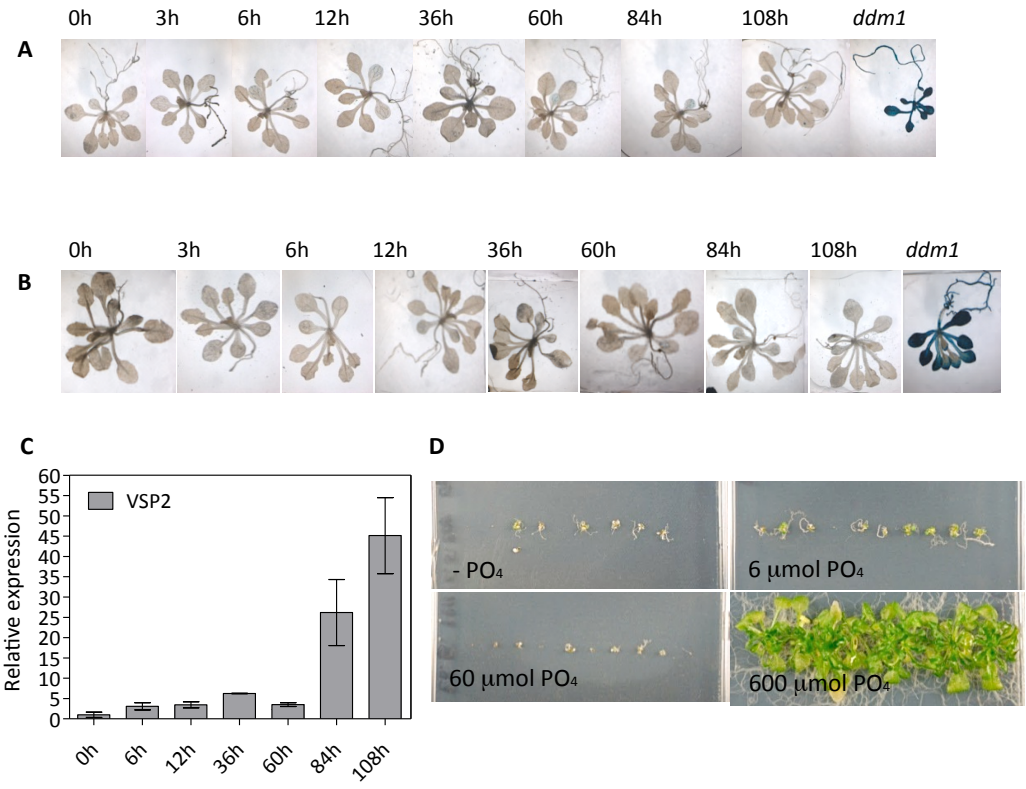

Figure 5. High light and low light intensity have no impact on TGS.
GUS staining of L5 plants after growth under **(A)** high light and **(B)** low light intensity. **(C)** Relative expression of VSP2 as control gene for high light stress. Values are normalized to UBC28, a gene with equal expression under all light conditions. Error bars indicate standard deviation of 3 technical replicates. **(D)** 30 days old seedlings grown on medium containing different concentration of phosphate (no/6/60/600 µmol $PO4^{-2}$).

3.2 The magnitude of heat stress response is influenced by the length of the light period

To gain insight into the regulation of heat stress response I tested potential factors influencing it, predominantly light and the circadian rhythm. Light is the most important energy source for plants; it affects numerous physiological and developmental aspects. Another important regulatory network is the circadian clock. Estimations suggest 16% of gene expression in Arabidopsis seedlings under control of the internal clock (Edwards *et al*. 2006).

During the first set of experiments a standard heat stress treatment of 30 h at 37°C (SH) was defined. Incubation started at the onset of the light phase (6.00 am). In

parallel experiments, the plants were alternatively subjected to the same heat period starting the incubation after a period of darkness of 2 days (DSH), or by changing the light regime during the heat exposure, harvesting the material at the end of the light (SHE) or the end of the dark period (SHM) (Figure 6A). In each case, the expression of GUS, TSI and COPIA 78 was quantified after 30 h. This enables to observe possible involvement of the circadian rhythm in the heat stress regulation.

GUS staining in the plantlets appeared equal regardless of the diverse preceding treatments of the plants (Figure 6B). The expression of all 3 quantified targets in SHE treated plants equalled that of SH. However, DSH and SHM displayed a decreased activation (Figure 6C).

I observed a change of expression of the reference gene UBC28 (AT1G64230), formerly used for normalization, under the diverse light regimes applied during heat treatment. Therefore, I investigated the expression of diverse commonly used housekeeping genes for normalisation under these stress conditions, including ACTIN2 (AT3G18780), ACTIN7 (AT5G09810), ROC3 (AT2G16600), EF1-α (AT5G60390), EIF4A1 (AT3G13920), UBC10 (AT5G53300) (data not shown). EIF4A1 displayed the most stable expression of all tested genes and was subsequently used as new reference gene, including the experiments in Figure 6C.

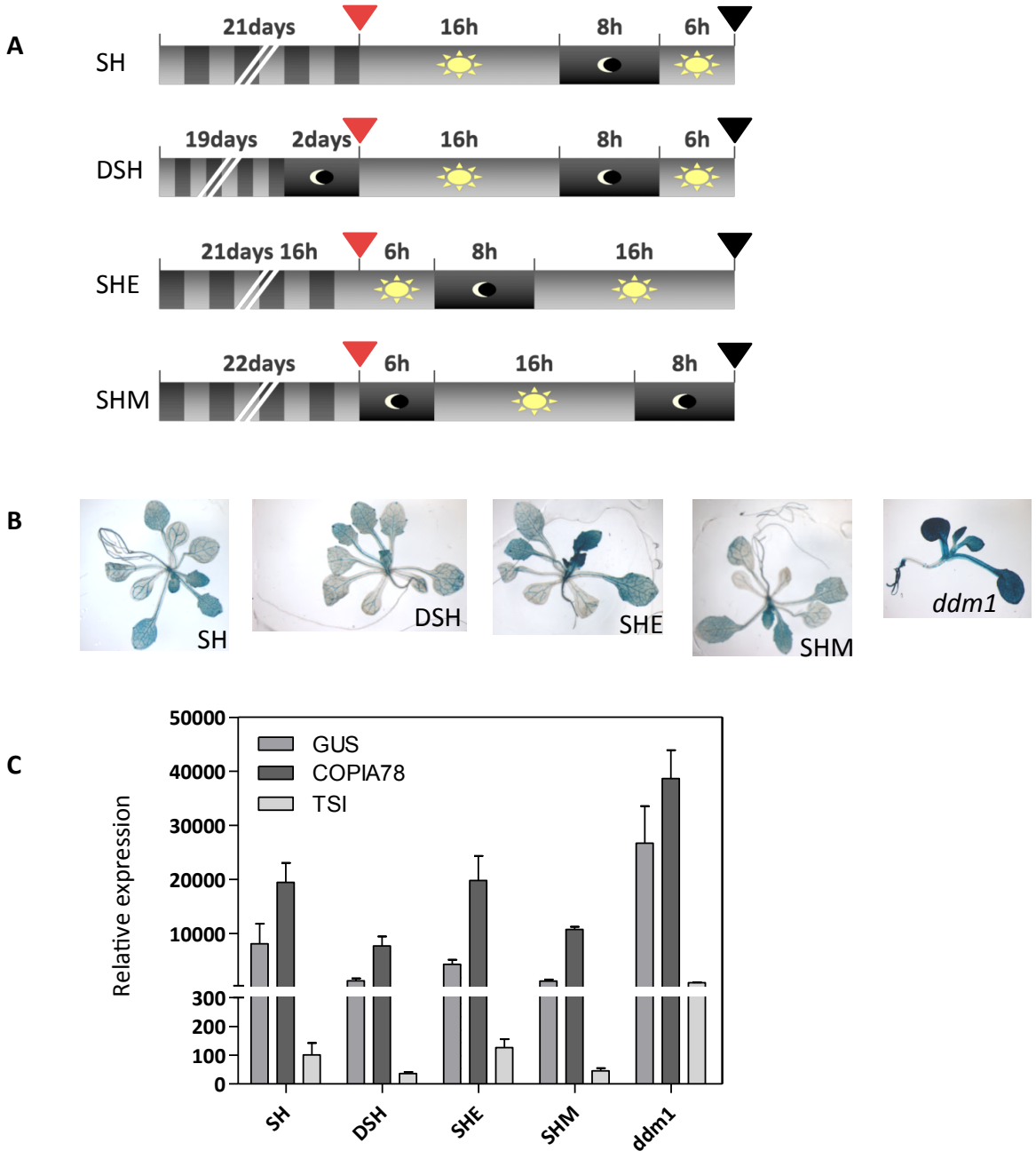

Figure 6. The amount of light influences the magnitude of heat stress-induced expression.

(A) Scheme of light conditions during the 30h of heat treatment. Red arrows indicate the beginning of heat stress treatments; black arrows indicate the harvesting time. SH: standard heat stress with a start at the beginning of the light phase; DSH: 2 days of darkness prior to standard heat stress; SHE: harvesting material after standard heat stress at the end of the light period (evening); SHM: harvesting material after standard heat stress at the end of the dark period (morning). **(B)** GUS staining and **(C)** expression analysis in L5 seedling. **(C)** Quantification of expression of GUS, retrotransposon COPIA78 and TSI by qRT-PCR after

treatments described in A. Values are normalized to EIF4A1, a gene with equal expression under all conditions tested here. Error bars indicate standard deviation of technical triplicates.

The previously described experimental set up included differences in the total hours of light during the heat stress period. SHM setup comprised 6h hours of light less compared to the others. Consequently, I wanted to differentiate whether the light duration or the preceding light period was causing the expression differences during heat stress. Therefore, I exposed the plants to continuous light (LL) or continuous dark (DD) during the heat stress period (Figure 7A). The maximum of expression for all three observed targets (TSI, COPIA 78 and GUS) was achieved during continuous light, even more prominent than after the standard heat treatment. Continuous darkness decreased expression levels compared to standard heat stress conditions (Figure 7C) Histochemical stainings provided a comparable result (Figure 7B). No expression of the TGS targets was seen in seedlings exposed to the same light conditions without high temperature stress.

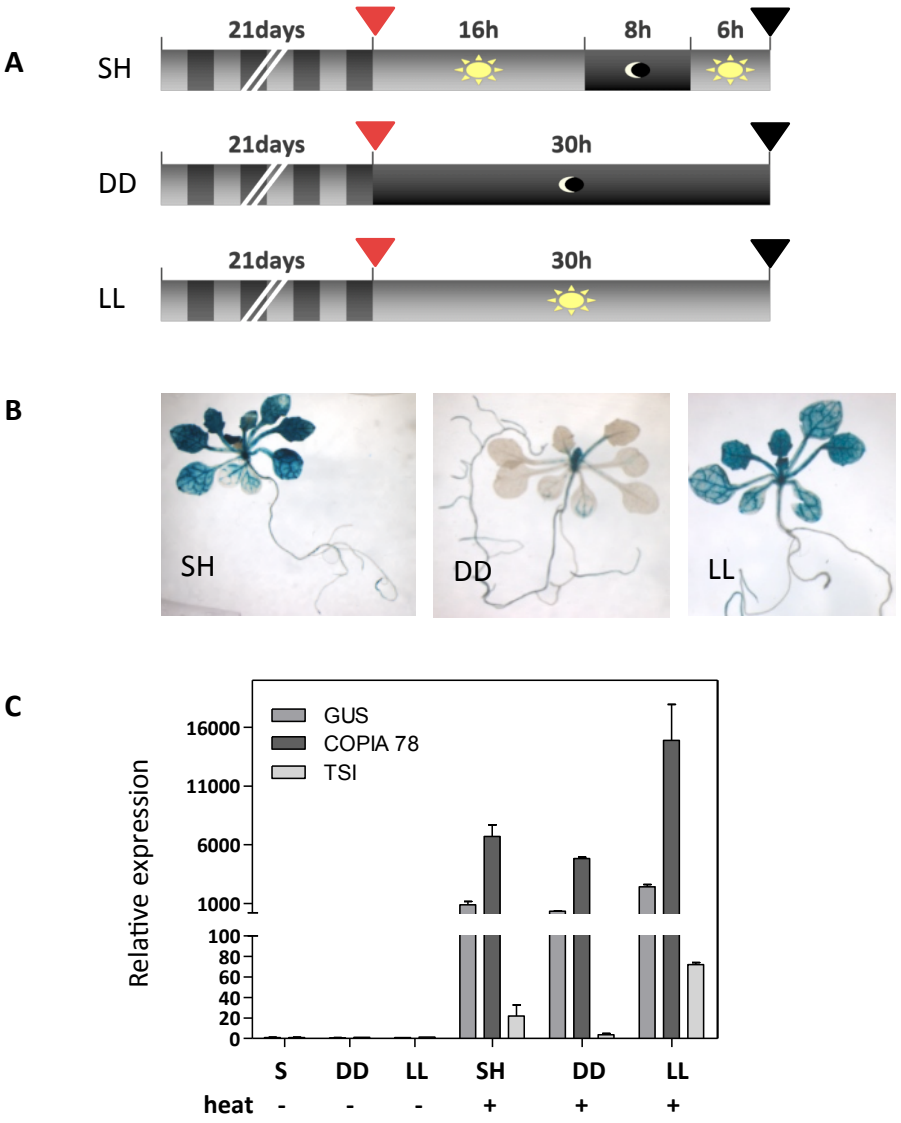

Figure 7. Continuous light increases the magnitude of heat stress-induced expression. **(A)** Scheme of light conditions during the 30h of heat treatment. Red arrows indicate the beginning of heat stress treatments; black arrows indicate the harvesting time. SH: standard heat stress with a start at the beginning of the light phase; DD: continuous darkness; LL: continuous light. **(B)** GUS staining and **(C)** expression analysis in L5 seedling. **(C)** Quantification of expression of GUS, COPIA78 and TSI by qRT-PCR after treatments described in A and in controls not heat-treated. Values are normalized to EIF4A1 , a gene with equal expression under all conditions tested here. Error bars indicate standard deviation of technical triplicates.

After finding that the amount of light influenced the extent of heat response, I tested next if the time point of light exposure is important under a regime of an equal

number of light hours. Therefore, the new experimental setup includes exposure to heat stress for 24 h divided in 12 h light followed by 12 h dark (LD) or vice versa (DL). As references I used DD and LL light conditions during 24 h of heat. No prominent differences compared to DD and LL in expression could be seen between LD and DL. (Figure 8C). To see if light or darkness has an impact on the expression during recovery, I applied light or dark for 12 h during the recovery phase without heat stress. As shown in Figure 4B, the expression levels of TSI returned to normal values after recovery of two days. COPIA 78 expression remains up-regulated after 12 h of recovery as shown before (Pecinka *et al*. 2010). In contrast, GUS expression returned to mock levels after 12 h recovery. Expressional recovery seems to be slightly faster under light than under dark and even increased with a light period previous to recovery phase (Figure 8D).

In summary, the presence of light influences the magnitude of expression during heat stress and subsequent recovery phase.

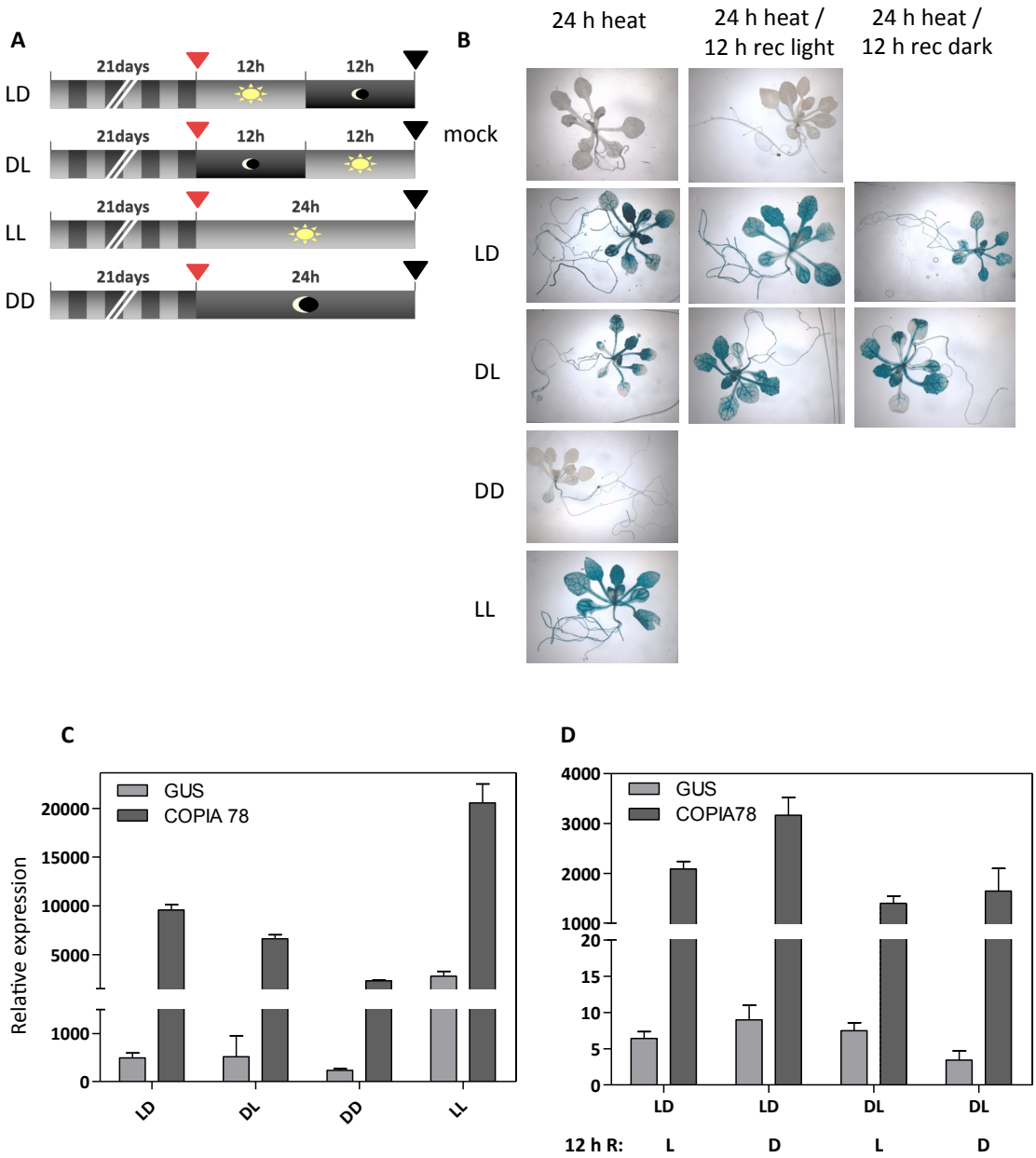

Figure 8. The photoperiod does not influence the magnitude of heat stress-induced expression.

(A) Scheme of light conditions during 24 h of heat treatment. Red arrows indicate the beginning of heat stress treatments; black arrows indicate the harvesting time. LD: 12h light – 12 h darkness; DL: 12 h darkness – 12 h light; DD: continuous dark ; LL: continuous light. (B) GUS staining of L5 seedlings directly after the heat treatments described in A and after 12 h recovery in ambient temperature in light or darkness. (C and D) Quantification of expression of GUS and COPIA78 by qRT-PCR after treatments and recovery described in A and B. Values are normalized to EIF4A1 . Error bars indicate SD of 3 technical replicates.

3.3 Quantification of transient heterochromatic de-condensation reveals natural variation and dependence on light

Additionally to the release of TGS, prolonged heat stress is concomitant with heterochromatin de-condensation. To quantify the loss of chromocenter organisation I used fluorescence in situ hybridisation (FISH). By labelling 180 bp pericentromeric satellite repeats it was possible to follow the dynamics of de-condensation induced by heat. Accordingly, 21-day-old L5 seedlings were subjected to standard heat stress conditions. Leaves were harvested at intervals of 6 hours up to 30 hours and a subsequent recovery phase of 2 days. FISH was performed to label the specific region of 180bp repeats with a red fluorescent dye. Additionally, I added DAPI, a DNA intercalating fluorescent stain, to mark the region of the whole nucleus in blue.

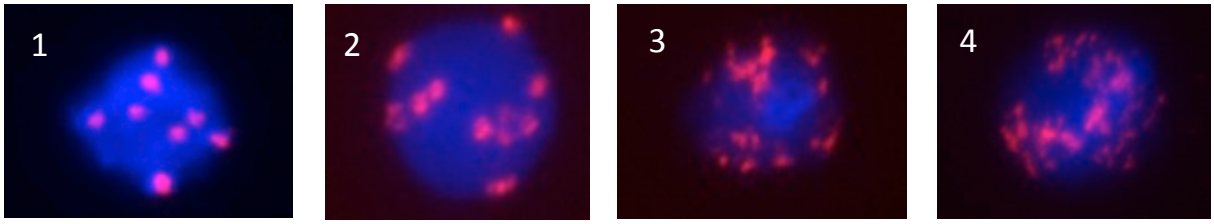

Figure 9. Heterochromatin condensation during heat stress is subdivided into four categories for quantification.

Fluorescence in situ hybridization in isolated nuclei with a centromeric repeat probe (Texas-Red) and DAPI counterstaining of DNA (blue). The categories indicate different degrees of de-condensation observed during heat stress treatments and subsequent recovery during 2 days.(1) fully condensed chromocenters; (2) less than four chromocenters de-condensed; (3) more than four chromocenters de-condensed; (4) all chromocenters de-condensed.

In the first quantification approach, four categories of condensation were distinguished as shown in Figure 9. The categories describe the condensation levels observed during heat after different time points. The categories include fully condensed chromocenters to fully de-condensed ones, in which no chromocenter was distinguishable anymore. For each time point, a minimum number of 200 nuclei was counted randomly to avoid bias, using a wide-field fluorescence microscope. Most of the fully condensed nuclei (category 1) were detected in the untreated

sample (Figure 10). After six hours, approximately 60% of the nuclei started to loosen their chromatin structure (category 2), and the first fully de-condensed nuclei (category 4) were observed. After 12 h the maximum of de-condensation seemed to be reached. Most of the nuclei recovered after 2 days, though a small percentage remained de-condensed.

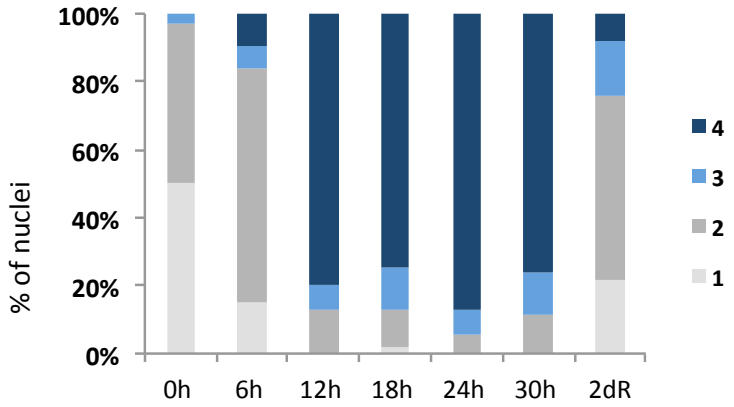

Figure 10. Chromocenters become transiently de-condensed during heat stress.
Percentage of nuclei (based on approximately 200 nuclei per treatment) with de-condensation classified according to Fig. 7 at different time points during exposure to heat (0-30 h) and after 2 days of recovery (2 d R).

Since a division into four categories does not reflect the gradual differences and was not always easy to apply, I wanted to increase the objectivity and reproducibility of this assay and to quantify the kinetics of decondensation in an automatic way. Therefore, an inverted wide-field microscope for fluorescence with a motorized xyz-stage was used. By programming the software, which also controlled the components of the microscope, it was possible to automatically acquire images of up to 200 nuclei per slide/time point. Pictures were taken for both DAPI and Texas Red (180bp-repeats) signals and were scanned at different positions on the slide.
To analyse the large amount of images taken, I used an algorithm of the program Definiens X, which recognized areas of DAPI signals as whole nuclei and 180bp signals only for the chromocenters (Figure 11).

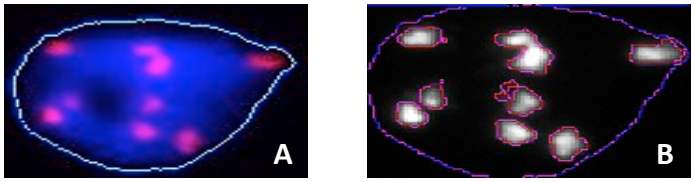

Figure 11. De-condensation can automatically be quantified by Definiens X.
(A) The algorithm detects the area of whole nuclei determined by DAPI (emitting wavelength: λ_{max} = 615 nm) and **(B)** FISH signals from the heterochromatin (emitting wavelength λ_{max} = 461nm).

The final outcome of this automatized method is a ratio of heterochromatic region in relation to the whole nucleus, with higher values indicating more de-condensation.
The boxplot in Figure 12 shows the de-condensation time course during heat stress and after recovery of 2 and 7 days. A significant increase from 0 h to 30 h was observed, with major steps from 0 to 6 h and between 12 and 18 h. Interestingly, overall condensation levels returned to mock status after 2 days of recovery. The expression of TGS target TSI correlates with the levels of de-condensation in the beginning, but reaches a plateau already after 18 h, while transcription is further increased (Figure 12).

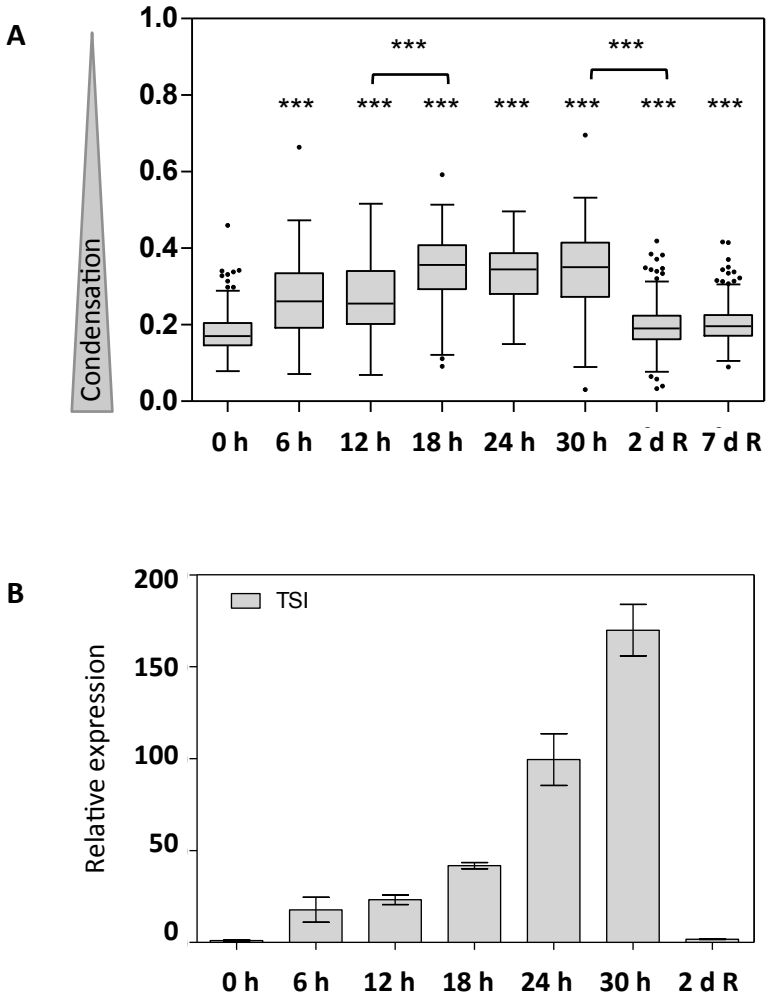

Figure 12. Transient de-condensation of chromocenters during heat stress parallels the transcriptional activation.

(A) The Boxplot summarizes the automatic quantification of heterochromatin de-condensation in approximately 200 nuclei in an interval of 6 hours during heat stress (0-30 h) and subsequent recovery of 2 and 7 days (2 d R and 7 d R). Whiskers: Tukey model (1,5x IQR). Statistically significant differences between starting point (0 h) and heat-stressed samples are indicated by asterisks (t-test, P value <0.0001). **(B)** Copy of Figure **4:** Relative expression of an endogenous TGS target (repeat TSI) at the same time points as A. Values are normalized to EIF4A1, a gene with equal expression under all conditions tested here. Error bars indicate standard deviation of 3 technical replicates.

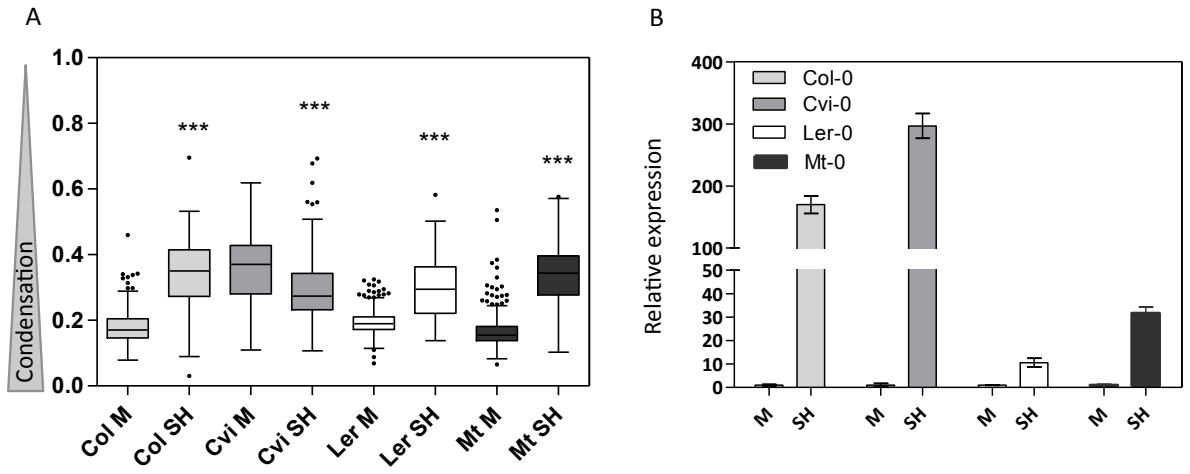

Figure 13. The degree of de-condensation and TSI expression after heat stress differs between ecotypes of Arabidopsis.

(A) De-condensation between mock (M) and standard heat stress (SH) treatments quantified in Arabidopsis ecotypes Col-0, Cvi-0, Ler-1 and Mt-0 as described in Figure 11. Whiskers: Tukey model (1,5x IQR). Statistically significant differences between mock and heat-stressed samples are indicated by asterisks (t-test, P value <0.0001). (**B)** Quantification of TSI after heat stress in different ecotypes determined by qRT-PCR and normalized to EIF4A1. Error bars indicate SD of 3 technical replicates.

Making use of the newly established automatic analysis method, chromocenter de-condensation was quantified for four different ecotypes of Arabidopsis, Columbia (Col-0), Cape Verde Islands (Cvi-0), Landsberg erecta (Ler-1) and Marturba (Mt-0). It was shown before that Cvi-0 features a higher de-condensation level under normal conditions than other ecotypes (Tessadori *et al*. 2009). My results confirmed this observation, shown in Figure 13. In contrast to Col-0, Ler-1 and Mt-0, which show a significant increase of de-condensation after 30 hours of heat, Cvi-0 showed no additional de-condensation. Furthermore the de-condensation was compared to transcriptional levels of TSI. Release of TGS was most prominent in Cvi-0, followed by Col-0. TSI was least expressed in Ler-0 after heat stress. Since I observed a shift of expression between heat stress treatment under continuous dark and continuous light, I investigated the aspect of de-condensation under those conditions. In fact, I observed a significant difference of decondensation, as demonstrated in Figure 13.

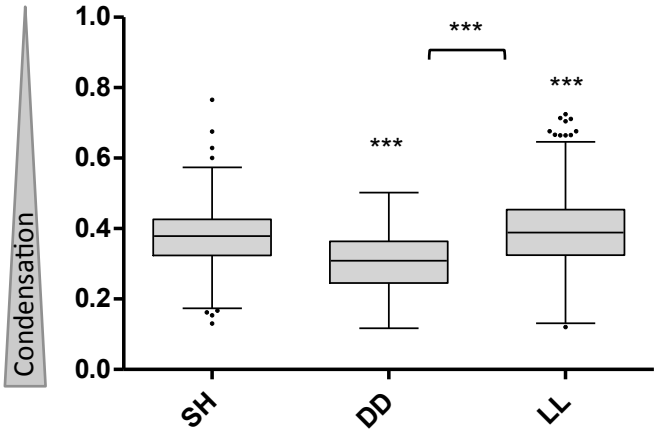

Figure 14. **Light conditions influence de-condensation in heat-stressed nuclei.**
De-condensation in L5 seedlings after standard heat stress treatments (SH), heat stress during continuous darkness (DD) or continuous light (LL) quantified as described in Figure 11. Whiskers: Tukey model (1,5x IQR). Statistically significant differences between samples are indicated by asterisks (t-test, P value <0.0001).

In summary, I established a quantitative and automated analysis of heterochromatin de-condensation and applied it to study kinetics of de-condensation induced by heat, differences between ecotypes and correlation with expression levels of TGS targets under heat stress. The presence of light influences the magnitude of de-condensation during heat stress.

4 Discussion

Work described in this thesis was based on the previous observation that long periods of heat stress interfere with epigenetic regulation, release transcriptional silencing from a number of genes and cause decondensation of heterochromatin (Pecinka *et al*. 2010). I have shown that other stress types do not have comparable effects, and that the transcriptional response is influenced by the amount of light to which the plants are exposed during heat stress. The results suggest an active and heat-specific mechanism.

Although a release of TGS was reported to occur during other stresses, for example freezing or UV irradiation (Lang-Mladek *et al*. 2010; Pecinka *et al*. 2010), decondensation of heterochromatin was not observed under similar conditions (Pecinka *et al*. 2010). Decondensation alone, that was observed in response to reduced light intensity (Tessadori *et al*. 2009), was not sufficient for the release of TGS, as I could also show under my settings. The specific effects of heat stress could therefore be due to a combination with oxidative stress. It is hypothesised that the increased decondensation levels in protoplast nuclei correlates with the level of oxidative stress in these cells (Ondrej *et al*. 2010), since adding antioxidant to the cells limited the decondensation. However, in my experiments, at least TGS was unaffected by highlight or oxidative stress, both supposed to activate the ROS pathway similar to heat stress. Studying the decondensation and transcriptional activation of silent genes in more detail under several ROS producing conditions might reveal whether both responses during heat stress are connected with oxidative stress. This is not necessarily so since there is evidence that different genes of the ROS gene network of Arabidopsis respond differently to distinct stress treatments (Mittler 2006). A role of nutritional limits, exemplified here by phosphate starvation, did not cause a release of TGS. However, there are many other possibilities for nutritional stress. However, they are difficult to analyse since withdrawal of macro- or micronutrients has delayed effects and also high impact on the vigour of the test plants, affecting metabolism and energy household in a complex way. So far, the epigenetic consequences of stress are most prominent and best characterized upon prolonged heat stress, but combinatorial effects are likely.

Applying diverse stresses to seedlings is also a matter of technical issues. The high light exposure was limited by the range of illumination in the Percival incubator, and high light is connected with increasing temperature. Although different methods were reported to overcome the additional temperature stress, like incubating detached leaves swimming in a water bath at constant temperature (Dunaeva and Adamska 2001), such methods cause other additional stress types and were therefore not suitable for my purpose.

Among the marker genes used to monitor and quantify TGS release, the retrotransposon COPIA78 seemed to be the most interesting candidate for heat stress response, because of its delayed resilencing after heat exposure (Pecinka *et al*. 2010). It was also shown to be differentially expressed upon heat exposure of different ecotypes of *Arabidopsis thaliana* (Vanja Cavrak, unpublished data). This could be correlated with different copy number and genetic polymorphisms. Another explanation for quantitative differences was provided by Vanja Cavrak, who found extra-chromosomal COPIA78 DNA (Ito *et al*. 2011) in different amounts in the ecotypes. COPIA 78 transcription and/or formation of the extra-chromosomal DNA during heat stress are likely coupled but not necessarily proportional. Therefore, COPIA78 quantification with qRT-PCR on cDNA is inaccurate. Subsequently, I used the well characterised endogenous repeats TSI and the GUS transgene, both not forming extrachromosomal DNA.

My experiments to investigate possible differences of epigenetic heat response in connection with day and night rhythm were based on the information that the temperature compensation of the circadian clock lasts only up to 27°C in *Arabidopsis thaliana* (McWatters and Devlin 2011). Clock genes remain expressed under extreme temperatures but their amplitude changes. For the heat-induced activation of TGS controlled genes, I observed independence from the preceding light phase but dependence on the amount of light during the stress exposure, with less response in the dark. This could be due to different energy levels: the adaptation of the whole organism to heat stress might be energy-consuming, and the lack of photosynthesis during darkness probably lowers energy resources even more. In addition to the differences in transcription, I observed the same correlation with light exposure for the chromatin decondensation response. Although decondensation and loss of nucleosomes could simply be passive consequences of destabilized structures by increased temperature, this is not very likely, considering the

requirement for long heat periods and the lag period before the effects become apparent. In vitro studies with histones bound to DNA, with and without adding chromatin remodelling factors and energy supply, would provide a possibility to distinguish between passive or active processes. So far, the results suggest that the epigenetic response to heat stress is rather an active process, and at least the restoration of silencing requires involvement of a chromatin remodelling factor (Pecinka *et al*. 2010).

During the initial manual analysis of the dynamic heterochromatin decondensation upon heat stress in approximately 200 nuclei, I observed that individual nuclei were not equally affected. Within the resolution of the sampling times, I found the first completely decondensed nuclei after 6 h, however with small numbers. On the other end of the scale, some nuclei remained decondensed even after 2 days of recovery. Also nuclei of untreated samples differ sometimes with regard to the condensation status of heterochromatin. The manual method is suitable to discover this heterogeneity, but it is not very reproducible, the categories are not always easy to separate, it can be subjective and is not suitable for large sample numbers. To perform the cytological analysis in a large scale and with sufficient biological replicates, the automated method developed here is helpful even if detailed information is partially lost. The progress of decondensation was similarly documented using both methods, but the automated evaluation revealed a more extended reconstitution of heterochromatin condensation during the recovery phase than documented previously (Pecinka *et al*. 2010). Therefore, the method should be chosen depending on the questions, and a combination of both methods could provide additional information.

My analysis documented a tight correlation between decondensation and transcriptional activation of the TSI repeats during heat exposure. However, the decondensation was analysed with the probe for the 180 bp repeat. Therefore, investigating more targets for both processes within the same samples would provide stronger evidence. Additionally, one needs to consider natural variation of heterochromatin organisation in non-stressed plants and in response to heat. Wild type plants of the ecotype Cvi-0 have less condensed chromocenters (Tessadori *et al*. 2009), and beside of genetic differences in the PHYB gene, a mutation in *HDA6* is responsible, the latter coding for a histone deacetylase that was shown to have a role in maintenance of TGS targets (Probst *et al*. 2004). Similar, yet undetected genetic

polymorphisms might be responsible for qualitatively or quantitatively different epigenetic responses to heat between ecotypes and await further investigations.

Release of TGS was neither connected with changes in DNA methylation nor with histone modifications (Pecinka *et al*. 2010). Therefore, further analysis should focus on the roles of chaperons and histone variants, such as those of H2A and H3. The activated TGS targets are usually all hypermethylated, and the mutual exclusion of DNA methylation and H2A.Z (Zilberman *et al*. 2008) makes the involvement of this variant unlikely. However, changes in DNA methylation and chromatin configurations have in common that they do not occur at the same level for all genes and are not sufficient for transcriptional activation (Pecinka and Mittelsten Scheid 2012). There is also evidence for cell-, tissue- or organ specificities. Preliminary data suggest a protective mechanism against heat stress-induced release of TGS in the apical meristem (Baubec *et al*., unpublished). Furthermore, less decondensation was observed in meristematic cells (Pecinka *et al*. 2010). The newly established quantification method for decondensation provides a useful tool to analyse this in more detail. A late separation of the germ line from somatic cells in plants provides the possibility to memorize epigenetic changes induced by environmental stresses even into the next generation (Mirouze and Paszkowski 2011), providing an adaptive advantage. However, transmission of such changes opposes the need to protect the integrity of the genome and epigenome. Transmission of genetic information is based on a good balance between conservation and change, and it is likely that epigenetic information is under a similar control providing stability and flexibility at the same time. Investigations of environmentally induced but heritable changes and protection mechanisms against them is an exciting topic of current research.

5 List of abbreviations

Table. 2: Abbreviations used in the text

CAF-1	CHROMATIN ASSEMBLY FACTOR 1
cDNA	Complementary DNA
Cen H3	Centromere H3
Col-0	Columbia-0
Cvi-0	Cape Verde Islands
DAPI	4', 6-diamidino-2-phenylindole
DD	Continuous dark
Ddm1	Deficient in DNA Methylation 1
DL	12 h dark followed by 12 h light
DNA	Deoxyribonucleic acid
DSH	Darkness previously to standard heat
FISH	Fluorescence in situ hybridization
GM	Germination medium
GUS	β-glucuronidase
HDA6	Histone deacetylase 6
LD	12 h light followed by 12 h dark
Ler-1	Landsberg erecta
LL	Continuous light
LTR	Long terminal repeat
mC	Methylcytosine
Mt-0	Marturba
PCR	Polymerase chain reaction
PHYB	PHYTOCHROME-B
PSR	Phosphate starvation response
PTMs	Posttranslational modifications
qRT-PCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive Oxygen Species
SH	Standard heat
SHE	Standard heat harvested in the evening
SHM	Standard heat harvested in the morning
si RNA	Short interference RNA
TE	Transposable element
TGS	Transcriptional gene silencing
TSI	TRANSCRIPTIONALLY SILENT INFORMATION
VSP2	VEGETATIVE STORAGE PROTEIN 2
X-Gluc	5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid cyclohexylammonium salt
Zh	Zurich

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