Identifying novel regulators of heat stress memory in *Arabidopsis thaliana*

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1. Abstract

Heat stress (HS) is a major abiotic stress that negatively affects plant growth and productivity. However, plants have developed various adaptive mechanisms to cope with HS, including the acquisition and maintenance of thermotolerance, which allows them to respond more effectively to subsequent stress episodes. HS memory includes type II transcriptional memory which is characterized by enhanced re-induction of a subset of HS memory genes upon recurrent HS. In this study, new regulators of HS memory in *A. thaliana* were identified through the characterization of *rein* mutants.

The *rein1* mutant carries a premature stop in CYCLIN-DEPENDENT-KINASE 8 (CDK8) which is part of the cyclin kinase module of the Mediator complex. *Rein1* seedlings show impaired type II transcriptional memory in multiple heat-responsive genes upon re-exposure to HS. Additionally, the mutants exhibit a significant deficiency in HS memory at the physiological level. Interaction studies conducted in this work indicate that CDK8 associates with the memory HEAT SHOCK FACTORs HSAF2 and HSFA3. The results suggest that CDK8 plays a crucial role in HS memory in plants together with other memory HSFs, which may be potential targets of the CDK8 kinase function. Understanding the role and interaction network of the Mediator complex during HS-induced transcriptional memory will be an exciting aspect of future HS memory research.

The second characterized mutant, *rein2,* was selected based on its strongly impaired *pAPX2::LUC* re-induction phenotype. In gene expression analysis, the mutant revealed additional defects in the initial induction of HS memory genes. Along with this observation, basal thermotolerance was impaired similarly as HS memory at the physiological level in *rein2*. Sequencing of backcrossed bulk segregants with subsequent fine mapping narrowed the location of *REIN2* to a 1 Mb region on chromosome 1. This interval contains the *At1g65440* gene, which encodes the histone chaperone SPT6L. SPT6L interacts with chromatin remodelers and bridges them to the transcription machinery to regulate nucleosome and Pol II occupancy around the transcriptional start site. The EMS-induced missense mutation in *SPT6L* may cause altered HS-induced gene expression in *rein2,* possibly triggered by changes in the chromatin environment resulting from altered histone chaperone function.

Expanding research on screen-derived factors that modify type II transcriptional memory has the potential to enhance our understanding of HS memory in plants. Discovering connections between previously identified memory factors will help to elucidate the underlying network of HS memory. This knowledge can initiate new approaches to improve heat resilience in crops.

2. Zusammenfassung

Hitzestress ist ein abiotischer Stressfaktor, der Pflanzenwachstum und Ertragsfähigkeit negativ beeinflusst. Pflanzen haben Anpassungsmechanismen entwickelt, einschließlich des Erwerbs und der Aufrechterhaltung von Thermotoleranz, die es ihnen ermöglichen auf wiederholte Stressereignisse effektiver zu reagieren. Das Hitzestress-Gedächtnis umfasst unter anderem verstärkte Re-Induktion von Gedächtnisgenen nach wiederholter Exposition (Typ II). In dieser Arbeit wurden anhand der Charakterisierung von Re-Induktionsmutanten (*rein* Mutanten) neue Regulatoren des Typ II Hitzestress-Gedächtnisses in *A. thaliana* identifiziert.

Die *rein1* Mutante weist ein vorzeitiges Stoppcodon in CDK8 auf, einer Untereinheit im Kinasemodul des Mediator Komplexes. *Rein1* Keimlinge zeigen ein beeinträchtigtes Hitzstress-Transkriptionsgedächtnis, sowie Defekte in der Aufrechterhaltung der Thermotoleranz auf physiologischer Ebene. Mittels Interaktionsstudien konnte gezeigt werden, dass CDK8 mit den im Hitzestress-Gedächtnis fungierenden Hitzeschockfaktoren HSAF2 und HSFA3 interagiert. Die Ergebnisse legen nahe, dass CDK8 zusammen mit HSFs eine Rolle bei der Aufrechterhaltung des Hitzestress-Gedächtnisses spielt, wobei letztere potenzielle Ziele der Kinasefunktion von CDK8 darstellen. Die Rolle und das Interaktionsnetzwerk des Mediatorkomplexes während der durch Hitzstress-induzierten transkriptionellen Gedächtnis-bildung und Aufrechterhaltung ist ein aufregender Aspekt zukünftiger Forschung.

Die zweite *rein* Mutante (*rein2*) wurde aufgrund einer stark beeinträchtigten transkriptionellen Re-Induktion nach wiederholtem Hitzestress für weitere Charakterisierungen ausgewählt. Dabei wurden zusätzliche Defekte in der initialen Induktion von Hitzestress-Gedächtnisgenen festgestellt. Die basale Thermotoleranz in *rein2* war in ähnlicher Weise beeinträchtigt wie das Hitzestress-Gedächtnis. Die Position von *REIN2* wurde mithilfe von Sequenzierung und Feinkartierung auf eine 1 Mb große Region auf Chromosom 1 eingegrenzt. Dieses Intervall enthält das Gen *At1g65440*, das für Histon-Chaperon SPT6L kodiert. Die Missense-Mutation in *SPT6L* könnte die Ursache für das veränderte Hitzestress-induzierte Transkriptionsmuster in *rein2* sein, möglicherweise aufgrund von einer abweichenden Chaperonfunktion und folglich Veränderung in der Chromatinumgebung.

Die Ausweitung der Forschung zu den in diesem Screening ermittelten Faktoren, die das Typ II Transkriptionsgedächtnis beeinflussen, hat das Potenzial, unser derzeitiges Verständnis des Hitzestress-Gedächtnisses in Pflanzen zu verbessern und Verbindungen zwischen zuvor entdeckten Gedächtnisregulatoren herzustellen. Dieses Wissen kann dazu beitragen neue Ansätze zur Verbesserung der Hitzeresilienz bei Nutzpflanzen anzustoßen.

3. Introduction

3.1 Thermotolerance in plants

High temperature is a major abiotic stress for plants, restricting vegetative and reproductive growth (Young *et al.*, 2004; Sakata *et al.*, 2000; Hasanuzzaman *et al*., 2013). Plants are exposed to seasonal and diurnal temperature fluctuations. Depending on different parameters, such as temperature range, duration of the stress or interplay with other stress factors, plants respond with different physiological and developmental changes (Hayes *et al.*, 2021). Warm ambient temperatures – temperatures within the physiological range of the species and only slightly above the optimum for plant growth – result in morphological adaptions in a process called thermomorphogenesis (Balasubramanian *et al.*, 2006; Koini *et al.*, 2009; Quint *et al*., 2016). Moderatly higher temperatures are perceived as heat stress (HS) and trigger acclimation responses that enhance the tolerance to a potential subsequent heat period (Mittler *et al.*, 2012; Yeh *et al.*, 2012). Severe HS – a temperature rise far beyond optimal growth temperature – causes cellular damage which eventually results in cell death (Vacca *et al.*, 2004; Larkindale and Knight, 2002). Multiple phenotypic assays have been developed in order to address these diverse physiological responses to HS and characterize the molecular basis of thermotolerance in plants, which will briefly be discussed in the following section.

3.1.1 Basal and acquired thermotolerance

Adapted to their sessile lifestyle, plants have an inherent ability to survive exposure to a sudden rise in temperature, far above optimal. This is referred to as basal thermotolerance (bTT). They gain improved thermotolerance by priming through a mild HS and a recovery phase of a few hours, which enables them to survive a subsequent severe HS (acquired thermotolerance (aTT)) (Mittler *et al.*, 2012). The heat thermotolerance of the plants depends on species, plant tissue, age and assay conditions. However, for *Arabidopsis* most thermotolerance studies so far favored the seedling stage, since multiple individuals can be grown side by side on petri dishes to the advantage of large sample sizes and equal stress conditions (Yeh *et al.*, 2012). A HS treatment of 44 - 45°C for durations of 15 to 60 minutes is the major, established way of studying bTT, displaying the full range between tolerable and lethal exposure. A prior treatment of seedlings with moderate HS (37 - 38°C for 1 hour), followed by a short recovery period (less than 2 hours), is used in assays analyzing aTT. That primes the plant to survive an otherwise lethal HS of 44°C.

3.1.2 Maintenance of acquired thermotolerance

Like other environmental stresses, HS is often recurring. To face these stress episodes, plants have the ability to actively maintain a primed state over time. This process is named maintenance of thermotolerance (maTT), also referred to as heat stress memory (HS memory) (Charng *et al.*, 2006; Stief *et al.*, 2014). It is caused by past HS, enabling the plants to prepare molecularly for a second, triggering high temperature stress. The plant presumably benefits from maintaining the primed state, thereby improving its ability to survive future stresses, which outweighs the cost (Hilker *et al.*, 2016). The maintenance of stress memory depends on the duration of priming and triggering stresses, as well as age and species of the plant. In *Arabidopsis* and rice, the priming state after a moderate HS is maintained over several days (Stief *et al*., 2014; Lin *et al*., 2014). Recently, a study showed HS memory in *Arabidopsis* to still be detectable for up to six days after the priming event (Friedrich *et al*., 2021).

3.2 Heat stress perception

Research is still ongoing to uncover how plants primarily sense HS. To anticipate high temperatures, they have to sense the ambient temperature and switch on suitable genetic programs. At high temperatures, plants initiate the heat shock response (HSR) which includes the activation of heat shock transcription factors (HSFs) and production of heat shock proteins (HSPs) in addition to other stress responsive proteins in order to cope with cellular damage (Vierling, 1991; Larkindale and Vierling, 2008). Although a comprehensive picture of temperature perception is still missing, different processes were proposed as potential heat sensing mechanisms.

3.2.1 Calcium signaling linking heat stress sensing and transcriptional regulators

Activation of certain plasma membrane channels and changes in the membrane fluidity are considered to function as primary heat sensing events (Sangwan *et al*., 2002; Horváth *et al*., 1998). Changes in the plasma membrane trigger a calcium ion (Ca^{2+}) influx from the apoplast into the cytoplasm via cyclic nucleotide-gated ion channels (CNGCs) within minutes in the *Physcomitrella patens* moss (Saidi *et al.*, 2009). Interestingly, *cngc2* and *cngc4* loss-of-function mutants in *Arabidopsis* exhibit deregulated thermotolerance, strengthening the idea that they are essential components of the thermosensory machinery controlling HSR (Finka *et al.*, 2012). Additionally, CNGC6 is a heat activated Ca^{2+} -permeable channel and *cngc6* mutants exhibit a decrease of HSP expression induction and thermotolerance (Gao *et al.*, 2012).

The Ca^{2+} in the cell is assumed to serve as a key messenger molecule that is further transduced into a proper HSR (Figure 1). Several different protein families in plants have been discovered to decode Ca^{2+} signals during various developmental processes and response to environmental stimuli, among them the calcium-modulated proteins (Calmodulins/CaMs), Calmodulin-like proteins (CMLs), Calcineurin B like proteins (CBLs) and Calcium-Dependent Protein Kinases (CDPKs) (Anderson *et al.*, 1980; Roberts & Harmon, 1992; Kudla *et al.*, 1999; Harmon *et al.*, 1987). One member of the CaM family, CaM3, participates in the heat transduction cascade (Liu *et al.*, 2005). CaM3 is induced upon HS and regulates several HS-related genes, presumably by activating phosphotransferases which regulate the activity of a key transcription factor in the HSR: CLASS A1 HEAT SHOCK FACTOR (HSFA1) (Liu *et al.*, 2005; Liu *et al.*, 2007; Liu *et al*., 2008). A recent study identified a CDPK from maize (ZmCDPK7) as a positive regulator of HS tolerance, interacting with sHSP17.4 and positively regulating its expression, as well as phosphorylation levels (Zhao *et al.*, 2021a). Limited information about the role of CDPKs in HS transduction is available up to now, but there is a suspected link between Ca^{2+} influx and the observed activation of mitogen-activated protein kinases (MAPKs). A publication indicates that the treatment with an antagonist of CDPKs inhibits the HS-induced activation of a MAPK from alfalfa (*Medicago sativa*) cells (Sangwan *et al.*, 2002). The MAPK cascade has a great impact on the phosphorylation and thereby activation status of transcription factors, splicing factors and protein kinases. The MAPK6 interacts and phosphorylates the HEAT SHOCK TRANSCRIPTION FACTOR A2 (HSFA2), which contributes to its nuclear localization during HS (Evrard *et al.*, 2013) (Figure 1). HEAT SHOCK TRANSCRIPTION FACTOR A4 (HSFA4) represents another HSF that is phosphorylated by multiple MAP kinases (Andrási *et al.*, 2019). However, the publication by Andrási *et al.* does not provide information of molecular or physiological effects of this modification. Additional phosphorylation sites were detected in HSFA4A, which are not substrates of MAP kinases, leaving room for other kinases that contribute to the regulation of HSFA4A.

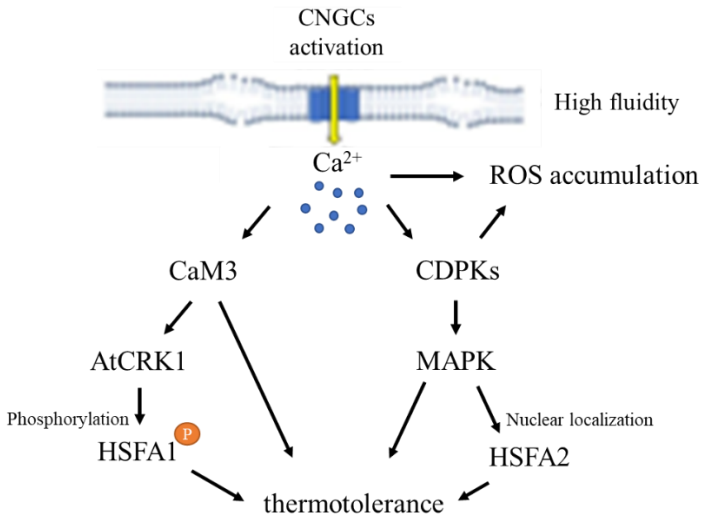

Figure 1. Contribution of heat-induced Ca2+ signaling to thermotolerance. Elevated temperatures cause alteration in plasma membrane fluidity, which may trigger Ca2+ influx into the cytosol mediated by calcium channels (CNGCs). Ca^{2+}-binding proteins, such as CaM3 and CDPKs are part of signal transduction pathways that lead to the activation of HS-responsive genes and improved thermotolerance. CaM3 regulates the expression of HSPs and activates CDPK-RELATED KINASE 1 (AtCRK1), a kinase which might regulate HSFA1 through phosphorylation. CDPKs trigger ROS accumulation and subsequent ROS signaling by activating RBOHD and activate the MAPK cascade, which contribute to the activation and localization of HSFs, like HSFA2. Modified from (Bourgine and Guihur, 2021).

3.2.2 Role of reactive oxygen species in heat signaling

Ca^{2+} homeostasis has an effect on the production of reactive oxygen species (ROS), which function as important downstream secondary messengers in the HSR (Ogasawara *et al.*, 2008; Larkindale *et al.*, 2005; Volkov *et al.*, 2006). ROS is a collective term for reactive molecules and free radicals derived from oxygen as byproducts of cellular metabolism, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hydroxyl radicals ($^{\bullet}OH$) (Halliwell, 2006; Das and Roychoudhury, 2014). While the harmful effects of excessive ROS caused by imbalance of production and scavenging have been extensively studied, their role as signaling molecules, important for plant development and stress responses is emerging (Bartoli, 2004; Dat *et al.*, 2003; Price *et al.*, 1989; Anjum *et al.*, 2015; Zandalinas *et al.*, 2020). An important role as a signaling molecule was assigned to H_2O_2 due to its relatively long half-time compared to its usual precursor O_2^- and its impact on tolerance to several environmental stimuli such as heat, salinity, drought and cold (Wang *et al.*, 2014; Uchida *et al.*, 2002; Hameed and Iqbal, 2014; Sun *et al.*, 2016; Prasad *et al.*, 1994; Hossain *et al.*, 2015) H_2O_2 is produced at various compartments of the plant, including the chloroplasts, peroxisomes, mitochondria and the apoplast (Smirnoff and Arnaud, 2019; Niemeyer *et al.*, 2021). During HS at least two ROS-

generating nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, the RESPIRATORY BURST OXIDASE HOMOLOGUE B and D (RBOHB and RBOHD), which are localized at the plasma membrane, contribute to the plants thermotolerance (Larkindale *et al.*, 2005). Observation show synergistic activation of RBOHD through a conformational change by phosphorylation and binding of Ca^{2+} via intrinsic helix–loop–helix structural domains, called EF-hand motifs (Ogasawara et al., 2008). Additionally, multiple studies show that in parallel to the aforementioned Ca^{2+}, H_2O_2 is an important factor for the induction of MAP kinases (Kovtun *et al*., 2000; Nakagami *et al*., 2006). The increasing H_2O_2 levels during HS also induce the expression of small HSPs (sHSPs), exemplarily shown on sHSP17.6 and sHSP18.2, which are essential components of the HSR (Volkov *et al.*, 2006).

3.2.3 Thermosensors in plants

Previous research suggests HSFs to function as heat or H_2O_2 sensors (Miller and Mittler, 2006). Research in mammalian cells and *D. melanogaster* shows heat- and H_2O_2-mediated trimerization of HSF1 (Zhong *et al.*, 1998; Ahn & Thiele, 2003). In line with these observations, H_2O_2 treatment has a similarly activating effect as HS and pH changes in *Arabidopsis*, causing trimerization and subsequent binding of HSFA1a to its DNA-targets. The DNA-binding can be reversed with reducing agents dithiothreitol and NADPH, supporting the claim that the process is dependent on that redox state (Liu *et al.*, 2013).

Several processes are known to be involved in warm ambient temperature sensing (ranging between 24°C and 30°C for *Arabidopsis*) (Hayes *et al.*, 2021). Considering an overlap in plant responses to different stages of heat, factors that were initially characterized to be involved in thermomorphogenesis or warm temperature response, might also be relevant for the HSR. One of the best characterized thermosensors is phytochrome B (phyB), a photoreceptor that is regulated by light and temperature cues (Legris *et al*., 2016). PhyB gets inhibited with increasing temperatures. Among other downstream signaling events, this inhibition leads to the accumulation of a basic helix–loop–helix (bHLH) transcription factor: PHYTOCHROME INTERACTING FACTOR 4 (PIF4). PIF4 is a key transcription factor regulating a large number of growth-related genes (Jung *et al.*, 2016; Legris *et al.*, 2016; Koini *et al.*, 2009). A role in thermosensing was attributed to another PIF, PHYTOCHROME INTERACTING FACTOR 7 (PIF7) (Chung *et al.*, 2020). *PIF7* translation is increased during higher temperatures, leading to the activation of key thermomorphogenesis genes. The enhanced translation originates in a specific secondary structure (hairpin loop) of the *PIF7* RNA, showing

that RNA thermoswitches provide another level of temperature signal perception. EARLY FLOWERING3 (ELF3), a component of the plant circadian clock, is another protein that functions as a molecular thermosensor (Ezer *et al.*, 2017). ELF3 is expressed at the end of the day and represses elongation growth-related target loci, like *PIF4* (Box *et al.*, 2015). This repression is temperature-dependent, considering that ELF3 binding to target loci decreases with increasing temperature. In addition to its regulation by phytochromes, ELF3 is also directly sensitive to temperature which depends on an intrinsic prion-like domain. This domain confers temperature responsiveness by mediating two conformations of ELF3: an active soluble form and an inactive form, where ELF3 molecules cluster together in condensates (liquid droplets) inside the nucleus with increasing temperatures. This mechanism of temperature-dependent phase separation presents a recently discovered process for responsiveness to environmental input (Jung *et al.*, 2020). Further, an epigenetic mechanism was proposed to be involved in thermosensing, based on the observation of dynamic exchange of histone variants with varying temperatures (Kumar and Wigge, 2010). Histone variants describe noncanonical histone proteins that provide a variety of different functions due to specific structural features (Probst *et al.*, 2020). A histone variant of Histone 2A, H2A.Z, which has an important role in regulating plant responses to environment (Lei and Berger, 2020), was shown to be evicted from nucleosomes in temperature-induced genes at warm temperatures (Kumar and Wigge, 2010). Later, it was reported that H2A.Z nucleosomes were not intrinsically temperature responsive, but rather depleted by a mechanism that requires HSFA1a (Cortijo *et al.*, 2017).

3.3 Factors involved in heat stress response

The immediate response to elevated temperature in plants is a well conserved cellular mechanism (Richter *et al.*, 2010). During the HSR, a rapid signal transduction chain eventually leads to the activation of HSFs that mediate the transient accumulation of HSPs, which have an essential role in withstanding extreme temperatures (Figure 2). Heat inducible HSPs are grouped into different classes depending on their approximate molecular weight in kilodalton (kDa): Hsp100, Hsp90, Hsp70, Hsp60 and small heat-shock proteins (sHsps) (Vierling, 1991; Kotak et al., 2007). sHSPs whose molecular weight ranges from 15 to 42 kDA, are highly abundant and stress-inducible (Sun *et al.*, 2002). Many heat inducible HSPs function as chaperones and counteract protein misfolding and aggregation, reducing cellular damage (Wang et al., 2004). In addition to HSFs, other factors and signaling pathways take part in the HSR and contribute to the plants thermotolerance.

3.3.1 Classes and functions of heat shock transcription factors

In contrast to invertebrate organisms, like *Drosophila* and yeast with only one HSF, vertebrates and plants express multiple forms of HSFs (Clos et al., 1990; Sorger and Pelham, 1987; Wiederrecht et al., 1987; Scharf et al., 2012). In comparison to the existence of only four HSF members in vertebrates, a large number of HSFs of up to 56 in wheat (Xue *et al.*, 2014) were identified in various plant species. They exhibit a well conserved modular structure with an N-terminal DNA-binding domain (DBD), which includes a central helix-turn-helix motif that specifically binds to heat shock elements (HSEs) in target promoters and an oligomerization domain with a heptad pattern of hydrophobic residues (HR-A/B region). Both domains are connected by a linker of variable length (15 to 80 amino acid residues) (Scharf *et al.*, 2012). Plant HSFs are divided into three classes: HSFA, B and C, classified according to structural features of the HR-A/B region and the linker. In *Arabidopsis*, 21 HSF genes are identified and have been assigned to three different classes (A, B, C) and 14 groups (A1-9, B1-4, C1), based on structural characteristics and phylogenetic comparison (Nover *et al.*, 2001).

Class A HSF members function as transcriptional activators, mediated by short AHA (activator) motifs in their C-terminal domains, which are not present in class B and C HSFs (Kotak *et al.*, 2004). Within the class A HSFs, the members of the A1 group are identified as master regulators of the HS response. First observed in tomato, the silencing of HSFA1 leads to severe defects in thermotolerance and plant development at elevated temperatures (Mishra *et al.*, 2002). Almost a decade later, by creation of a quadruple mutant with the knockout of all four existing HSFA1s in *Arabidopsis*, the importance of HSFA1a, A1b,A1d and A1e could be revealed as redundantly working master transcriptional regulators of the HSR as well (Liu *et al.*, 2011; Yoshida *et al.*, 2011). Recent studies, aiming to understand the regulation of the HSFA1 transcription factors in *Arabidopsis*, have indicated that HSP70s and HSP90s repress HSFA1 activity and prevent their translocation to the nucleus under normal growth conditions. The repression is regulated by an interaction of the HSPs with a temperature-dependent repression domain in HSFA1 (Ohama *et al.*, 2016; Zhao *et al.*, 2021b).

Additional layers of regulation are involved in the activity of the HSFA1s and their full downstream transcription cascade. Among them (de)phosphorylation of the HSFA1 proteins. Both the A-type cyclin-dependent kinase CDKA1 and the CALCIUM-DEPENDENT KINASE (CDPK)-RELATED KINASE 1 (AtCRK1) control the binding activity of HSFs to HSEs by phosphorylation of HSF1 (Reindl *et al.*, 1997; Liu *et al.*, 2008). Furthermore, an interaction

between the PROTEIN PHOSPHATASE 7 (PP7) and HSFA1a was reported *in vivo* and presents another plausible candidate in regulation of HSFA1 activity (Liu *et al.*, 2007).

The activation of the HSFA1 master regulators triggers the immediate upregulation of more than 200 HS-responsive genes, including a variety of HSPs and several key transcription factors (Liu *et al.*, 2011). Among the most notable targets of HSFA1 are the heat-inducible transcription factors *HSFA7, DREB2A (DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN 2)* and *HSFA2*, which act as secondary regulators of a subset of heat-shock upregulated genes and are essential for HSR. DREB2A and its homolog DREB2B were initially identified as proteins that are rapidly induced by high-salt and dehydration stress (Nakashima et al., 2000). They regulate the expression of drought stress-responsive genes by recognizing a dehydration-responsive element, which is a 9-bp long conserved sequence (Liu *et al.*, 1998). Later a dual role for DREB2A could be revealed, discovering that an overexpression of a constitutive active form of DREB2A leads to an upregulation of many HS-related genes such as *HSFA3* and *HSPs*, resulting in an increased thermotolerance (Sakuma *et al.*, 2006). *HSFA2* is strongly activated by HSFA1s and functions foremost in the phase after the immediate HSR by mediating the long-term induction of several HS-responsive genes. Loss of *HSFA2* has no effect on the acquisition of thermotolerance, but severely impacts the plant's capacity of maintaining the primed state (Charng *et al.*, 2007; Lämke *et al.*, 2016). HSFA3 has a similar function to HSFA2, regulating the sustained expression of HS-responsive genes (Friedrich *et al.*, 2021).

HSFB1 and *HSFB2* are further targets of the HSFA1 proteins (Liu *et al.*, 2011). A highly conserved tetrapeptide motif (LFGV) can be identified in all HSFB family members (except HSFB5), and functions as a repressor domain (Ikeda and Ohme-Takagi, 2009). HSFB1 and HSFB2 target similar groups of genes like the class A HSFs and an *hsfb1 hsfb2* double mutant exhibits higher levels of their target genes *HSFA2* and *HSFA7a* under extended HS conditions, suggesting that they are part of the plants attenuation system after the massive upregulation of heat-inducible genes (Ikeda *et al.*, 2011).

3.3.2 Roles of WRKY, bZIP, NAC and MBF1 transcription factors in the heat stress response

Additional transcription factor families that are not related to the classical HSFs, have been reported to be part of the heat shock response, including members from the WRKY, basic leucine zipper (bZIP), NAM-ATAF1,2-CUC2 (NAC) and MULTIPROTEIN BRIDGING FACTOR 1 (MBF1) families. The WRKY transcription factor family has been extensively studied and plays a critical role in response to various biotic and abiotic stresses (Jiang *et al.*, 2017). There is evidence for WRKY25, WRKY26, WRKY33 and WRKY39 to be heat-responsive and positively regulate thermotolerance. Overexpression of these factors leads to enhanced tolerance to heat (Li *et al.*, 2011, Li *et al.*, 2010). WRKY25-overexpressing plants were recently reported to be more tolerant to salt and oxidative stress, suggesting a role for WRKY25 in mediating intracellular H_2O_2 levels. Considering the strong increase of ROS levels during heat and salt stress, the oxidizing conditions might trigger the involvement of WRKY factors during HS (Doll *et al.*, 2020)

The bZIP transcription factors bZIP28, bZIP17 and bZIP60, classically known for their involvement in the unfolded protein response, are activated by HS (Gao *et al.*, 2008; Che *et al.*, 2010; Deng *et al.*, 2011). bZIP28 is a membrane-associated transcription factor that contributes to heat tolerance by upregulation of HS-responsive genes. Induced by heat, bZIP28 is cleaved by regulated intramembrane proteolysis, which results in a redistribution of the factor from the endoplasmic reticulum (ER) to the nucleoplasm, where it activates HS-responsive genes (Gao *et al.*, 2008). This heat-dependent relocalization to the nucleus can be observed for bZIP17 as well. The activation of bZIP60 in contrast involves splicing of bZIP60 microRNA (miRNA), mediated by the ER-membrane-associated protein INCREASED ORGAN REGENERATION 1 (IRE1), which leads to deletion of the transmembrane domain and subsequently a nuclear-localized, active bZIP60 (Nagashima *et al.*, 2011).

The NAC transcription factors are one of the largest plant-specific transcription factor families, containing more than 100 members in *Arabidopsis*. They are characterized by a highly conserved N-terminal region that has DNA-binding ability and is associated with protein binding and dimerization (Duval *et al.*, 2002). The NAC transcription factor NAC019 binds to promotors of several HSFs (Figure 2). In correlation with reduced heat induction of HSFs, transcripts of several HSPs are decreased following HS conditions in the *nac019* mutant, while an overexpression of NAC019 leads to increased thermotolerance (Guan *et al.*, 2014). NAC WITH TRANSMEMBRANE MOTIF 1-LIKE 4 (NTL4) is a NAC transcription factor that is

induced by high temperatures and is considered to be participating in the HS response. NTL4 triggers H_2O_2 accumulation, which serves as an important signaling component for programmed cell death in HS conditions (Lee *et al.*, 2014).

The MBF1 proteins are highly conserved transcriptional co-activators. In contrast to MBF1a and MBF1b, MBF1c expression in *Arabidopsis* is specifically induced upon response to various stimuli, including heat, pathogen infection, salinity, drought, H_2O_2 and hormone treatment (Suzuki *et al.*, 2005). MBF1c is a key player in thermotolerance and regulates the expression of essential players in the HS response like DREB2A, HSFBs and several zinc finger proteins (Figure 2). MBF1c likely functions through its putative DNA-binding element in the C-terminus, which is an unexpected finding, considering the observations from other systems, where it functions as a non-DNA-binding transcriptional co-activator (Suzuki *et al.*, 2011). Insect and yeast MBF1 proteins interact with DNA-binding proteins and increase their DNA binding activities, thereby mediating transcriptional activation (Takemaru *et al.*, 1997; Takemaru *et al.*, 1998; Kabe *et al.*, 1999). This suggests that MBF1c other than MBF1a and AtMBF1b evolved to function in stress tolerance regulation in plants.

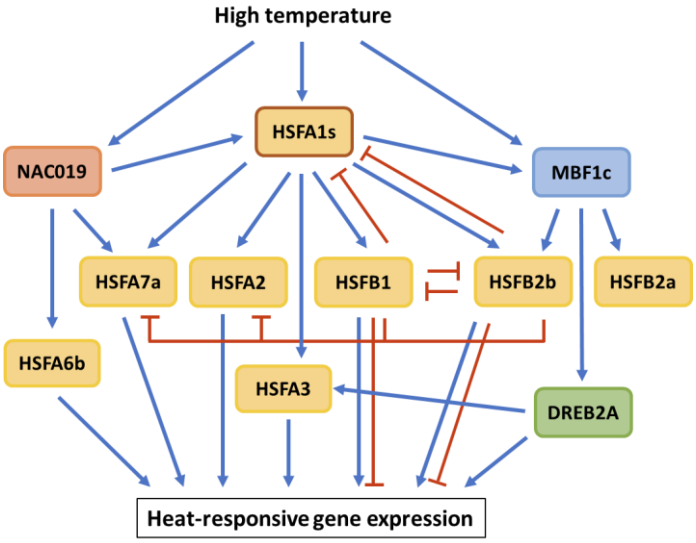

Figure 2. Interaction network of HSFs and key transcription factors regulating heat-responsive gene expression. HSFA1s are the central regulators during the HSR. They coordinate the expression of downstream transcription factors like HSFA7a, HSFA2, HSFA3, HSFBs and MBF1C, which upregulate the expression of HS-responsive genes. MBF1c functions upstream of essential regulators of the HSR, among them DREB2A and HSFBs. HSFB1 and HSFB2b negatively regulate the expression of several HSF genes. They are activated by MBF1C and HSFA1s, forming a regulatory loop that fine-tunes the expression of HS inducible transcription factors. NAC019 binds to promoters of several HSFs (HSFA1a, HSFA6b and HSF7a) and positively influences thermotolerance. Modified from (Haak *et al.*, 2017).

3.3.3 Signaling processes involved in thermotolerance

An important component in acquisition of thermotolerance is the effective accumulation of HSPs. Consequently, plants, which lack the molecular chaperone HEAT SHOCK PROTEIN 101 (HSP101), are severely impaired in their ability to acquire thermotolerance in *Arabidopsis*, maize and tomato (Hong and Vierling, 2000; Nieto-Sotelo *et al.*, 2002; Yang *et al.*, 2006). Despite that, the production of HSPs alone does not fully account for aTT. The existence of mutants, which are defective in aTT, however not impaired in the accumulation of HSPs, are prove of that (Hong *et al.*, 2003; Larkindale *et al.*, 2005). The thermotolerance of plants depends on various effects of HS on cell functions. Exemplarily, changes to the fluidity and permeability of the membrane, as well as alterations in enzyme activity, affecting the metabolic homeostasis influence thermotolerance. Furthermore, membrane and protein damage lead to the production of ROS and subsequently oxidative stress signaling. Although there is a number of signaling pathways that contribute to both bTT and aTT, there are also pathways that seem specific or critical for only one of them (Larkindale *et al.*, 2005). In the study by Larkindale *et al.*, the contribution of various components to thermotolerance is studied by examining 46 *Arabidopsis* mutants, defective in various signaling pathways, including abscisic acid (ABA), salicylic acid (SA), ethylene and oxidative burst signaling. While mutants defective in ethylene signaling and the antioxidant pathway displayed normal aTT, they exhibited strong defects in bTT. In line with these findings, one of the key regulators of thermotolerance - MBF1C, is specifically required for bTT, while negligible for aTT and functions upstream of ethylene signaling (Suzuki *et al.*, 2005; Suzuki *et al.*, 2008). ABA signaling mutants are affected in both thermotolerance assays, though they display a more severely reduced survival in tests for aTT as opposed to bTT (Larkindale *et al.*, 2005).

3.3.4 Role of ascorbate peroxidases in heat stress response

It is extremely important for plants to tightly regulate ROS homeostasis and to keep non-toxic levels of H_2O_2 in order to function as a signaling molecule by balancing its production and scavenging. Overproduction and accumulation of ROS can lead to oxidative stress and cause severe damage to cell components (Sharma *et al.*, 2012). Oxidized lipids alter intrinsic membrane properties and disrupt their function as a barrier, which is followed by the oxidation of proteins, DNA and RNA. Oxidation in proteinogenic amino acids affects protein characteristics, which negatively influence their enzymatic and physiological activities. Excessive ROS levels can damage nuclear, mitochondrial and chloroplastic DNA. Both the

sugar and base fractions are susceptible to oxidation, which can cause breaks in DNA strands, modification or removal of nucleotides with the potential for mutagenic alterations and DNA-protein crosslinks (Sharma *et al.*, 2012; Demidchik, 2015).

To avoid oxidative stress, plants have various non-enzymatic and enzymatic antioxidants to regulate the ROS concentration (Mittler, 2002; Das and Roychoudhury, 2014). Key ROS-scavanging enzymes include catalases, superoxide dismutases and ascorbate peroxidases (APXs). Due to the high affinity of APXs to H_2O_2, utilizing ascorbate as an electron donor for the reduction into water, they are considered to play an important role for H_2O_2 scavenging and signaling. APXs are found in various organisms, including higher plants, algae, cyanobacteria and even insects (Groden and Beck, 1979; Miyake *et al*., 1991; Mathews *et al.*, 1997). Eight isozymes have so far been identified in *Arabidopsis*, which are localized in different cellular compartments. They are thereby classified as cytosolic (APX1, APX2, APX6), chloroplastic (stromal (sAPX) and thylakoid (tAPX)) and peroxisomal (APX3, APX4, APX5) isoforms (Kubo *et al.*, 1992; Santos *et al.*, 1996; Jespersen *et al.*, 1997; Panchuk *et al*., 2002). Increased temperature leads to a significant accumulation of two members of the APX family in *Arabidopsis*: the two cytosolic isoforms APX1 and APX2. *APX1* is constitutively expressed and further upregulated in response to diverse abiotic and biotic stimuli, including ozone and sulfur dioxide treatment, high light stress and wounding (Kubo *et al.*, 1995; Karpinski *et al*., 1997; Davletova *et al.*, 2005; Maruta *et al.*, 2012). In contrast, the other cytosolic isoform APX2, which is also induced upon several abiotic stimuli, is almost undetectable in stress-free conditions. The induction of *APX2* upon HS, however, is massive. Up to a 1.000-fold increase of *APX2* transcript levels is observable, while *APX1* expression only increases about 2- to 4-fold, following exposure to elevated temperatures (Panchuk *et al.*, 2002). This suggests an important role for APX2 during the HSR. Both *APX* genes contain HSEs in their respective promotors (Santos *et al.*, 1996; Storozhenko *et al.*, 1998; Schramm *et al.*, 2006). A conserved HSE in the *APX1* promotor is essential for its induction under heat shock conditions and interaction of the HSE with a recombinant tomato HSF indicates that it constitutes an HSF-binding site (Storozhenko *et al.*, 1998). Using microarray expression profiles of wildtype (WT) and HSFA2 knock-out mutants, *APX2* was identified as a target of HSFA2. This can be traced back to HSFA2 binding to a specific HSE dimer within the promotor region of *APX2* (Schramm *et al.*, 2006).

3.4 Heat stress priming and memory in plants

Stress priming describes the process by which an organism emerges as prepared (primed) for a modified response to future stress incidents (Hilker *et al.*, 2016; Bruce *et al*., 2007). Memory, the retention of information from priming events over stress-free intervals enables a modified response to further stress incidents. It is not only found in vertebrates but also in bacteria, fungi and plants (Kordes *et al.*, 2019; Runde *et al*., 2014; Guhr and Kircher, 2020; Andrade-Linares *et al*., 2016). Priming of plant responses to pathogen and herbivore attacks (Engelberth *et al.*, 2004; Bandoly *et al*., 2015) as well as various abiotic stressors, such as heat, drought, salinity, and cold have been reported (Ding et al., 2012; Charng et al., 2007; Sani et al., 2013; Zuther *et al.*, 2018). Priming and stress memory is exhibited through reversible phenotypic changes that result from different modifications like transcriptional priming, epigenetic modifications or specific hormonal or metabolic signatures without changes in the DNA sequence itself (Hilker *et al.*, 2016). The duration of stress memory varies from days to months or can be transmitted to the direct offspring (intergenerational memory) and following generations (transgenerational memory) depending on the underlying memory mechanisms (Wibowo *et al.*, 2016; Rasmann *et al.*, 2012). For somatic abiotic stress memory, which is limited to one generation, a duration of several days to weeks was observed in multiple studies (Ding *et al.*, 2012; Lämke *et al*., 2016; Singh *et al.*, 2014; Lämke and Bäurle, 2017). The transcriptional memory of stress-related genes, which is commonly used as a read-out of stress memory, often correlates with changes at the chromatin level. Chromatin is a complex that is formed within the nucleus, consisting of DNA and associated proteins. The basic unit of chromatin, the nucleosomes, consist of approximately 147 base pairs of DNA wrapped around eight histone proteins (Luger *et al.*, 1997). Nucleosomes provide a variety of possibilities for modifications which can impact the chromatin organization and DNA accessibility for the transcriptional machinery or regulatory proteins, thereby allowing specific gene expression patterns. Besides posttranslational modifications of histone tails, positioning of nucleosomes and incorporation of specific histone variants impact the chromatin structure (Zentner and Henikoff, 2013; Struhl and Segal, 2013; Probst *et al.*, 2020). In past years, multiple studies have shown chromatin-based mechanisms to be a key feature of stress memory (Singh *et al.*, 2014; Sani *et al.*, 2013; Brzezinka *et al.*, 2016). While some chromatin regulated processes are stress-specific, assessed by current knowledge, common molecular features of somatic stress memory emerge with progressive stress memory research. Hyper-methylation of histone 3 lysine 4 (H3K4), for example, has been identified as a common feature in response to different abiotic stress cues (Ding *et al.*, 2012; Lämke *et al.*, 2016; Feng *et al.*, 2016; Lämke and Bäurle, 2017).

3.4.1 Factors involved in heat stress memory

Like other environmental stresses, HS is often recurring. To face these stress episodes, plants have the ability to actively maintain a primed state. This process is referred to as HS memory (Charng *et al*., 2006; Stief *et al.*, 2014). It is caused by past HS, enabling the plants to prepare molecularly for a second, triggering stress incident. The plant presumably benefits from maintaining the primed state, thereby improving its ability to survive future stresses, which outweighs the cost (Hilker *et al.*, 2016). The maintenance of stress memory depends on the duration of priming and triggering stresses, as well as age and species of the plant. In *Arabidopsis* and rice, the priming state after a moderate HS is maintained over several days (Stief *et al.*, 2014; Lin *et al.*, 2014). Recently, a study showed HS memory in *Arabidopsis* to still be detectable for up to six days after the priming event (Friedrich *et al.*, 2021). While the molecular basis of the immediate response to heat has intensively been studied, less is known about the processes during HS memory in plants.

Two types of transcriptional memory are dominant after a priming HS: type I and type II transcriptional memory (Lämke *et al.*, 2016; Bäurle, 2018) (Figure 3). Type I transcriptional memory is present in a set of genes that are not only significantly activated upon HS, but whose expression is sustained during stress memory (Stief *et al.*, 2014). Type II transcriptional memory is found in genes, whose expression levels also rise during initial stress and increase even more so after re-induction, following the triggering stress (Lämke *et al.*, 2016; Liu *et al.*, 2018). Among genes exhibiting either type of transcriptional memory after HS *APX2* and *sHSP*s are found. This indicates the importance of chaperone function, as well as ROS-scavenging in maTT. Another occurrence of a very similar transcriptional stress memory is found in dehydration stress genes of *Arabidopsis* and maize, which also behave in distinct memory response patterns upon repeated drought stress (Ding *et al.*, 2012; Ding *et al*., 2013; Ding *et al.*, 2014).

Since early research in the field, one key protein in both types of transcriptional memory has been found in HSFA2. It is one of the major factors that drive HS memory not only on the molecular, but also physiological level (Charng *et al.*, 2007; Lämke *et al.*, 2016). *Hsfa2* mutants are specifically impaired in maintenance and not the acquisition of thermotolerance. In line with this observation, the majority of HS memory-related genes do not show a significant impairment in the initial transcription induction upon HS. Like HSFA2, the HSP HEAT-STRESS-ASSOCIATED 32-KD PROTEIN (HSA32) is specifically required for maintaining thermotolerance after acclimation treatment (Charng *et al.*, 2006). However, a later study shows

a deficiency in bTT in *hsa32* mutants, indicating a broader role of HSA32 in HSR (Charng *et al.*, 2007). Another factor, specifically involved in maTT, is ROTAMASE FKBP1 (ROF1), a member of the FK506 BINDING PROTEIN (FKBP) family. ROF1 forms a complex with HSP90.1 and is required for the ongoing accumulation of HSFA2-targeted *sHSPs*, whose expression is essential for survival during heat (Meiri and Breiman, 2009).

Lastly, the miRNA pathway contributes to HS memory. Several miRNAs are not only strongly upregulated during HS, they impact the overall survival of the plant physiologically as well (Stief *et al.*, 2014; Guan *et al.*, 2013; Lin *et al.*, 2018). Specifically, *miR156* isoforms, are required to maintain expression levels of HS memory-related genes during maTT and their overexpression has a prolonging effect on HS memory (Stief *et al.*, 2014). MiR156 targets SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE (SPL) genes, which have a role in limiting organ initiation and consequently their suppression might help the plant counteract the growth inhibition effects of HS (Stief *et al.*, 2014; Wu *et al.*, 2009; Xu *et al.*, 2016).

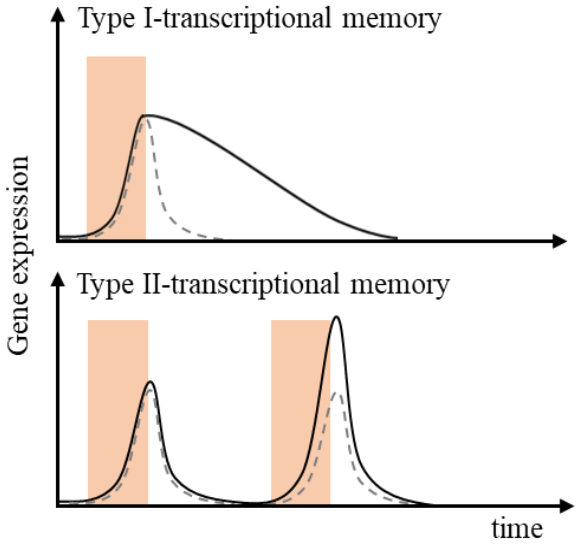

Figure 3. Transcriptional behavior of HS memory genes after stress exposure. Type I transcriptional memory genes show sustained activation after a priming HS. Memory genes that have type II transcriptional memory are induced upon a first stress and exhibit enhanced re-induction upon the second stress exposure separated by a period of stress-free conditions. The grey dotted lines represent the transcriptional behavior of a HS-inducible non-memory gene and the orange bars represents stress exposure.

3.4.2 Epigenetic processes involved in heat stress memory

Throughout recent years, the importance of epigenetics in HS memory, such as nucleosome remodeling and chromatin modifications, is becoming more and more evident (Lämke *et al.*, 2016; Friedrich *et al.*, 2021; Brzezinka *et al.*, 2016). An HS-dependent deposition of di- and trimethylation of lysine 4 of histone 3 (H3K4me2/me3) is observed at HS memory loci (Lämke *et al.*, 2016). H3K4 hyper-methylation, especially H3K4me3, is an epigenetic signature that has often been associated with transcriptionally active genes (Santos-Rosa *et al.*, 2002; Zhang *et al.*, 2009). This chromatin modification is also found in context of other abiotic stress memory processes: a rise of H3K4 hyper-methylation levels is observable at dehydration memory genes

(Ding *et al.*, 2012) and hyperosmotic stress-related genes (Sani *et al.*, 2013) after their respective priming stresses. This indicates that H3K4 hyper-methylation might commonly function as a mark of recently active genes and influence the transcriptional re-activation of selected genes upon recurring stress. In the context of HS, the deposition of these chromatin marks is dependent on the action of HSFA2. While HSFA2 dissociates from HS memory-associated genes within the first few hours after acclimation, the chromatin marks persist at those loci and decrease slowly during maintained HS memory (Lämke *et al.*, 2016).

3.4.2.1 Role of FGT proteins in heat stress memory

In search of additional regulators of HS memory, a forward genetic screen was performed, to identify proteins with major function in type I transcriptional memory after HS. Three mutants, later *forgetter (fgt)*, were identified based on their deficiency in sustained expression of the HS memory gene *HSA32* (Brzezinka *et al.*, 2016; Urrea Castellanos *et al.*, 2020; Friedrich *et al.*, 2021). Similar to *hsfa2* mutants, *forgetter3* (*fgt3)* mutants exhibit an HS memory specific phenotype. However, unlike *hsfa2*, which is impaired in both transcriptional memory types, *fgt3* is only deficient in the sustained induction of HS memory-associated genes. *FGT3* encodes *HSFA3* and positively influences H3K4 hyper-methylation. It forms heteromeric complexes with HSFA2 and additional HSFs (HSFA1A, 1B, 1D, HSFA7A and HSFA6B). Considering that HSFs form trimers and hexamers, there is a high probability for HSFA2 and HSFA3 to work together with a variable third HSF. This might provide more flexibility towards environmental stress events (Friedrich *et al.*, 2021).

FORGETTER 1 (FGT1) is an orthologue of *Drosophila* Strawberry notch (Sno), which is a conserved helicase protein (Majumdar *et al.*, 1997). Loss of function of FGT1 results in HS memory defective phenotypes (Brzezinka *et al.*, 2016). FGT1 interacts with the chromatin remodeling proteins BRAHMA (BRM), CHROMATIN-REMODELING PROTEIN 11 (CHR11) and CHROMATIN-REMODELING PROTEIN 17 (CHR17). Chromatin remodeling describes changes in the organization of nucleosomal DNA, impacting the accessibility for DNA replication, DNA repair and selective gene expression (Song *et al.*, 2021). To gain access to and thusly activate genes, the transcription machinery requires open chromatin. Occupancy by nucleosomes – bundles of DNA and protein – plays a major part in how cells can control opening and closing of chromatin: low nucleosome occupancy results in open chromatin. In complex with BRM, CHR11 and CHR17, FGT1 is necessary to maintain open chromatin by mediating low nucleosome occupancy around the first nucleosome in the gene body (+1

nucleosome) at HS memory associated genes and ensures sustained gene expression after HS. Thus, nucleosome occupancy is another epigenetic regulatory mechanism in HS memory, additionally to histone modifying marks.

FGT2, encoding a plasma membrane located type 2C protein phosphatase (PP2C), isolated in the same screen, shows involvement in HS memory as well, likely by acting in lipid signaling or membrane composition dynamics (Urrea Castellanos *et al.*, 2020). Overall, the screen indicates that multiple regulatory layers are contributing to HS memory in plants.

3.4.2.2 Transgenerational heat stress memory

Whilst the aforementioned, somatic memory only lasts several days, there are also a few reports of transgenerational HS memory in plants (Migicovsky *et al.*, 2014; J. Liu *et al.*, 2019). The progeny of repeatedly heat stressed plants, exhibits an altered epigenome and physiology, such as accelerated flowering. The process behind transgenerational HS memory is poorly understood. It is reported, however, that inhibition of post transcriptional gene silencing as well as trans-acting small interfering RNA (tasiRNA) are involved in the transmission of HS memory to the progeny. The process involves HS-activated HSFA2, which targets the H3K27me3 demethylase gene *RELATIVE OF EARLY FLOWERING 6 (REF6)*, which in turn recruits BRM. Together, REF6 and BRM promote the reduction of H3K27me3 levels at the *HSFA2* locus, leading to its upregulation. In a positive feedback loop, HSFA2 triggers the upregulation of *REF6* and *BRM*. This regulatory loop activates an E3 ubiquitination ligase, SUPPRESSOR OF GENE SILENCING 3 (SGS3)-INTERACTING PROTEIN 1 (SGIP1). SGIP1 regulates a transgenerationally transmitted ubiquitination and degradation of SGS3, leading to lowered tasiRNA biogenesis. As a consequence, *HEAT-INDUCED TAS1 TARGET 5 (HTT5)* is upregulated. High expression levels of HTT5 promote early flowering and attenuated disease immunity. Depletion of repressive H3k27me3 marks at the *HSFA2* locus along with upregulation of *HSFA2*, are transmitted to the unstressed progeny, as well as the associated phenotypes (Liu *et al.*, 2019). HS induces the transcription of the copia-like retrotransposon ONSEN, that evolutionally acquired an HSE for activation by HSFs, like HSFA1 and HSFA2 (Ito *et al.*, 2011; Cavrak *et al.*, 2014). Its accumulation, however, is counteracted by the action of siRNAs. Mutants compromised in siRNA biogenesis exhibit transgenerationally, inherited retrotransposition and increased copy number of *ONSEN*. These results add to the observation in *Liu et al. 2019*, suggesting an important role of the siRNA pathway in regulating the inheritance of HS memory.

3.5 The Mediator complex

Mediator is a conserved multiprotein complex which is an essential component of the transcription machinery in all eucaryotes, functioning as a link between transcription factors and the RNA polymerase II (Pol II). The existence of Mediator was first discovered in yeast in 1990 whose presence is necessary for activator-mediated transcription (Kelleher *et al.*, 1990; Flanagan *et al.*, 1991).

The structure of yeast Mediator was visualized by electron microscopy, revealing that the complex is organized into three major modules, namely the "head", "middle" and "tail" module, each consisting of multiple Mediator subunits (MED subunits). Together they form the core Mediator (cMed) (Asturias *et al.*, 1999). In 2014, cryo-electron microscopy provided insights into the modular organization of Mediator and led to 3D models of the head and middle modules (Wang *et al.*, 2014). The head and middle modules fold back on one another to form a triangular structure (Figure 4). Both, the head (MED6, MED8 and MED17) as well as part of the middle module (the knob domain, consisting of MED4, MED7, MED14, MED31) interact with the carboxy-terminal domain (CTD) of Pol II (Nozawa *et al.*, 2017; Harper & Taatjes, 2018). Together they build the upper portion of the complex, while the tail module appears as a distinct dense structure at the base (Figure 4). Head, middle and tail domains are linked by the MED14 subunit, which serves as the central backbone of the entire complex. The tail module has been reported to mediate interactions with various transcription activators (Zhang *et al.*, 2004; Thakur *et al.*, 2009; Ansari *et al.*, 2012). The loss of tail module function in yeast was shown to affect the expression of genes involved in nutrient/metabolic transport and stress response which suggests a role of the tail module in regulation of genes that are differentially expressed in environmental stress conditions (Ansari *et al.*, 2012).

Results from various studies aiming to resolve the structure of Mediator revealed that the complex exists in multiple functionally distinct forms (Liao *et al.*, 1995; Han *et al.*, 1999; Chang *et al.*, 1999; Y. Liu *et al.*, 2001). This can partly be explained by differences in the methodical approaches used for the purification of the complex, but also flexibility in subunit composition. The best example of this high, structural variability is the reversible association of a fourth Mediator module, the CYCLIN DEPENDENT KINASE 8 (CDK8) kinase module (CKM), consisting of four subunits, MED12, MED13, CDK8 and CYCLIN C (CYCC), that can interact with cMed (Liao *et al.*, 1995; Borggrefe *et al.*, 2002). The association with the CKM delivers regulatory information to Pol II by interacting with different transcription factors (Dannappel *et al.*, 2019). 25 subunits, including the CKM, have been identified in yeast. Research

continually led to the discovery of Mediator in different organisms, including metazoans and plants. On one hand, the cumulative findings reveal a high variability of its exact composition in regard to amount and sequence homology of subunits. On the other hand, a similarity of the overall modular organization of this complex emerged and led to a unified Mediator subunit nomenclature in all species (Bourbon *et al.*, 2004).

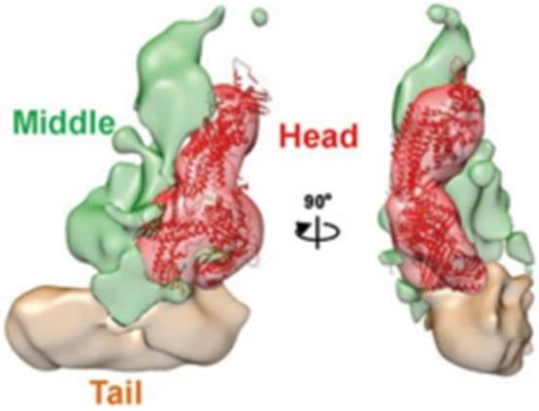

Figure 4. Three-dimensional (3D) reconstructions of cMed from *S. cerevisiae* in two different angles. The head (green) and middle (red) modules of the Mediator complex fold back on one another. Together they form the upper portion of cMed. The crystal structure of the head module from (Robinson *et al.*, 2012) is fitted into the 3D model (red ribbon). The tail module (brown) appears as a distinct dense domain at the base. Figure taken from (Wang *et al.*, 2014).

3.5.1 The Mediator complex in plants

In 2007, a Mediator complex was successfully purified from plants with 21 conserved subunits and six additional plant-specific subunits, named MED32-37 (Bäckström *et al.*, 2007). One year later, two of the six plant-specific subunits, MED32 and MED33 were identified as homologues of the yeast tail module subunits MED2 and MED5, respectively (Bourbon, 2008). The CKM was not purified with the *Arabidopsis* Mediator complex, which was attributed to the conditional and reversible nature of the interaction with Mediator. However, the existence of homologous genes to the four subunits of the CKM was confirmed by bioinformatic approaches and additionally, another putative subunit, MED30, was identified in *A. thaliana* using this approach (Bourbon, 2008). A recent publication delivered experimental validation of the existence of the CKM by co-purification of all its four predicted subunits together with cMed (Guo *et al.*, 2021). In this study, the existence of the four plant specific MED subunits 34-37 were not confirmed. On the contrary, MED37 was found as a background protein that also showed up in the control sample and did not interact with any other mediator subunit. Two homologous histone acetyltransferases, HISTONE ACETYLTRANSFERASE OF THE CBP FAMILY 1 and 5 (HAC1 and HAC5), were additionally identified as plant-specific Mediator subunits. Adding up the findings from biochemical purification and bioinformatic studies in recent years, the plant Mediator complex, including the CKM, is currently estimated to consist of 34-36 subunits.

3.5.2 Role in plant development

Many plant mediator subunits are associated with various roles in regulating development and response to different abiotic and biotic stresses. Analysis of tissue and developmental stage specific expression of the Mediator subunits in *Arabidopsis* and rice indicated a role for several subunits during developmental transitions (Mathur *et al.*, 2011). Mediator subunits from the CKM (MED12 and MED13) were reported to regulate the transition from juvenile to adult stage (vegetative phase change). Mutations of the two CKM subunits led to growth arrest resulting from altered expression of the phase-transition-associated miRNA, miR156 and miR172 (Gillmor *et al.*, 2014; Buendía-Monreal & Gillmor, 2016). Three other Mediator subunits, MED17, MED18 and MED20a are also involved in vegetative phase change, having a role in the biogenesis of several miRNAs, among them miRNA159 (Kim *et al.*, 2011).

The first Mediator subunit found to regulate the switch from vegetative to reproductive growth (flowering transition) was MED25, originally identified as PHYTOCHROME AND FLOWERING TIME 1 (PFT1). MED25 acts in the photoperiod pathway downstream of phyB regulating the expression of the flowering promoting gene FLOWERING LOCUS T (FT) (Cerdán and Chory, 2003). Another well characterized Mediator subunit involved in floral transition is MED18. MED18 directly binds to promotors of the key floral repressor FLOWERING LOCUS C (FLC) and plays a critical role in its repression to contribute to the flowering timepoint (Zheng *et al*., 2013; Lai *et al.*, 2014). MED25 and MED18 are only two of several Mediator subunits that impact flowering time. The floral transition is a process that seems to be strongly impacted by Mediator, as various mutants from all four Mediator modules exhibit a late flowering phenotype. Their impact on flowering control includes almost all known genetic pathways: the photoperiod pathway, vernalization, the autonomous pathway and the gibberellic acid (GA) pathway (Kidd *et al.*, 2009; Gillmor *et al.*, 2014; Knight *et al.*, 2008; Kim *et al.*, 2011).

3.5.3 Role in biotic stress responses

The Mediator complex is an essential regulatory component of the plant's immune response pathway. A considerably high number of Mediator subunits were found to contribute to the defense response in plants. MED25 was one of the first Mediator subunits found to be involved in the defense response to pathogen infection. The higher susceptibility to the necrotrophic fungal pathogen *Alternaria brassiciola* in the *med25* mutant is a result of decreased jasmonic acid (JA)-induced expression of defense genes, like PLANT DEFENSIN 1.2 (PDF1.2) (Kidd

et al., 2009). Interaction of MED25 with the transcriptional activator MYC2 suggests that MED25 acts as a coactivator to promote JA-triggered gene expression by recruiting Pol II to promotors of MYC2 targets (Chen *et al.*, 2012). Multiple other transcription factors, including members from the key JA-associated protein family of APETALA2/ETHYLENE RESPONSIVE FACTOR (AP2/ERF) interact with MED25, emphasizing its key function in the JA-induced transcriptional changes (Çevik *et al.*, 2012). Similar to MED25, MED8 was found to be required for necrotrophic pathogen resistance. The *med8* mutant however additionally shows a strong delayed flowering phenotype leaving the possibility of secondary effects that affect the immune response (Kidd *et al.*, 2009). MED18 is another subunit that is involved in the plant immune response, inhibiting the progression of necrotrophic pathogen infection. Interestingly, MED18 operates mechanistically different from MED25 and MED8 and functions independently of JA-mediated gene expression response. Instead, MED18 interacts with structurally distinct key transcriptional regulators that impact the ABA response, flowering and plant immunity. For instance, the interaction between MED18 and the transcriptional repressor YIN YANG 1 (YY1) mediates the suppression of disease susceptibility genes (thioredoxins and glutaredoxin). In addition to its repressing function, MED18 also has an activating role in the immune response and regulates the upregulation of defense-related gene *PEPTIDE TRANSPORTER 3 (PTR3)*. The disruption of *MED18* correlates with decreased Pol II occupancy and significantly changed histone 3 lysine 36 tri-methylation (H3k36me3) at *PTR3* and other target loci of MED18 (Lai *et al.*, 2014). The different roles of MED18 regarding its contribution to basal transcription as part of the Mediator complex on one hand and its highly specific roles in either suppression or activation of gene expression depending on the interaction partner on the other hand, illustrate the essential and diverse impact of Mediator on plant development and stress response.

Three subunits from the tail module, MED14, MED15 and MED16, have been associated with resistance to pathogens. Not only do *med16* mutants exhibit enhanced susceptibility to necrotrophic funghi and the biotrophic bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000, but MED16 also plays a role in systemic acquired resistance (SAR) which describes the process of long-term protection from pathogen attacks. SAR is triggered by a localized infection response that is spread through the plants tissue to confer resistance upon a secondary infection. The process is mediated by the signal molecule SA which induces the key regulator protein NONEXPRESSER OF PR GENES 1 (NPR1). *med16* mutants exhibit a reduced accumulation of NPR1 protein levels after the treatment with an SA-analog (Zhang *et al.*, 2012). Similar to MED16, MED14 and MED15 play a role in SA-signaling and turned out

to be positive regulators of SAR as well, pointing out an important role of the mediator tail module in both, the basal plant immunity and SAR (Zhang *et al*., 2013; Canet *et al.*, 2012). A recent study which examined the function of Mediator subunits in *Oryza sativa* also found that OsMED16 and OsMED25 silencing decreased the resistance to the fungus *Magnaporthe grisea* (Zhang *et al.*, 2021)

An interaction between MED25 and the CKM subunit CDK8 suggested a role for the Mediator kinase module in the plant immune response (Zhu *et al.*, 2014). Indeed, a positive regulatory role for CDK8 regarding the expression of JA-responsive genes like PDF1.2 and AGMATINE COUMAROYLTRANSFERASE (AACT1) was found. The positive role of CDK8 in resistance to the necrotrophic pathogen *Alternaria brassicicola* is dependent on the kinase activity. A single amino acid substitution that disrupts the phosphorylation activity of CDK8 results in susceptibility to the fungal pathogen. However, CDK8 also has a role in the negative regulation of resistance to the pathogen *Botrytis cinerea* independently of its kinase function. The study from Zhu *et al.* revealed that CDK8 has a function in the cuticle development. The *cdk8* mutant exhibits increased cuticle permeability and reduced thickness, resulting in a weaker physical barrier against pathogens. Higher resistance to *B. cinerea* in the *cdk8* mutant was suggested to result from CDK8's function in regulation of cuticle permeability which favors the faster recruitment of antifungal compounds to the infection site. Both *med12* and *med13* exhibited similar phenotypes in regard to the susceptibility to both pathogens, suggesting a general role of the CKM in the immune response (Zhu *et al.*, 2014). In a study that analyzed the function of HISTONE MONOUBIQUITINATION1 (HUB1) in plant defense, MED21 was found as an interaction partner. HUB1 and its homologue HUB2 mediate the monoubiquitination of histone 2B (H2B), which is a posttranslational modification associated with active gene transcription (Roudier *et al.*, 2011; Woloszynska *et al.*, 2019). HUB1 and MED21 expression is induced by chitin which is a pathogen-associated molecular pattern (PAMP) derived from the fungal cell wall. Both, *hub1* and RNA interference lines with reduced MED21 expression showed similar enhanced susceptibility phenotypes to necrotrophic pathogens. Thus, MED21 might be the mediator between chromatin modification and regulation of Pol II in transcription of genes that are relevant for pathogen resistance (Dhawan *et al.*, 2009).

3.5.4 Role in abiotic stress responses

Several subunits of the Mediator complex operate in abiotic stress responses such as drought, high salinity, cold and heat stress. The *Med19a* mutant shows lower tolerance to drought stress with 60% less survival rate compared to wild type plants after dehydration and subsequent rewatering. The mutant phenotype was explained by a reduced sensitivity to stomatal closure rate, which is a process triggered by ABA. ABA-regulated gene expression is indeed partially affected in the mutant and the expression level of the ABA-responsive gene RESPONSIVE TO DESICCATION 29B (RD29B), which is known to accumulate in drought stress and positively regulates drought stress tolerance in plants, was downregulated in the *med19a* mutant (Li *et al*., 2018; Liu *et al.*, 2020).

In contrast to MED19a, MED25 negatively regulates drought resistance. MED25 interacts with DREB2A, which operates both as transcriptional repressor and activator. While *dreb2a* mutants are sensitive to drought, *med25* mutants show the opposite phenotype and are drought resistant. MED25 binds to a region in DREB2A between its activation domain and its C-terminal repression domain. It was proposed that MED25 keeps the repression domain of DREB2A accessible for interaction with another Mediator subunit to mediate repression of DREB2A-activated genes. In high salinity conditions MED25 acts together with two transcriptional activators, ZINC FINGER HOMEODOMAIN 1 (ZFHD1) and a MYELOBLASTOSIS (MYB)-like protein to function as a positive regulator of salt stress resistance in contrast to its role as a negative regulator of drought resistance (Elfving *et al.*, 2011).

Another positive regulator of drought is CDK8. CDK8 associates with the ERF/AP2 transcriptional activator RELATED TO AP2 6 (RAP2.6) which is involved in plant development and tolerance to abiotic stresses through the ABA signaling pathway. RAP2.6 interacts with the protein kinase SUCROSE NONFERMENTING 1-RELATED PROTEIN KINASE 2-6 (SnRK2.6), an important player in ABA-induced stomatal closure, while an interaction between CDK8 and SnRK2.6 was only observed in presence of RAP2.6. It was suggested that their combined action functions as a shortcut to control transcriptional activation upon ABA and drought by directly linking important signaling pathway contributors to the Pol II machinery through CDK8 (Zhu *et al.*, 2020) (Figure 5). A study that investigated mitochondrial to nuclear communication (retrograde signaling) found that CDK8 participates in integrating multiple environmental signals with plant development. The findings revealed that a mutation in *CDK8* results in an impaired response to H_2O_2 and cold treatment (Ng *et al.*, 2013). An interaction with SNF1 KINASE HOMOLOG 10 (KIN10), a subunit of the Sucrose

Nonfermenting 1-Related Protein Kinase 1 (SnRK1) complex, which has a pivotal role in balancing signals for stress, growth, energy balance and metabolism, suggests that CDK8 and KIN10 cooperate to regulate stress-related genes, like *ALTERNATIVE OXIDASE 1* (*AOX1*), via a phosphorylation cascade (Baena-González *et al.*, 2007; Ng *et al.*, 2013) (Figure 5).

Three Mediator subunits from the tail module MED16, MED14 and MED2 participate in cold stress. Before MED16 was identified as a Mediator subunit, it was characterized as SENSITIVE TO FREEZING 6 (SFR6) to emphasize its involvement in freezing tolerance (Knight *et al.*, 2009). The *sfr6/med16* mutant suffers severe damage after cold acclimation at 4°C with subsequent freezing and recovery compared to wild type plants, which were unaffected by this treatment. The disrupted freezing tolerance is correlated with a deficiency in expression of several cold-regulated (COR) genes which harbor a C repeat/dehydration-responsive element (CRT/DRE) motif (Knight et al., 1999; Boyce et al., 2003). The reduced COR induction in *sfr6/med16* upon cold exposure did not result from a decrease of the C-repeat binding factor (CBF) transcription factor which is known to control the COR gene expression. Also, CBF recruitment to the COR gene promoters as well as epigenetic changes in form of increased histone acetylation and reduction of nucleosome density following cold exposure at COR gene promoters were not affected in the mutant. Instead, the phenotype resulted from a drastic decrease of Pol II recruitment to cold-inducible target genes of CBF. While Pol II occupancy increased significantly after cold treatment, *srf6/med16* failed to initially recruit Pol II, while the Pol II progression was not affected. MED14 and MED2, which were selected due to close proximity to MED16 based on yeast Mediator interaction studies, operate in cold acclimation as well (Hemsley *et al*., 2014; Guglielmi *et al.*, 2004). A reduced resistance to cold stress could also be observed in *O. sativa* after silencing *OsMED16.* Lower levels of OsMED16 resulted in reduced expression of cold-responsive genes and high levels of malondialdehyde (MDA), a physiological index for the degree of cell membrane injuries and lipid peroxidation (Zhang *et al.*, 2021).

A recent study analyzed the involvement of Mediator in HS which focused on the two structural hub subunits, MED14 and MED17. Disruption of both genes led to decreased bTT and aTT (Ohama *et al.*, 2021). Two additional subunits, MED16 and MED18, were tested regarding their role in thermotolerance to address the specificity of the observed phenotype and both did not show any defects in the HS treatments. 24% of HS-inducible genes did not get properly induced in *med14* and *med17*, among them key HS transcription factors like DEB2A, HSFA7B, while HSP gene expression was not decreased after HS treatment compared to WT (Ohama *et al.*, 2021).

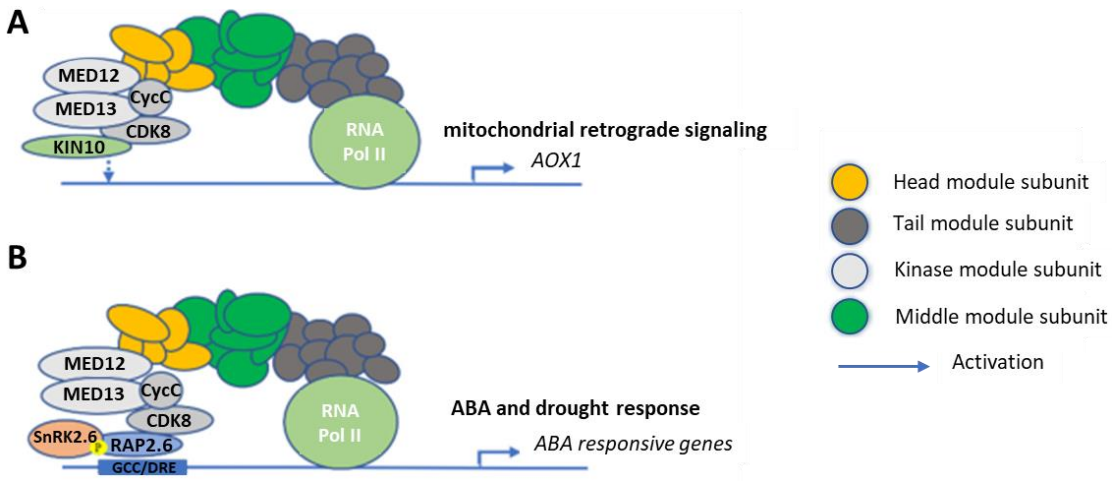

Figure 5. Two mechanisms of CDK8-mediated transcriptional regulation in abiotic stress response A CDK8 is involved in mitochondrial retrograde signaling. In association with KIN10, CDK8 positively regulates stress-responsive genes, like *AOX1*. B CDK8 interacts with the transcriptional activator RAP2.6. Together with the kinase SnRK2.6, they positively regulate transcription of ABA-responsive genes. A proposed model suggests that RAP2.6 is phosphorylated by SRK2.6 and associates with CDK8 to recruit Pol II to its target promoters (GCC/DRE motif). Figure modified from (Chong *et al.*, 2020).

3.5.5 Role in transcriptional memory

In budding yeast, *INOSITOL-1-PHOSPHATE SYNTHASE (INO1)* exhibits transcriptional memory and requires Mediator for epigenetic transcriptional poising (D'Urso *et al.*, 2016). Upon induction, *INO1* changes its subnuclear localization. While it is distributed randomly in the nucleoplasm under repressing conditions in presence of inositol, transcriptional activation by inositol starvation leads to a translocation of *INO1* to the nuclear periphery where it is stably maintained over several generations after returning to repressing conditions (Brickner *et al.*, 2007). After returning to repressing conditions, *INO1* does not only exhibit memory in form of retaining at the nuclear periphery but also shows a faster rate of transcriptional re-activation after subsequent activation. *INO1* re-activation is dependent on a promoter *cis*-acting memory recruitment sequence (MRS) which is targeted by the transcription factor SUPPRESSOR GENE FOR FLOCCULATION (SFL1) that binds specifically during memory (Light *et al.*, 2010). Different chromatin states at the *INO1* locus can be observed depending on the transcriptional activity (D'Urso *et al.*, 2016). While repressed, *INO1* is hypoacetylated and unmethylated on H3K4. Activation leads to both, hyper-acetylation and di- and trimethylation of H3K4. Upon repression followed by the above-described memory phase, the acetylation and H3K4me3 marks are lost, while H3K4me2 levels persist. Furthermore, during the memory

phase, Pol II and general transcription factors, forming the preinitiation complex (PIC), are still associated with the *INO1* locus. KIN28, a member of the kinase module belonging to the multisubunit TRANSCRIPTION FACTOR IIH (TFIIH), however, was absent during memory. Consequently, the CTD of Pol II was found in an unphosphorylated state which was interpreted as an inactive, poised Pol II form that mediates the process of re-activation upon subsequent inositol starvation (Light *et al.*, 2013). The continuous PIC recruitment and Pol II binding was found to be regulated by the Mediator complex. Interestingly, Mediator does not only bind to *INO1* during activating conditions to mediate the assembly of the PIC, it could further be observed that a different composition of the complex takes an active role in transcriptional memory. The memory-related Mediator complex included the Cdk8 module which was absent under activating conditions. INO1 re-activation was slower in cells lacking the yeast CDK8 SUPPRESSOR OF SNF1 3 (SSN3), while the initial activation was unaffected, suggesting a specific role in memory conditions and transcriptional poising of Pol II. The authors point out a conserved role of CDK8 in transcriptional memory in mammalian cells as well. Gamma interferon (IFN-y) has been shown to prime mammalian cells for enhanced transcription of specific genes upon a second stimulus (Gialitakis *et al.*, 2010). Similar to what was observed in yeast, CDK8 was found to associate specifically with genes that exhibit transcriptional memory after removal of the IFN-y stimulus suggesting a conserved function in transcriptional memory for CDK8 (D'Urso *et al.*, 2016).

The *Arabidopsis* MED25 subunit has a positive role in JA-mediated stress response (Chen *et al.*, 2012). Dehydration as well as treatment with JA were shown to prime a subset of memory genes for enhanced re-induction upon a subsequent dehydration stress. Both, priming by JA and drought treatment correlate with accumulation of MED25 at certain dehydration memory genes (Liu & Avramova, 2016). MED25 gets recruited by MYC2, a key transcription factor in JA-/drought-related transcription. While MED25 remained associated with the memory genes, MYC2 dissociated after priming. Elevated phosphorylation of serine 5 of Pol II CTD (Ser5P) correlated with MED25 binding, presumably facilitating the faster re-induction upon a recurrent stress incident. The direct interaction between MYC2 and MED25 suggested a mechanism where Mediator is recruited to specific dehydration memory genes after priming to initiate the assembly of a paused or stalled PIC.

4. Research Aims

The aim of this study was to perform a forward mutagenesis screen on a *pAPX2::LUC* transgenic *A. thaliana* line to identify mutants with a modified re-induction pattern (type II transcriptional memory) after the triggering HS. This screen was intended to further elucidate the mechanistic basis of HS memory in plants. To accomplish this goal, the following aspects were addressed in the scope of this thesis:

- Identify several re-induction (*rein*) mutants and select the most promising mutants for further analysis.
- Identify potential candidate genes for the *rein* mutants and further confirm the candidates using additional alleles and complementation.
- Perform further characterization of the loss-of-transcriptional memory phenotype with respect to gene expression of selected HS memory genes.
- Characterize the role of *rein* mutants in HS memory at the physiological level by evaluating the seedling survival under different types of HS regimes.
- Investigate the interaction of the REIN factors with known chromatin-based regulators of HS-induced type II transcriptional memory, such as HSFA2, to analyze the molecular mechanism.

5. Results

5.1 Luciferase-based screen to identify regulators of type II transcriptional memory

HS memory is associated with transcriptional memory of a subset of genes. The memory gene *APX2* is heat-inducible and exhibits type I transcriptional memory after HS. At the same time, it is also one of a subset of memory genes that shows type II transcriptional memory upon a second HS stimulus, i.e. enhanced re-induction upon recurrent HS. To gain insights into type II transcriptional memory regulation and find new layers of HS memory control, a complementary genetic screen was initiated with a *pAPX2::LUC* reporter. The mutagenesis screen was done based on the published *Arabidopsis* transgenic line *pAPX2::LUC* which expresses 593 bp of the promoter from memory gene *APX2* fused to the luciferase gene from the common eastern firefly *Photinus pyralis* (Figure 6 A) (Liu *et al.*, 2018).

The following section describes the experimental approach of this screen leading to the identification of several potential re-induction (*rein*) mutants with altered *pAPX2::LUC* expression after repeated HS.

5.1.1 Visualization of type II transcriptional memory with a *pAPX2::LUC* reporter line

Type II transcriptional memory of *APX2* after a priming HS (37°C for 1 h) is strong compared to other HS memory genes and lasts for at least 5 days with a gradual decrease of hyper-inducibility when increasing the length of the recovery phase (Friedrich et al., 2021).

Initially, it was analyzed whether the transcriptional behavior of *APX2* can be mimicked with the *pAPX2::LUC* reporter line to evaluate its suitability for a subsequent mutagenesis screen. *pAPX2::LUC* transgenic seedlings were subjected to three different HS treatments, aiming to test visualization of induction (T), sustained expression following a single HS event (P) and hyper-inducibility upon repeated HS (P+T) in luciferase reporter assays (Figure 6). By varying the amount of recovery days after P and between the P+T HS treatments, the *pAPX2::LUC* HS memory, duration and optimal conditions for the intended subsequent mutant screen were tested (Supplemental Figure S1).

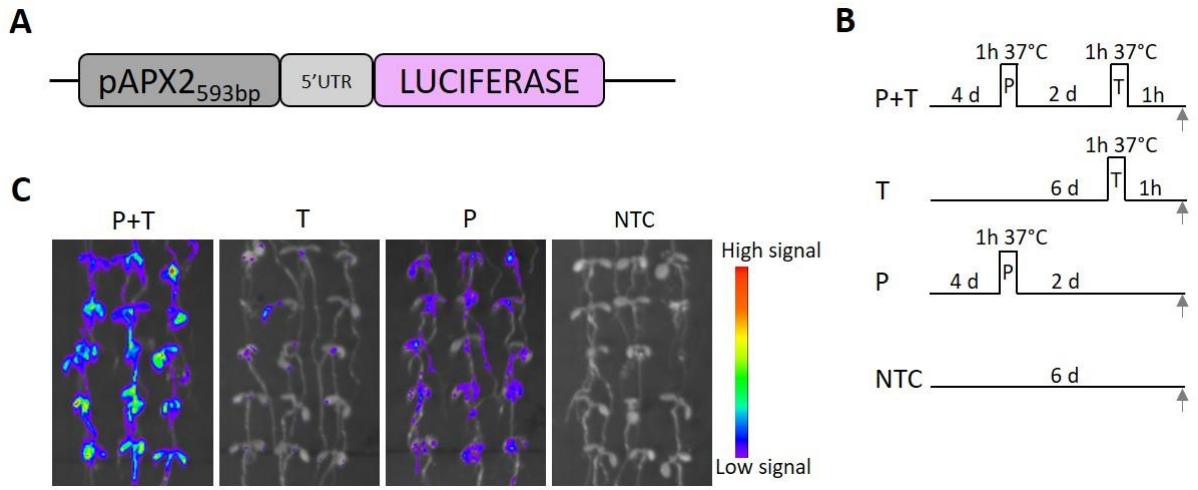

Figure 6. Transcriptional memory of *APX2* upon HS that can be visualized with a *pAPX2::LUC* reporter line. **(A)** Schematic representation of the *pAPX2::LUC* reporter gene. **(B)** Schematic representation of the HS treatments performed in A. The arrow symbolizes the timepoint of luciferase imaging. **(C)** Bioluminescence signal of the reporter construct *pAPX2::LUC* was assayed after repeated (P+T), single (T,P) HS treatments and in non-treated control (NTC) seedlings. The false color scale of relative luciferase activity is shown on the right.

Two days of recovery phase between the two HS treatments of 37°C were found to be suitable for displaying the hyper-induction of *APX2* (Figure 6 C). Hyper-induction of the *pAPX2::LUC* reporter gene upon repeated HS was clearly distinguishable from its induction upon a single HS, indicated by strong luciferase signals in P+T conditions compared to those after T or the remaining signal from P two days before visualization. While no more luciferase signal was detectable after 3 days of recovery following P, hyper-induction was still observable with 4 days of recovery between P and T treatment (Supplemental Figure S1).

5.1.2 Isolation of mutants with defective type II transcriptional memory

To identify regulators involved in type II transcriptional memory of *APX2*, repeated HS were performed on approximately 30 seedlings per EMS mutagenized M2 line following P+T conditions (Figure 6 B, top row) and luciferase signal was compared to the parental *pAPX2::LUC* line.

Out of 1077 screened M2 lines, 200 lines contained one or multiple seedlings with reduced *APX2::LUC* signal in P+T conditions compared to the parental line. These seedlings were transferred to soil and self-fertilized to produce the M3 generation. The M3 was retested to

validate the mutant luciferase phenotype in P+T conditions and to examine the initial induction and sustained activation of *pAPX2::LUC* (P, T) conditions. In 41 out of 200 selected (of which 58 were retested) M3 lines, the mutant phenotype originally observed in the M2 generation was confirmed (Figure 7).

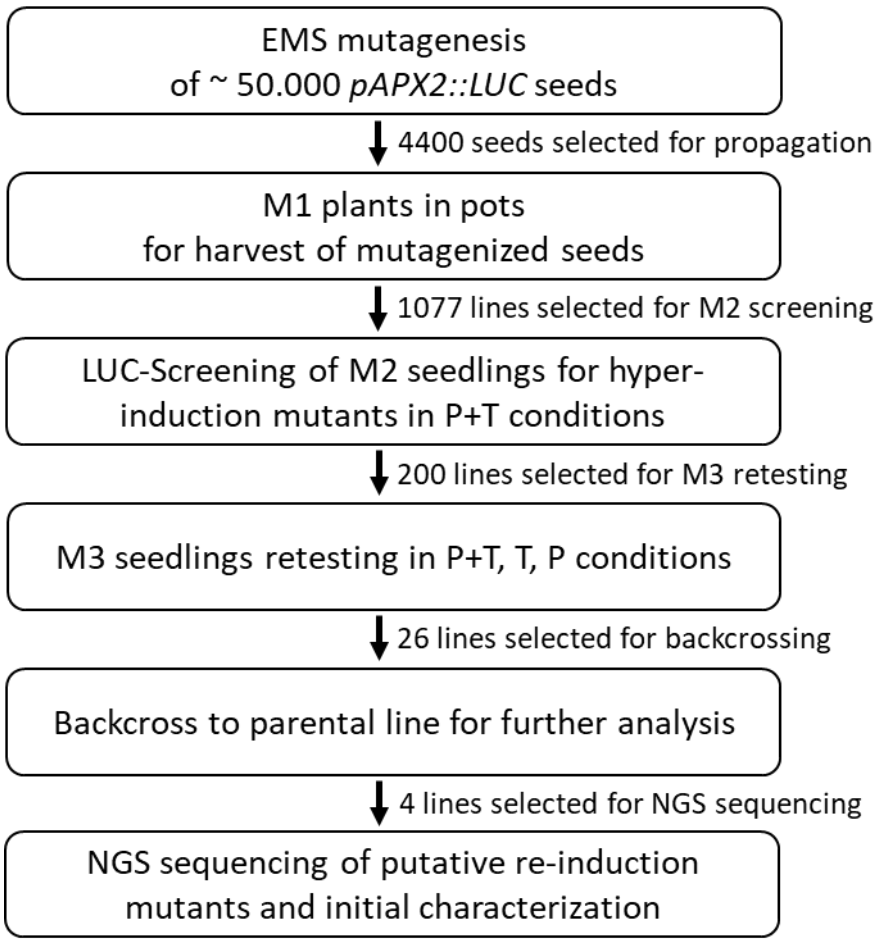

Figure 7. Outline of mutagenesis screen for modifiers of *pAPX2::LUC* expression in type II transcriptional memory. Approximately 4400 M1 plants, derived from mutagenized *pAPX2::LUC* transgenic seeds, were grown in the greenhouse and self-fertilized. M2 seedlings were tested for luciferase activity in P+T conditions. Individual seedlings from M2 lines with reduced *pAPX2::LUC* expression were selected and self-fertilized. Seedlings of M3 lines were re-tested for hyper-induction (P+T), induction (T), and sustained expression (P) of *pAPX2::LUC*. Hyper-induction mutants were backcrossed to the parental line for sequencing (with bulked segregants from the BC1F2 population) and further characterization (with homozygous BC1F3 plants).

Mutant lines with altered *pAPX2::LUC* expression in the M3 retesting conditions (P, T and P+T) were selected during the screening process. They were grouped in two different categories based on their luciferase phenotype after single and repeated HS treatments. Category A

contained lines with memory-specific phenotypes, specifically affected in hyper-induction of p*APX2::LUC*, while induction upon priming with a single HS was unaffected. From 41 confirmed M3 lines, 16 lines were sorted into category A, exemplified by the lines *134.5*, *144.7* and *177.2* (Figure 8).

To analyze the induction of *APX2::LUC* in more detail, a two-step acclimation HS (ACC) was performed to boost expression of the transgene (Figure 8, T conditions). Additionally, the recovery time after the priming HS was prolonged from one to four hours to exceed the detection threshold. This was done since the initial test with the parental line had shown that after one hour recovery, luciferase activity was barely detectable (Figure 6 A, T conditions).

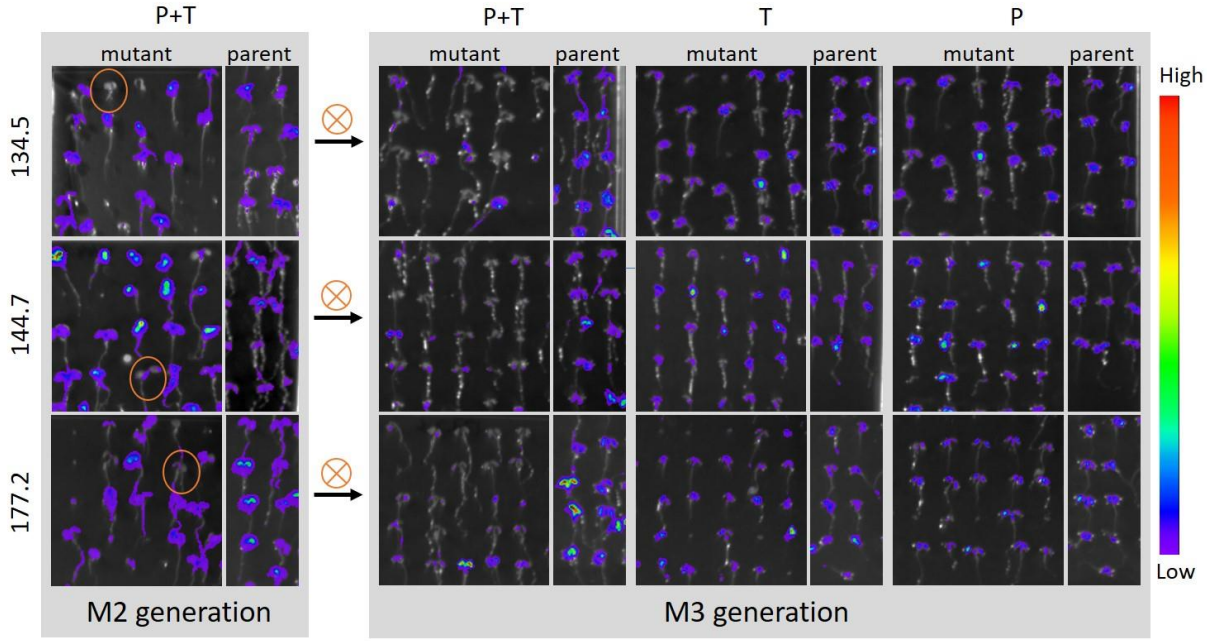

Figure 8. Three potential HS memory mutant lines (*134.5, 144.7* and *177.2*) of category A with reduced re-induction of *pAPX2::LUC* expression. *pAPX2::LUC*-derived bioluminescence was assayed after repeated HS (P+T) in the segregating M2 generation. Individual seedlings with reduced signal (orange circle) compared to the parental line were self-fertilized (x). In the M3 generation, seedlings were subjected to different HS regimes. P+T, HS 37°C for 1 h on day 4, 2 days recovery, HS 37°C for 1h on day 6, 1h recovery, luciferase imaging. T, ACC treatment (37°C for 1h, 1.5 h recovery, 44°C for 45 min) on day 6 with 4 h recovery before luciferase imaging. P, HS of 37°C for 1 h on day 4, 2 days recovery, luciferase imaging.

Category B comprised mutants affected in sustained and/or enhanced re-induction as well as initial induction of *pAPX2::LUC*. The majority of re-tested M3 lines were classified into category B and exhibited stronger defects in regard to reduced *APX2::LUC* expression after HS treatment compared to lines of category A (Figure 9).

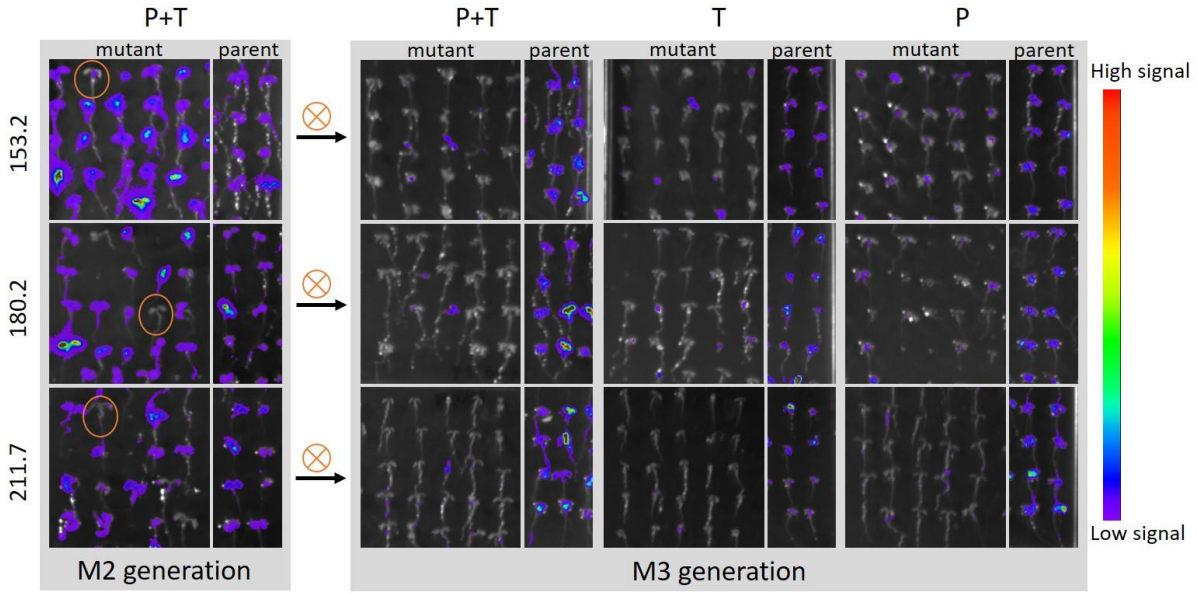

Figure 9. Three representative HS memory mutant lines (*153.2, 180.2, 211.7*) of category B with reduced *pAPX2::LUC* induction and hyper-induction. Luciferase activity of *pAPX2::LUC* was captured in segregating *rein* M2 lines next to the parent. Individuals with decreased luciferase levels compared to the parental line were selected (orange circle) for self-fertilization (x). The resulting M3 generation was assayed after different HS treatments to analyze enhanced re-induction (P+T), priming (T) and sustained induction (P). For detailed HS treatment conditions, see description in Figure 8.

26 M3 lines which exhibited reduced *pAPX2::LUC* expression following repeated HS from both categories were backcrossed to the parental line. In backcross-derived F2 populations (BC1F2), segregation of the mutant phenotype was assayed to analyze the dominance of the mutation. While six lines did not show segregation after backcrossing, all other BC1F2 populations segregated in a ratio of 3:1 indicating that the mutant phenotypes are caused by a heritable recessive mutation in a single locus.

The segregating BC1F2 generation of selected mutants was utilized for Next Generation Sequencing (NGS) to find the causal mutations in these lines. Additionally, seedlings with a low luciferase signal phenotype from these BC1F2 lines were selected for propagation. The resulting homozygous F3 population (BC1F3) was utilized for further analysis including expression analysis and physiological HS assays.

5.1.3 HSFA2 is required for hyper-induction of *pAPX2::LUC*

In parallel to the forward mutagenesis screen for identification of new regulators involved in HS memory, a well characterized HS memory-specific mutant, *hsfa2* (SALK_008978), was crossed into to the *pAPX2::LUC* line. HSFA2, which is dispensable for aTT, but specifically required for HS memory, is required for type I and type II transcriptional memory of HS memory genes. To visualize the effect of the *HSFA2* mutation on the expression of *pAPX2::LUC*, bioluminescence in seedlings of homozygous F3 progeny was assayed after repeated HS exposure (Figure 10).

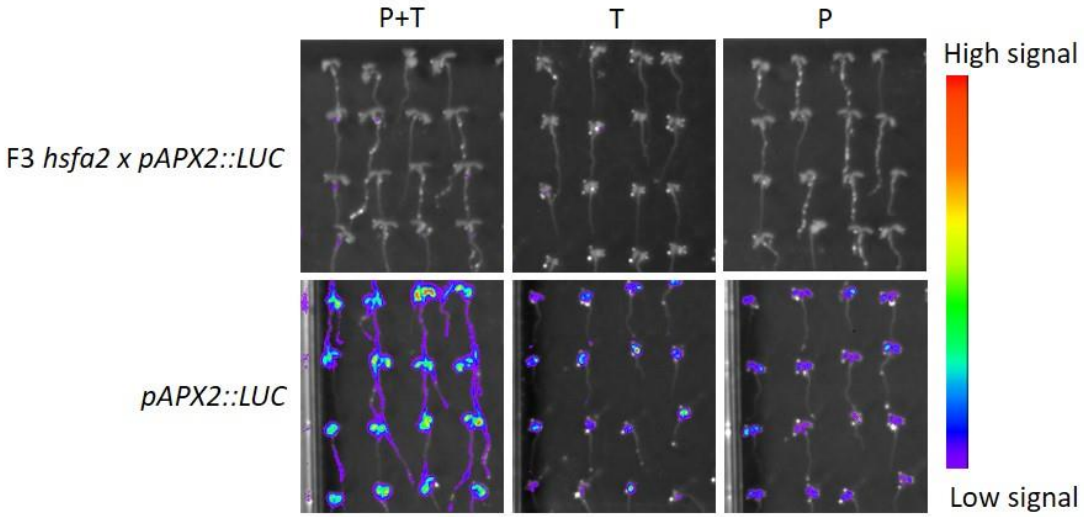

Figure 10. Introduction of the *hsfa2* mutant into *pAPX2::LUC* results in strong reduction of *pAPX2::LUC* activity after HS treatment. Luciferase reporter activity assessed in seedlings from homozygous F3 population of a *hsfa2* x *pAPX2::LUC* cross next to the parental line. Signal intensity was visualized after ACC on day 4 with 4 h recovery (T) or 2 days recovery (P) before imaging and after repeated HS (P+T) of each 37°C for 1 h on day 4 and 6 with 1 h recovery before imaging.

Unexpectedly, introduction of the *HSFA2* mutation into transgenic seedlings carrying *pAPX2::LUC* resulted in a strong reduction of *pAPX2::LUC* expression not only after repeated, but also single HS exposure (Figure 10), thus resembling category B mutants.

These results were supported by *rein* mutant line *180.2* which exhibited a mutation in the *HSFA2* gene displaying similar phenotype in luciferase assays after HS treatments (Figure 11 A). Mutant line *180.2*, which expressed almost no luciferase after single or repeated HS, was found to have barely detectable *HSFA2* transcript levels in addition to reduced *APX2* and *LUC* transgene expression following HS (Figure 11 B). By Sanger sequencing, a mutation

was detected in the splice acceptor site of the single intron in the *HSFA2* sequence (Supplemental Figure S2). The utilized primers for *HSFA2* mRNA detection flanked the intron (Supplemental Table S7) and consequently nonsense-mediated mRNA decay (NMD) as a result of the splice site mutation and a potential frameshift that leads to a premature stop codon could explain the absence of *HSFA2* transcripts in reverse transcription–quantitative polymerase chain reaction (RT-qPCR) analysis.

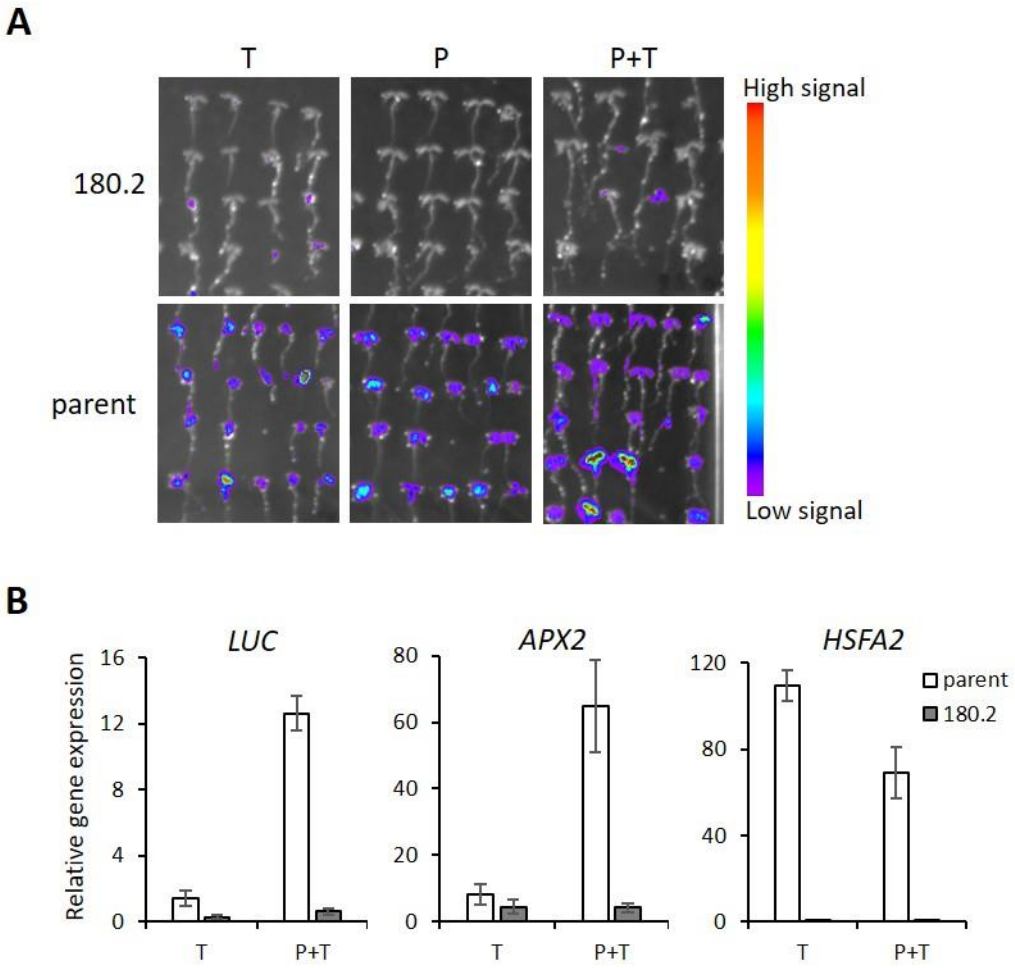

Figure 11. *rein* mutant line *180.2* exhibits reduced *pAPX2::LUC* expression and lower *APX2* and *HSFA2* after HS treatment. A Luciferase reporter activity was detected after an ACC with 4 h recovery (T), 52 h recovery (P) and after repeated HS (P+T) in the *180.2* mutant line compared to the *pAPX2::LUC* parental line. **B** Gene expression was analyzed by RT-qPCR normalized to *TUB6* expression**.** Plant material was collected from single (T) or repeated (P+T) HS-treated seedlings from line *180.2* and the parental line. Error bars show standard deviation from 3 biological replicates.

The *hsfa2* mutant displays a strong impairment of sustained induction and enhanced re-induction of all identified HS memory genes, shown in earlier studies (Lämke *et al.*, 2016; Liu *et al.*, 2018). Initial induction of *APX2* by a single HS treatment (37°C for 1 h), however, is not affected by absence of HSFA2 (Charng *et al.*, 2007). It was reported that the expression was rather suppressed in the mutant during prolonged HS and the recovery phase after HS, indicating that HSFA2 is not required for the acquisition of thermotolerance, but specifically for its maintenance. This may explain strongly reduced luciferase activity in T conditions in *hsfa2* mutants, taking into account that a minimum of 4 h recovery phase after the HS treatment was necessary to accumulate enough luciferase signal for detection using the NightOwl system.

The decision which mutants to select for further investigation was made according to the strength of the luciferase phenotype upon repeated HS treatments and consequently the confidence to select mutants for NGS sequencing in a BC1F2 segregating population. Furthermore, introduction of the *hsfa2* mutant revealed that not only category A, but also category B possibly contains candidates specifically involved in HS memory.

5.2 REIN1 is required for HS memory

Several putative *rein* mutants identified in the *pAPX2::LUC* forward genetic screen were chosen for initial investigation in respect to their role in HS response. Four mutant lines were selected for NGS sequencing using pools of individuals with exclusively mutant and WT phenotypes obtained from BC1F2 populations. Subsequently two of these lines were chosen for initial characterization within the scope of this thesis. The characterization of the *rein1* mutant will be described in the following section.

Some of the data, including the luciferase phenotype, physiological phenotype, and the interaction study with HSFAs, will be published in a manuscript in the near future.

5.2.1 The *rein1* mutant shows reduced re-induction of *pAPX2::LUC* after repeated HS

In mutant line *137.6*, a strong decrease in *pAPX2::LUC* signal, especially after repeated HS, was observed in the homozygous M3 and BC1F3 generations (Figure 12 A). The phenotype was stably inherited over generations and segregated as a recessive mutation in a the BC1F2 generation (Figure 12 B). The phenotype was confirmed in the homozygous BC1F3 generation and the mutant line *137.6* was renamed *rein1*.

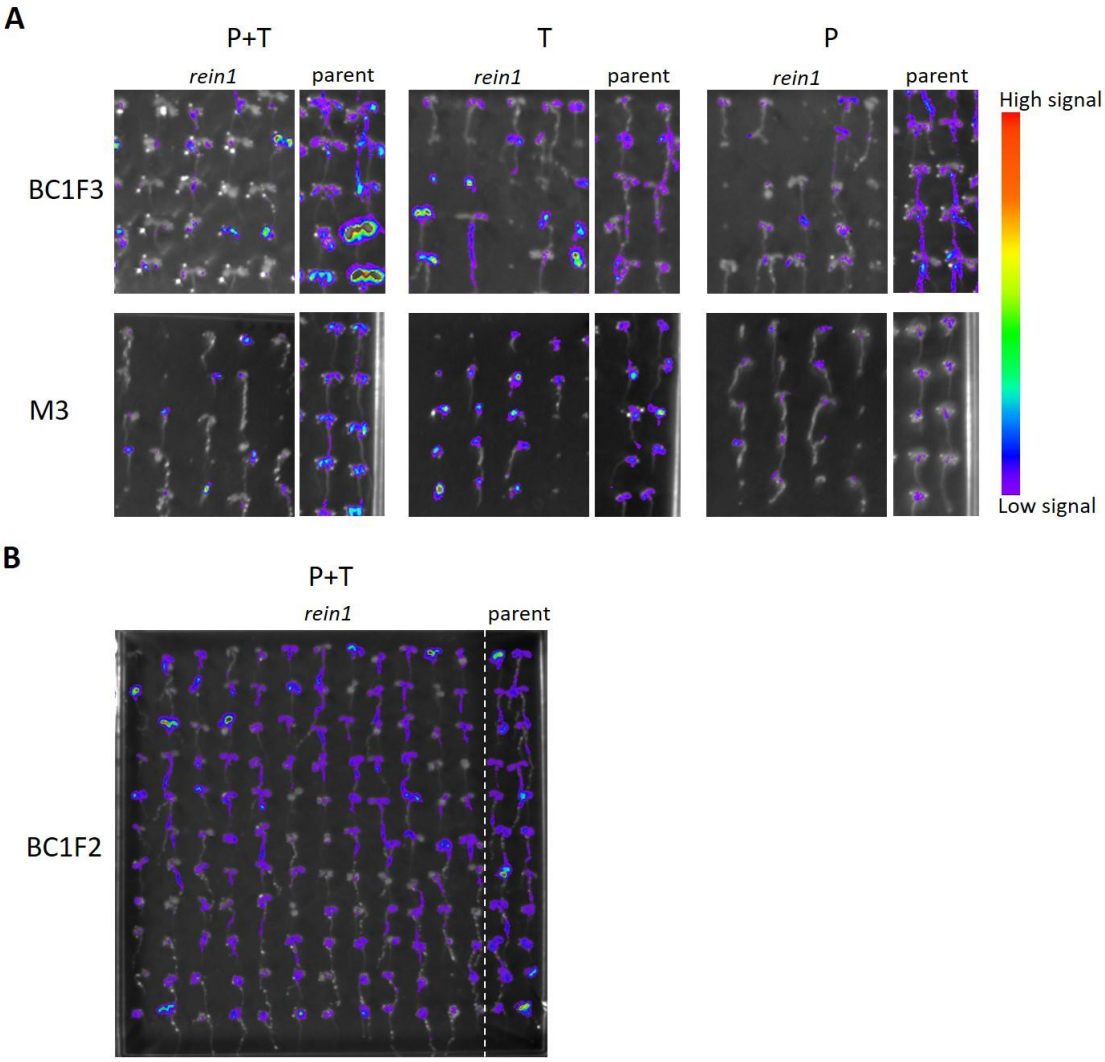

Figure 12. *pAPX2::LUC*-derived bioluminescence in seedlings from the M3 and BC1F3 generation of *rein1* following HS exposure compared to the parental line. A Luciferase activity of seedlings from *rein1* and the parental line *pAPX2::LUC* (parent) after different HS regimes. P+T, repeated HS on day 4 and day 6 with 1h recovery before imaging. T, a single HS on day 4 with 5 h recovery prior to imaging. P, a single HS on day 4 with 52 h of recovery prior to imaging. HS treatment of the seedlings was done at 37°C for 1h. **B** Luciferase activity in *rein1* segregating BC1F2 generation after repeated HS (P+T).

Luciferase activity assays indicated that *rein1* was deficient in both types of transcriptional memory (Figure 12 A). The mutation in *rein1* had a strong effect on type II transcriptional memory exhibited by a strong decrease of luciferase signal in P+T conditions, while type I transcriptional memory was also mildly affected, measured after two days of recovery (P).

In comparison to the strong effect that the *HSFA2* mutation had on luciferase expression after HS exposure, the mutation in *rein1* seemed to have an overall less severe effect on HS memory

of *APX2* and did not lead to complete loss of signal, but rather a strong decrease. The *rein1* mutant was classified into category A since the majority of *rein1* seedlings had the ability to induce *APX2* upon a priming HS in luciferase assays aimed at analyzing the induction of *APX2* (T).

5.2.2 *rein1* carries a mutation in *CDK8*

To identify the mutation causing the observed defect in transcriptional memory of *pAPX2::LUC* in luciferase assays following HS exposure, the segregating *rein1* BC1F2 population was used to generate two sequencing pools. To this end, plant material from 150-200 seedlings with exclusively mutant and wild type-like luciferase expression phenotype, respectively, were collected separately. The samples were used for library preparation, NGS was performed and analysis of the sequencing data was done by Dr. Christian Kappel (University of Potsdam).

Single Nucleotide Polymorphism (SNP) allele frequencies in the mutant and WT pool were calculated (Figure 13). This was done on the assumption that the causal change occurs with the highest frequency among all EMS-induced changes in the pool of bulked mutants, making it possible to identify the interval and candidate SNPs with the causal mutation.

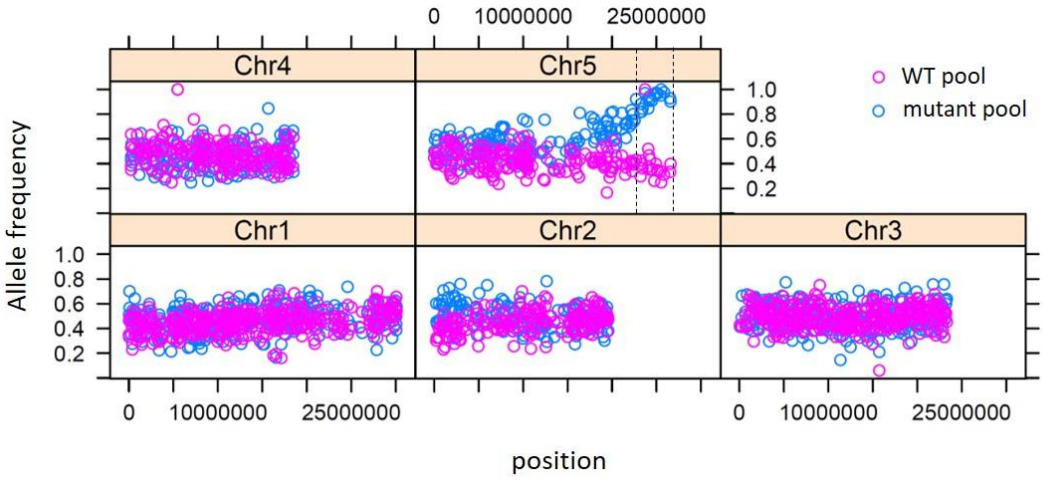

Figure 13. Allele frequencies of *rein1* mutant versus wild type markers from BC1F2 mutant (blue) and BC1F2 wild type (purple) pools. Variants are shown as dots with their position in base pairs along the five chromosomes. Dashed lines mark the interval containing the mutation causing the *rein1* mutant phenotype.

At the end of chromosome 5, a region of distorted segregation with variants enriched in the F2-mutant pool, but not in the F2-WT pool was detected (Figure 13). At around 25 megabase pairs

(Mbp) on this chromosome, the high mutant allele frequency peaked at 1.0 and consequently the surrounding region was selected for investigation into the causal mutation.

EMS treatment caused several SNPs in the putative region between 23.5 Mb and 26.5 Mb of chromosome 5 (Supplemental Table S5). Three mutations located in this interval were considered candidates due to their potential of having a strong impact on protein function. These included three guanine to adenine changes, which resulted in two missense mutations in *At5g50270* and *At5g61510* and one premature STOP codon in *At5g63610* (Table 1).

Table 1. Putative candidate genes identified in the *rein1* mutant to contain a locus conferring type II transcriptional memory of *APX2*. Position is given as base pair position on chromosome 1.

Position	Change	Gene ID	Target name	Description	Mutation effect
24258686	G → A	AT5G60270	LECRK-I.7	L-TYPE LECTIN RECEPTOR KINASE I.7	missense variant Arg309Lys
24737095	G → A	AT5G61510	AT5G61510	GroES-like zinc-binding alcohol dehydrogenase family protein	missense variant Leu404Phe
25463785	G → A	AT5G63610	CDK8	CYCLIN-DEPENDENT KINASE E;1	Stop gained Gln425*

The mutation in *At5g60270* which encodes L-TYPE LECTIN RECEPTOR KINASE I.7 (LECRK-1.7) resulted in a change from arginine to lysine at amino acid 309 and occurred between two predicted conserved domains for this protein: A protein kinase domain (accession National Center for Biotechnology Information (NCBI): cl21453) and a legume lectin domain (accession NCBI: pfam00139). The other candidate missense mutation occurred in *At5g61510* which encodes a putative NADP-dependent oxidoreductase. The mutation was located in the second to last amino acid, causing a change from leucine to phenylalanine.

The third candidate mutation in the list affected *At5g63610* that encodes CYCLIN-DEPENDENT KINASE E1 (CDK8). The guanine to adenine substitution in the *CDK8* gene introduced a nonsense mutation leading to a premature stop codon at residue 425 at the protein level (Figure 14). Nonsense mutations resulting in premature stop codons are considered severe mutations since they can alter stability and function of transcripts and proteins.

Figure 14. A schematic model of the CDK8 protein structure with the position of the amino acid change introduced in *rein1*. CDK8 contains a S_TKc domain (Serine/Threonine protein kinases, catalytic domain), predicted in Simple Modular Architecture Research Tool (SMART, accession number: SM000220). The mutation in *rein1* leads to an amino acid change at position 425 from glutamine to a premature stop codon.

CDK8 is the plant homologue of the mammalian and yeast CDK8 which phosphorylates the CTD of Pol II (Hengartner *et al.*, 1998). CDK8, a part of the CKM, functions as a separable accessory module of Mediator, a conserved multi-subunit complex bridging transcriptional regulators to the Pol II machinery (Malik and Roeder, 2010; Bourbon, 2008). Several recent reports revealed the contribution of plant Mediator subunits to regulation of gene expression in various physiological processes, including plants defense, developmental transition and stress response (Yang *et al.*, 2016). Therefore, the mutation causing a premature stop codon in *CDK8* was considered a likely candidate causing the mutant phenotype in *rein1* in the luciferase assays.

5.2.3 *rein1* is allelic to *cdk8*

A complementation test was performed to validate the SNP in *CDK8* was indeed causing the luciferase phenotype in *rein1* mutants. A previously described independent *cdk8* T-DNA insertion line (GABI_564F11) was used to confirm that *rein1* and *cdk8* were allelic. The luciferase phenotype of F1 seedlings resulting from two independent crosses between *rein1* and *cdk8* was assessed (Figure 15). The seedlings were grown for 6 days before they were subjected to repeated HS. Two control crosses between the *pAPX2::LUC* parental line and *cdk8* and between Col-0 and *rein1* were performed in parallel to the complementation cross to evaluate the effect of reduction of the *pAPX2::LUC* transgene and the heterozygous state of *rein1* or *cdk8* in the F1 generation on luciferase signal (Figure 15).

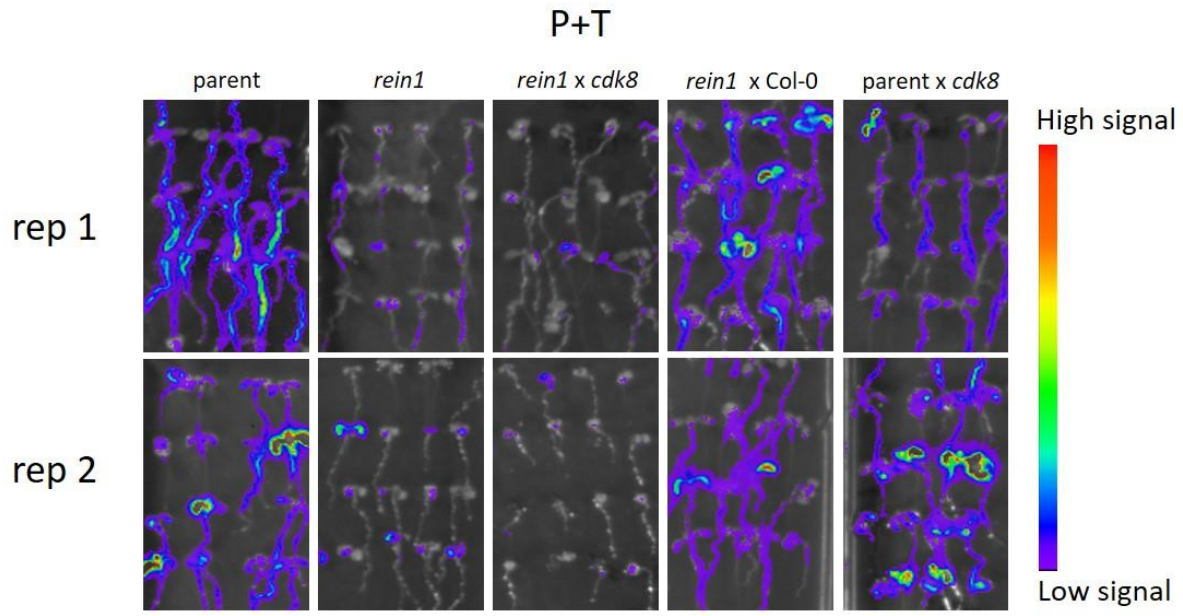

Figure 15. Complementation cross confirms allelism of *rein1* and *cdk8*. *pAPX2::LUC*-derived bioluminescence was measured in in the F1 generations from *rein1* and *cdk8* crosses next to the parent (*pAPX2::LUC)* and *rein1* mutants after repeated HS. F1 seedlings from two control crosses were included in the assay, consisting of parent x *cdk8* and *rein1* x Col-0. Results from two independent crosses and assays are shown. The false color scale of relative luciferase activity is shown on the right.

Luciferase activity in F1 seedlings of the *rein1* x *cdk8* complementation cross was strongly reduced after repeated HS compared to the signal of the parental line. The signal reduction was comparable to that in homozygous *rein1* mutants (Figure 15). It was also observed that both control crosses, parent x *cdk8* and Col-0 x *rein1* expressed *pAPX2::LUC* similarly to the parental line. This ruled out that the signal reduction resulted from the heterozygous state of the *pAPX2::LUC* transgene in *rein1* x *cdk8* plants. Furthermore, the *cdk8* x parent cross showed that introduction of a single *cdk8* mutant allele in the *pAPX2::LUC* background alone did not cause a strong reduction of the luciferase signal. Consequently, lack of complementation in the F1 hybrids from the *cdk8* x *rein1* cross in luciferase assays indicated that the two mutants are allelic and thus the stop-gain mutation in *CDK8* was causing the hyper-induction-deficient phenotype in *rein1* mutants.

5.2.4 The expression of HS-inducible memory genes is affected in *rein1* and *cdk8* mutants

The decrease in sustained expression and the reduced re-induction of *pAPX2::LUC* in *rein1* mutants following HS exposure indicated an involvement of *CDK8* in HS memory. To test whether reduced expression of the luciferase transgene mimicked decreased endogenous *APX2* transcript levels, RT-qPCR was used to analyze expression levels of *APX2* following HS treatment in *rein1* and *cdk8* T-DNA mutant seedlings (Figure 16). Additionally, transcriptional behavior of four other HS memory genes was tested to investigate whether *CDK8* function was specifically required for the regulation of *APX2* or expanded to other HS-responsive genes. *MYO-INOSITOL-1-PHOSPHATE SYNTHASE 2* (*MIPS2*), *CYTOCHROME P450 81D11* (*CYP81D11*), *XYLOGLUCAN ENDOTRANSGLYCOSYLASE 6* (*XTR6*) are induced upon a single HS and were selected due to their robust type II transcriptional memory behavior (+/++ class) (Liu *et al.*, 2018). *LONG CHAIN ACYL-COA SYNTHETASE 9* (*LACS9*) is also classified as type II transcriptional memory gene. In contrast to the other selected memory genes, it does not respond to a single priming HS, but is activated upon a triggering HS with prior priming stimulus (0/+ class) (Liu *et al.*, 2018).

Additionally, two of the tested genes, *APX2* and *XTR6* exhibit sustained expression after HS and were selected to gain more information on the role of CDK8 in type I transcriptional memory. Transcript levels in the *hsfa2* mutant were measured at the same time as *rein1*, *cdk8*, Col-0 and the *pAPX2::LUC* parental line, representing a factor that is specifically required for transcriptional HS memory.

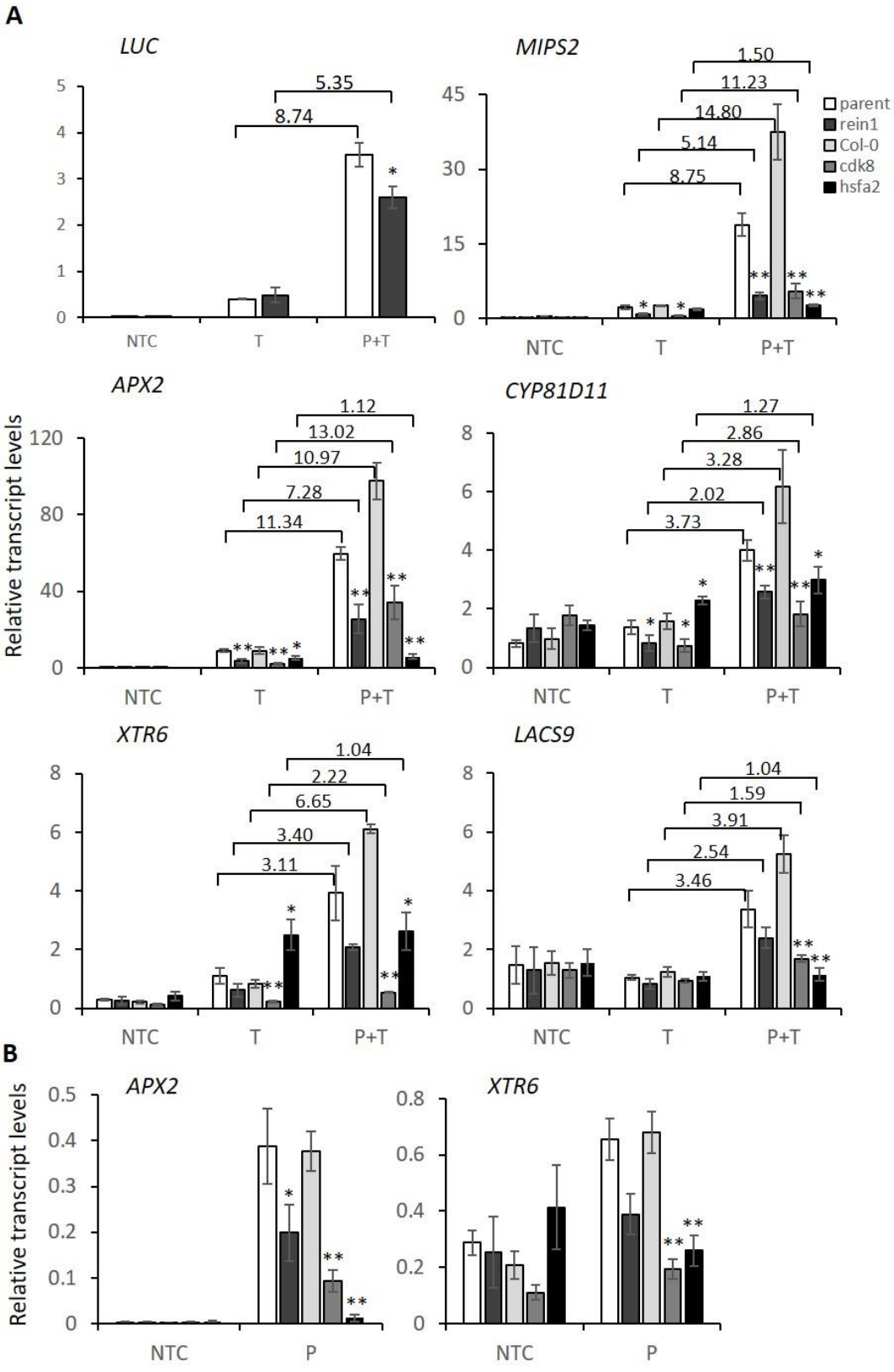

Figure 16. Transcript levels of HS memory genes *APX2*, *MIPS2*, *CYP81D11*, *LACS9* and *XTR6* are reduced in *rein1* and *cdk8* mutants after repeated HS. Plant material was collected from HS-treated *rein1* seedlings (BC1F3 generation), grown next to *pAPX2::LUC* (parent) and from *cdk8* and *hsfa2* mutants, grown next to WT background Col-0. Gene expression was analyzed by RT-qPCR. **A** Samples were collected from non-treated (NTC),

triggered plants on day 6 (T) as well as primed and triggered (P+T) plants. **B** Sustained expression was analyzed for *APX2* and *XTR6* in plants primed on day 4, 52 hours later (P). All transcript levels were normalized to the *At4g26410* transcript levels. Data were obtained from three independent biological replicates. Error bars show standard deviation. The (P+T)/T ratio for each gene for *pAPX2::LUC*, *rein1*, Col-0 and *cdk8* is indicated. The significance of the difference between *pAPX2::LUC* and *rein1* and Col-0 and *cdk8*, respectively, was analyzed by a two-sided Student's t-test (*$p < 0.05$, **$p < 0.01$).

The transcript levels of the selected genes were analyzed immediately after the end of a single HS on day 6 to test induction (T), two days after a single HS on day 4 to assess sustained expression (P), after repeated HS with two days of recovery in between the two treatments to test hyper-inducibility (P+T) and in non-HS conditions (NTC). The re-induction of the transgene *pAPX2::LUC* (LUC) was significantly reduced in *rein1* mutants compared to the parental line. Both, *rein1* and *cdk8* mutants also displayed a strong reduction in expression of endogenous *APX2* after repeated HS (P+T), suggesting that reduced luciferase activity in *rein1* mutants was indeed a result of reduced transcriptional activity of the HS memory gene (Figure 16 A).

The initial induction (T) of all tested +/++ class HS-memory genes (*APX2*, *MIPS2*, *CYP81D11*, XTR6) was impaired in both the *rein1* and *cdk8* mutants (Figure 16 A). No such effect was observed in the *LUC* transgene, where only the re-induction was affected. *Hsfa2* mutants exhibited a trend towards lower induction for two HS memory genes, *APX2* and *MIPS2*, at this sampling timepoint, however this was not statistically significant. The observed reduction in initial gene expression in *rein1* and *cdk8* was found to be statistically significant, as was the decrease in hyper-induction. Comparative analysis revealed lower fold changes (P+T / T) in the *rein1* and *cdk8* mutants in the majority of the selected loci as compared to their respective background, indicating a trend towards decreased re-induction despite the initial induction being affected. Compared to the *hsfa2* mutant, where hyper-induction was completely absent, *rein1* and *cdk8* mutants still exhibited responsiveness to a second HS.

The transcript levels of *APX2* and *XTR6* in *rein1* and *cdk8* under P conditions were lower compared to the respective wild type. This indicated that type I-transcriptional memory was also affected by mutation of *CDK8* (Figure 16 B).

In addition to the HS memory genes, expression levels of *HSP101* and *HSFA2*, which peak quickly after HS treatment but do not display type I- or type II-transcriptional memory were tested in *rein1* and *cdk8* mutants. HSP101 is required in the acute response to heat, resulting in

a deficiency of both bTT and aTT. HSFA2, in contrast, is dispensable for the above-mentioned HS tolerances at the physiological level and is specifically required for maTT.

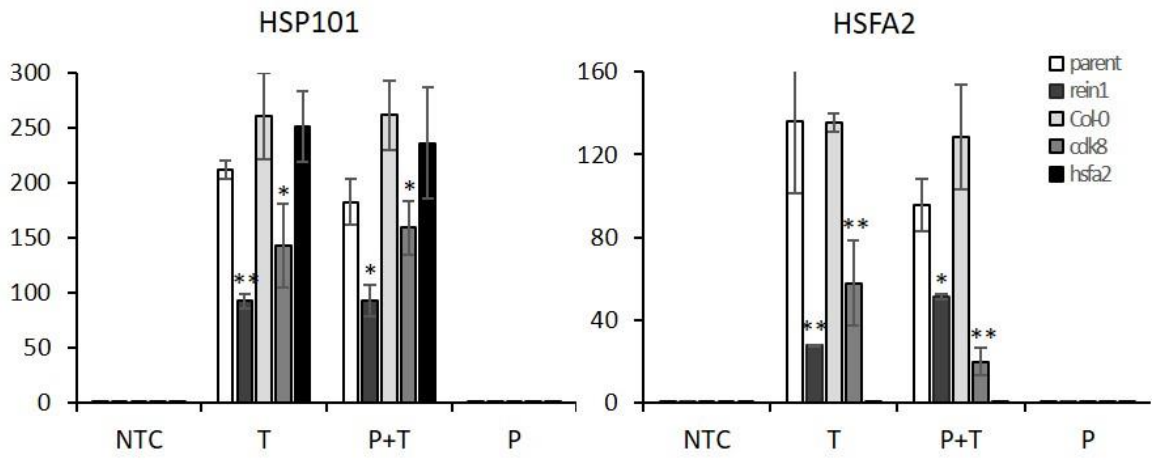

Figure 17. Expression levels of non -memory genes *HSP101* and *HSFA2* upon heat in *rein1, cdk8* and *hsfa2* compared to their respective WT backgrounds *pAPX2::LUC* (parent) and Col-0. Samples were taken from non-treated (NTC), triggered plants on day 6 (T), primed plants on day 4, sampled on day 6 (P) and primed and triggered (P+T) plants. HS treatment of the seedlings was done at 37°C for 1h and gene expression was analyzed by RT-qPCR. Transcript levels were normalized to *At4g26410* expression. Data was obtained from three independent biological replicates. Error bars show standard deviation.

While upregulation of *HSP101* after a HS treatment of 37°C is unchanged in *hsfa2* mutants, results showed that *cdk8* and *rein1* exhibited a significantly lower *HSP101* expression in comparison to their respective background (Figure 17). Likewise, transcriptional expression of *HSFA2* upon HS was reduced in *rein1* and *cdk8* mutants as well.

This suggests that CDK8 is not exclusively involved in the regulation of transcriptional memory after HS, but also has a role in activation of gene expression.

5.2.5 *rein1* and *cdk8* mutants exhibit reduced physiological HS memory

To analyze whether the observed deficiency in type I and type II transcriptional HS memory in gene expression and luciferase assays in *rein1* mutants correlated with an impairment in thermotolerance at the physiological level, survival rates after HS treatments were evaluated. To specify in which parts of the HS response REIN1/CDK8 was involved, different HS regimes were performed which included assays testing the mutants ability for bTT, aTT and maTT.

To further assess the extent of involvement in these different parts of HS response, two mutants were used as controls in the assays: *hsp101*, known to be strongly impaired in bTT and *hsfa2* which specifically exhibits defects in maTT. Additionally, an independent *cdk8* T-DNA line was tested alongside *rein1*.

Seedlings of *rein1* next to the parental line, as well as *cdk8* and *hsfa2* alongside their wild type background Col-0 were subjected to ACC prior to a triggering HS treatment at 44 C° for 70 - 110 min after a recovery phase of three days. Two weeks after the triggering HS, survival rates of the tested genotypes were evaluated (Figure 18 A) and quantified by classification into four phenotypic survival classes: healthy, affected, severely affected and dead (Figure 18 B, Figure 31).

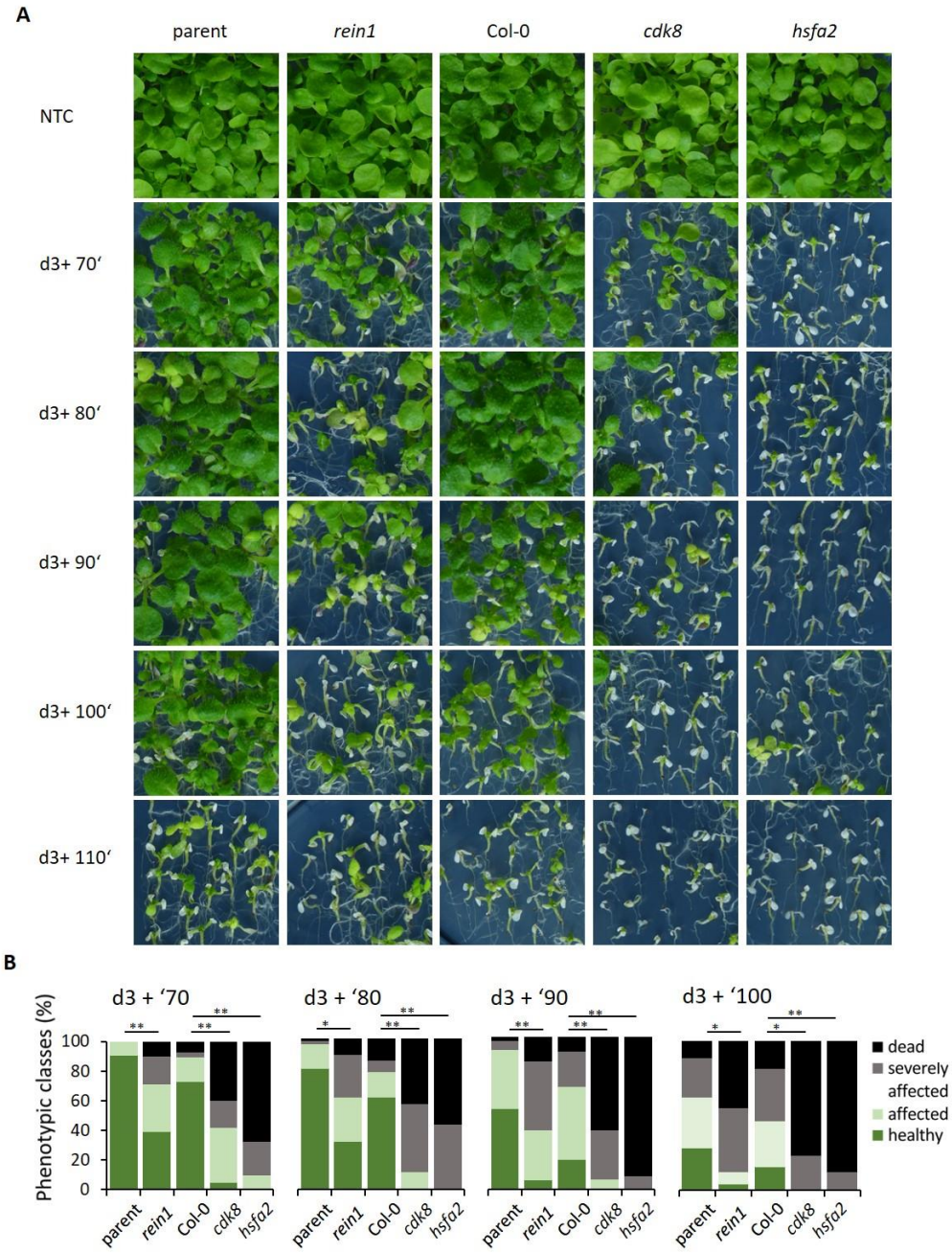

Figure 18. *rein1* and *cdk8* mutants are defective in maTT. Phenotyping and classification of *rein1*, *cdk8* (GABI_564F11) and *hsfa2* mutant seedlings were performed and compared to their respective WT backgrounds *pAPX2::LUC* (parent) and Col-0 in HS memory assays. The seedlings of the indicated genotypes received a full ACC (1h 37°C, 90 min recovery, 44°C for 45 min) 5 d after germination and were treated with a tester HS (44 °C) of different length (70 – 110 min) 3 d later. Pictures were taken after 14 d of recovery after the triggering HS. **A** Photographs of one representative HS assay of three biological replicates are shown. **B** Seedlings were categorized into four phenotypic classes according to the level of damage (dead, severely affected, affected and healthy). *hsfa2* mutants which have a reported HS memory phenotype were used as control and indicator for the strength of the phenotype (Charng *et al.*, 2007; Lämke *et al.*, 2016). Fisher´s exact test was used to compare the class distribution between mutants and their respective genetic background lines, *, $p<0.05$, **, $p<0.01$, $n = 3$.

The maTT assay showed that both, *rein1* and *cdk8* mutants, displayed a deficiency in survival (Figure 18 A), indicating that their HS memory is affected in comparison to the control lines. The quantification of phenotypic survival classes confirmed these observations (Figure 18 B).

5.2.6 The acquisition of thermotolerance is only mildly affected *rein1* and *cdk8* mutants

To elucidate further if REIN1/CDK8 is specifically required for HS memory or whether it has an additional role in early responses to heat, the mutant seedlings aTT was examined. The plants were pre-treated with moderately high but non-lethal temperatures (37°C for 45 min) prior to exposure to severe HS (44°C) with a duration of 140 – 220 min (Figure 19).

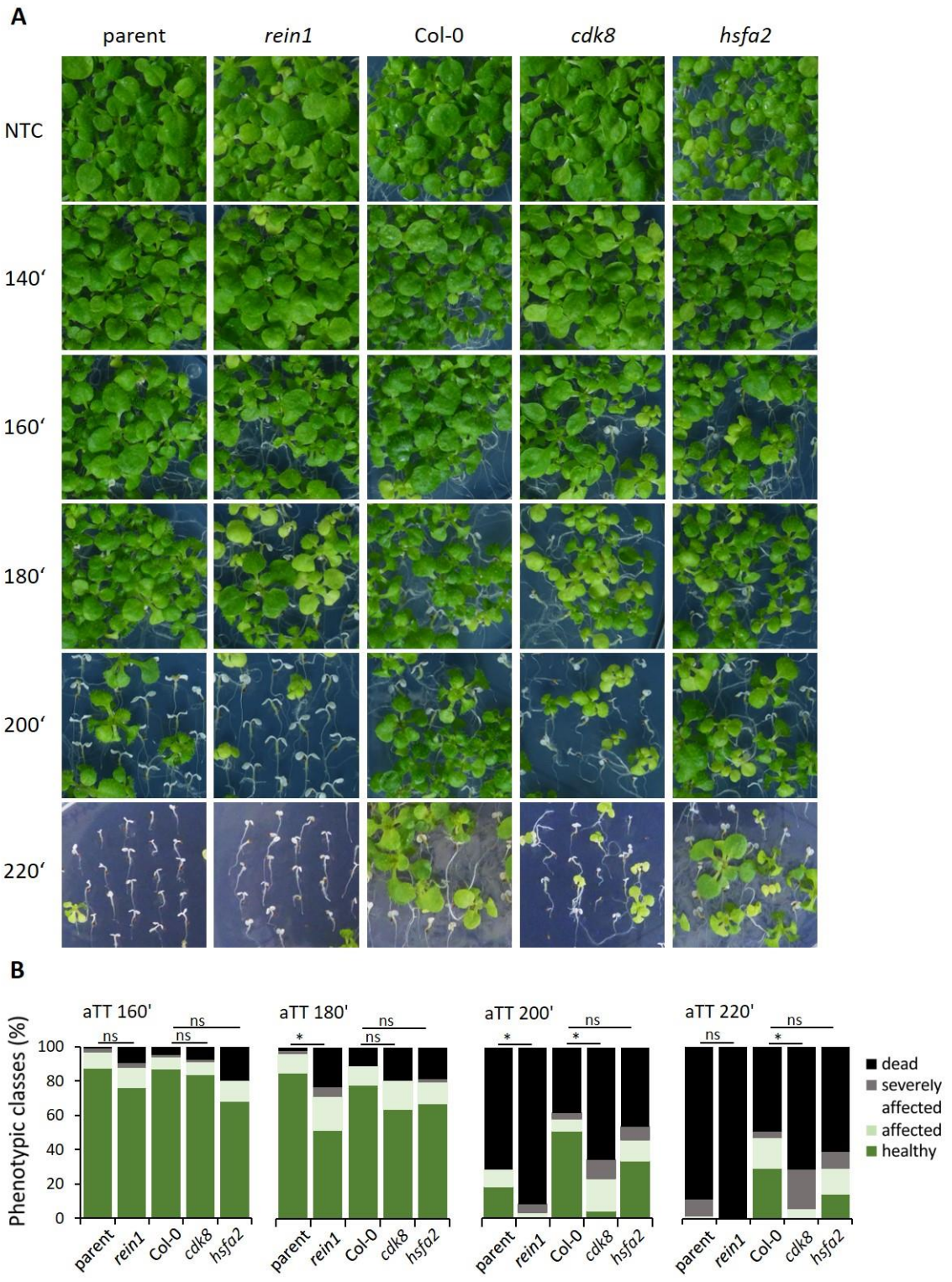

Figure 19. *rein1* and *cdk8* mutants show slightly decreased survival in aTT assays. 5 days old seedlings of the indicated genotypes were treated with a HS of 37° C for 60 min, then recovered for 90 min at RT followed by a second HS of 44°C of the indicated length. Pictures were taken after 14 d of recovery post HS treatment. **A**: One representative of three biological replicates is shown. **B**: Seedlings were categorized into four phenotypic classes according to level of damage (dead, severely affected, affected and healthy). Fisher´s exact test was used to compare the class distribution between mutants and their respective genetic background lines. *, $p<0.05$, **, $p<0.01$, n = 3

Categorization into the four survival classes 14 days after HS treatment showed that both, *rein1* and *cdk8* mutants were able to acquire thermotolerance, since a 37°C priming HS enabled both wild type and mutant backgrounds to survive a triggering HS at 44°C for up to 160 min with similar rates of unaffected and mildly affected seedlings (Figure 19 B). A longer duration of the 44°C triggering HS, however, led to a higher proportion of affected or dead seedlings in *rein1* and *cdk8* mutants compared to their respective backgrounds. Differences in survival between *rein1* and seedlings from the parental line were observed after treatment with a triggering HS of 180 min duration, whereas distribution of *cdk8* seedlings in the phenotypic classes did not differ from Col-0 at that timepoint. It was noted that the *pAPX2::LUC* parental line and Col-0, which served as wild type controls in the assays, exhibited a slightly different ability to acquire thermotolerance: while 90 % of seedlings from the parental line were dead after exposure to a 220 min long tester HS, only 50% of Col-0 plants died. In accordance with this observation, differences of survival rates between *cdk8* and Col-0 seedlings in the aTT assay were observed at the next tested timepoint with a tester HS of 200 min.

The impairment in aTT was rather slight in *rein1* and *cdk8* mutants and only detectable by application of a prolonged triggering HS in aTT assays. In contrast, the observed defects in the maTT were much more severe.

5.2.7 The basal thermotolerance is not affected in *rein1* and *cdk8* mutants

To test for involvement of REIN1/CDK8 in bTT, which allows plants to withstand sudden increases in temperature, *rein1* and *cdk8* mutant seedlings, alongside their respective genetic backgrounds and bTT-deficient *hsp101* mutants, were exposed to 44°C for a duration between 15 to 30 minutes (Figure 20). The survival of seedlings was examined 14 days after treatment.

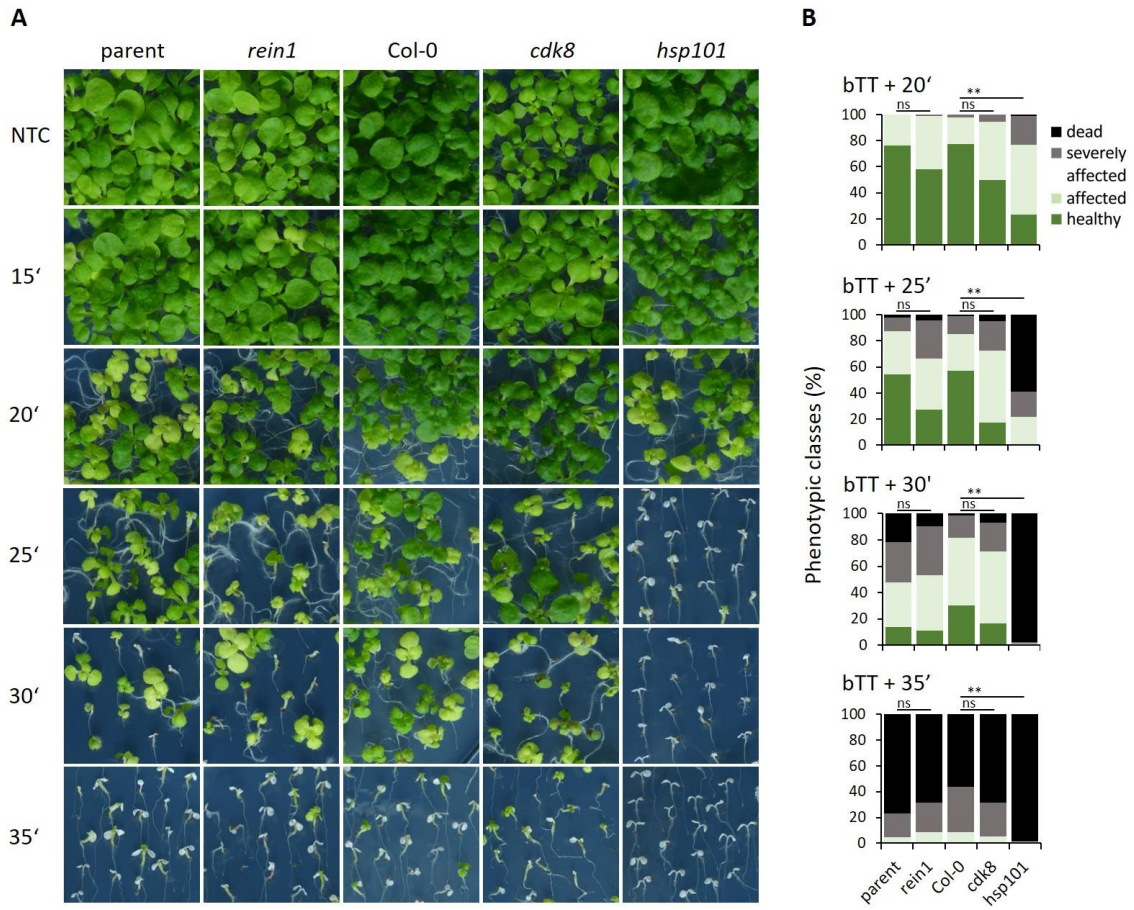

Figure 20. Basal thermotolerance is unaffected in *rein1* and *cdk8* mutants. 5 days old seedlings of indicated genotypes were treated with a severe HS of 44 ° C for a duration of 15 – 35 min and left for 60 min at RT before return to the growth chamber. Pictures were taken after 14 d of recovery post HS treatment. **A**: One of three biological replicates is shown. **B**: Categorization into four phenotypic classes, sorted by the level of damage (dead, severely affected, affected and healthy) after HS treatment for 20, 25, 30 and 35 minutes averaged over three biological replicates. Fisher´s exact test was used to compare class distribution between mutants and respective genetic background lines, *, $p<0.05$, **, $p<0.01$.

The quantification of survival classes did not reveal any differences in survival rates between the tested lines, while the *hsp101* control plants exhibited a death rate of almost 100 % after 25 minutes of HS exposure (Figure 20 B). Thus, *rein1* and *cdk8* mutants did not display reduced bTT.

Combining the observations from all performed HS assays, the data indicated that REIN1/CDK8 may not be involved in immediate response to heat that allows plants to withstand lethal temperatures for short periods of time. The strong deficiency in the maTT assays revealed a role for REIN1/CDK8 in HS memory on a physiological level. Additionally, the slight impairment at later timepoints in the aTT assays suggested that REIN1/CDK8 may additionally contribute to priming as well.

5.2.8 CDK8 interacts with memory HSFs

The results of the survival assays and gene expression studies indicated that CDK8 functions in HS memory at the physiological level and regulates the transcription of HS responsive (-memory) genes. To learn more on how CDK8 operates in contributing to HS memory, it was investigated whether there was direct interaction with two known HS memory factors, HSFA2 and HSFA3. HSFA2 und HSFA3 were found to be interaction partners and promote transcriptional memory by recruiting histone H3K4 hyper-methylation to HS memory loci (Lämke *et al.*, 2016; Friedrich *et al.*, 2021).

The interaction was tested in a Split Luciferase Complementation Assay (SLCA) using a ß-Estradiol-inducible vector system with N- and C-terminal parts of sea pansy *Renilla reniformis* luciferase (RLUC), referred to as N-RLUC and C-RLUC (Supplemental Figure S3, Schatlowski *et al.*, 2010). CDK8 was genetically fused to N-RLUC while the potential interaction partners (either HSFA2 or HSFA3) were fused to the C-RLUC fragment. The two fusion proteins were transiently transformed into *Nicotiana benthamiana* leaves and after β-Estradiol treatment and luciferase substrate infiltration, luminescence resulting from reconstituted luciferase activity, was measured (Figure 21).

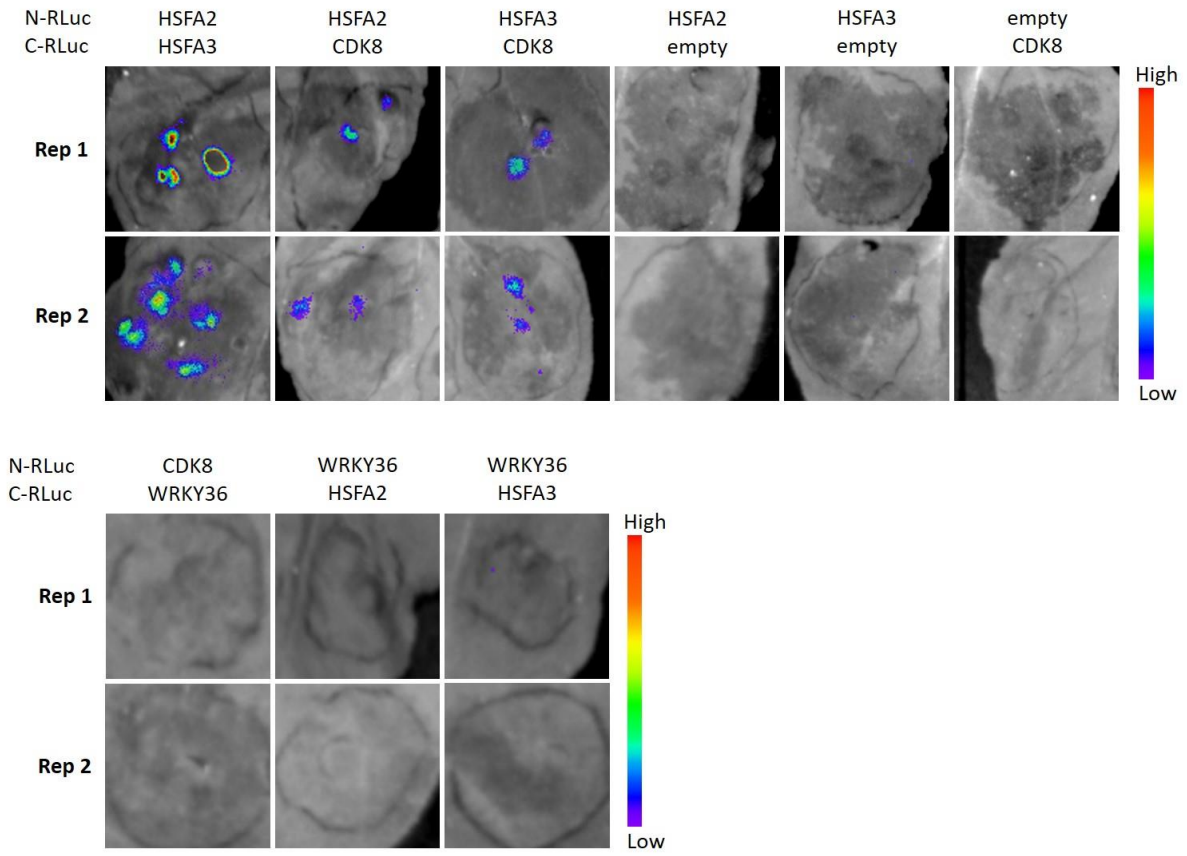

Figure 21. Interaction studies of CDK8 and HSFs using transient SLCA in tobacco leaves. Agrobacteria containing the split RLuc destination vectors were co-infiltrated into tobacco leaves. Expression of the indicated fusion proteins was induced by brushing infiltrated leaves with a ß-Estradiol solution (100 µM ß-Estradiol and 0.1 % Tween in water) and incubating for 24 h. For luminescence detection, leaves were infiltrated with 10 µM ViviRen live cell substrate (Promega). After incubation for 10 min in darkness, luminescence was detected with a NightOwl system for 30 min. The false color scale of relative luciferase activity is shown on the right. The experiment was performed in duplicate (Rep1 and Rep2).

HSFA2 and HSFA3 interact (Friedrich *et al.*, 2021). Thus, the interaction was used as a positive control for SLCA (Figure 21). Interaction between CDK8 and both HSF proteins was detected 24 h after induction of the fusion proteins with β-Estradiol in form of luminescent spots spreading from the infiltration site. The signal intensity resulting from this combination was lower than the signal received from co-infiltration of HSFA2 and HSFA3. The luminescence signal was reproducible and no signal was detected from the combinations expressing either one of the tested proteins of interest with the empty complementing luciferase half. Additionally, CDK8, HSFA2 or HSFA3 were co-expressed with WRKY DNA-BINDING PROTEIN 36 (WRKY36). WRKY36 reportedly does not interact with CDK8, which was tested in co-immunoprecipitation (Co-IP) and yeast two-hybrid (Y2H) experiments (Chen *et al.*,

2019). The WRKY36 fusion in combination with CDK8 or either HSFA fusion did not result in any luciferase signal accumulation, indicating that the signal gained from CDK8 and HSF combinations resulted from a specific interaction.

Additionally, the interaction between CDK8 and the two HSFs was tested by *in vitro* pulldown assays (Figure 22). CDK8-HALO in combination with HSFA2-FLAG or FGT3-FLAG fusion proteins were co-translated by using the TnT Coupled Wheat Germ Extract System (Promega). A pulldown was performed with anti-HALO beads and the presence of the potential HSF interaction partner was analyzed using an anti-FLAG antibody.

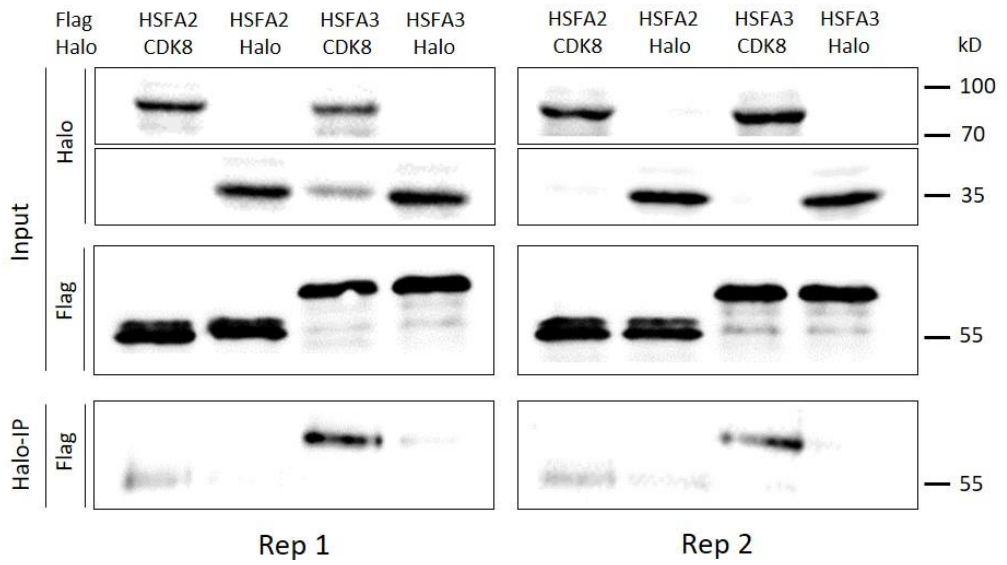

Figure 22. *In vitro* pulldown assay shows interaction between CDK8 and HSFA proteins. A HALO-tagged CDK8 protein and either FLAG-tagged HSFA2 or HSFA3 protein were co-translated *in vitro* and subsequently purified with anti-HALO beads. Samples were analyzed by SDS-Page and immunoblotting using an anti-FLAG antibody. HALO-Tag alone was used as a negative control.

Using *in vitro* pulldown assays, an interaction between CDK8 and both HSFA2 and HSFA3 was detected, as both HSFAs were pulled down together with CDK8 in both replicates (Figure 22). Although HSFA2 protein enrichment was lower than HSFA3, the protein enrichment was still higher than the background signal obtained from HALO-tag without fusion to CDK8. Combined with results from the SLCA, the obtained data suggested that CDK8 interacts with the HS memory factors HSAF2 and HSFA3.

5.3 Characterization of the *rein2* mutant

The second characterized *rein* mutant, mutant *rein2*, was found to have a strong reduction of *pAPX2::LUC* activity following HS exposure in luciferase assays (Figure 23). The following section describes the initial characterization of *rein2* and fine mapping results after NGS that narrowed down the genomic interval where the mutation is located.

5.3.1 *pAPX2::LUC*-derived bioluminescence after recurring HS is reduced *rein2* mutant

In the M2 generation of line *144.1*, multiple seedlings with reduced *pAPX2::LUC* derived bioluminescence were identified, arguing for the presence of a heritable mutation (Supplemental Figure S4). Line *144.1*, which was renamed *rein2*, was initially classified into category A, with unaffected induction of *pAPX2::LUC* in the M3 generation and a strong defect in hyper-induction compared to the *pAPX2::LUC* parental line (Figure 23).

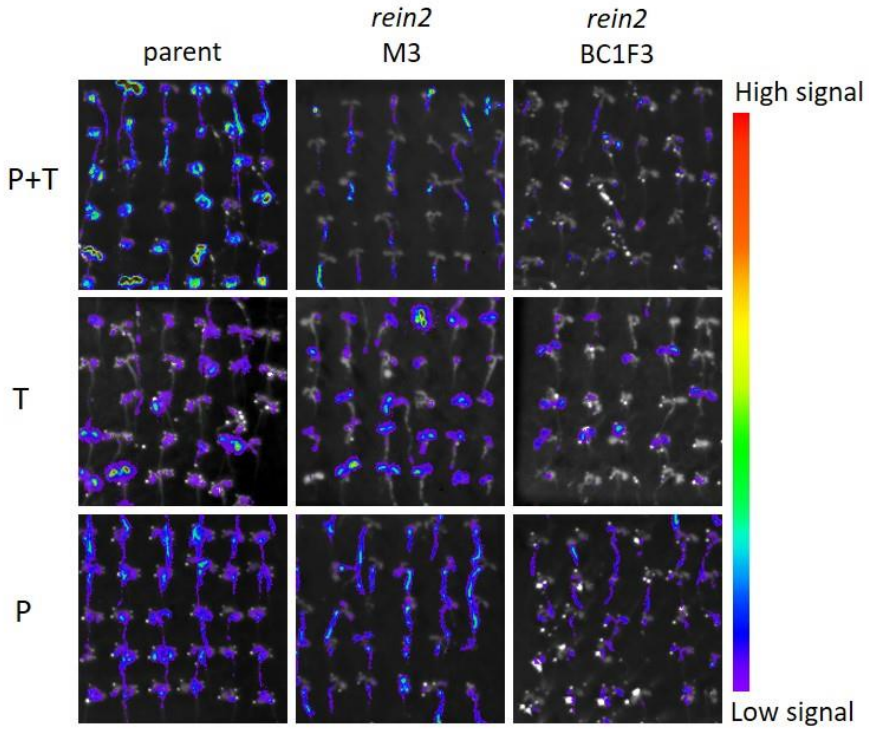

Figure 23. *pAPX2::LUC* derived bioluminescence in seedlings from mutant line *rein2* next to parental line after HS treatment. 5 days old seedlings were treated with a priming HS of 37°C for 1 h and signals were either measured 5 h (T) or 58 h (P) after recovery and after a second triggering HS of 1 h at 37°C with 1 h recovery at RT before signal detection (P+T), respectively. The luciferase assay was performed with seedlings from the M3 and the BC1F3 generation. A false color scale of relative luciferase activity is shown on the right.

The M3 generation of mutant line *rein2* displayed comparable luciferase signal to the parental line after a single HS treatment (T). Signal reduction was observed after repeated HS conditions (P+T). Additionally, sustained induction of *pAPX2::LUC* (P) was mildly affected in the *rein2* mutant line. A reduced priming capacity of the transgene in individuals was not evident in the M3 generation, however, lowered luciferase signals in P conditions were observed in individual seedlings of the BC1F3 generation (Figure 23).

5.3.2 The *rein2* mutant is deficient in thermotolerance

Seedlings from the BC1F3 generation of line *rein2* were used to test whether the mutation impacts thermotolerance at the physiological level. The seedlings were treated with a full ACC before subjecting them to a tester HS of 44°C for different lengths from 70 to 110 minutes. The *pAPX2::LUC*, wildtype (Col-0) and *hsfa2* lines were included as controls. The capacity for survival representing the plants HS memory was captured 2 weeks after recovery from the tester HS and quantified (Figure 24 B).

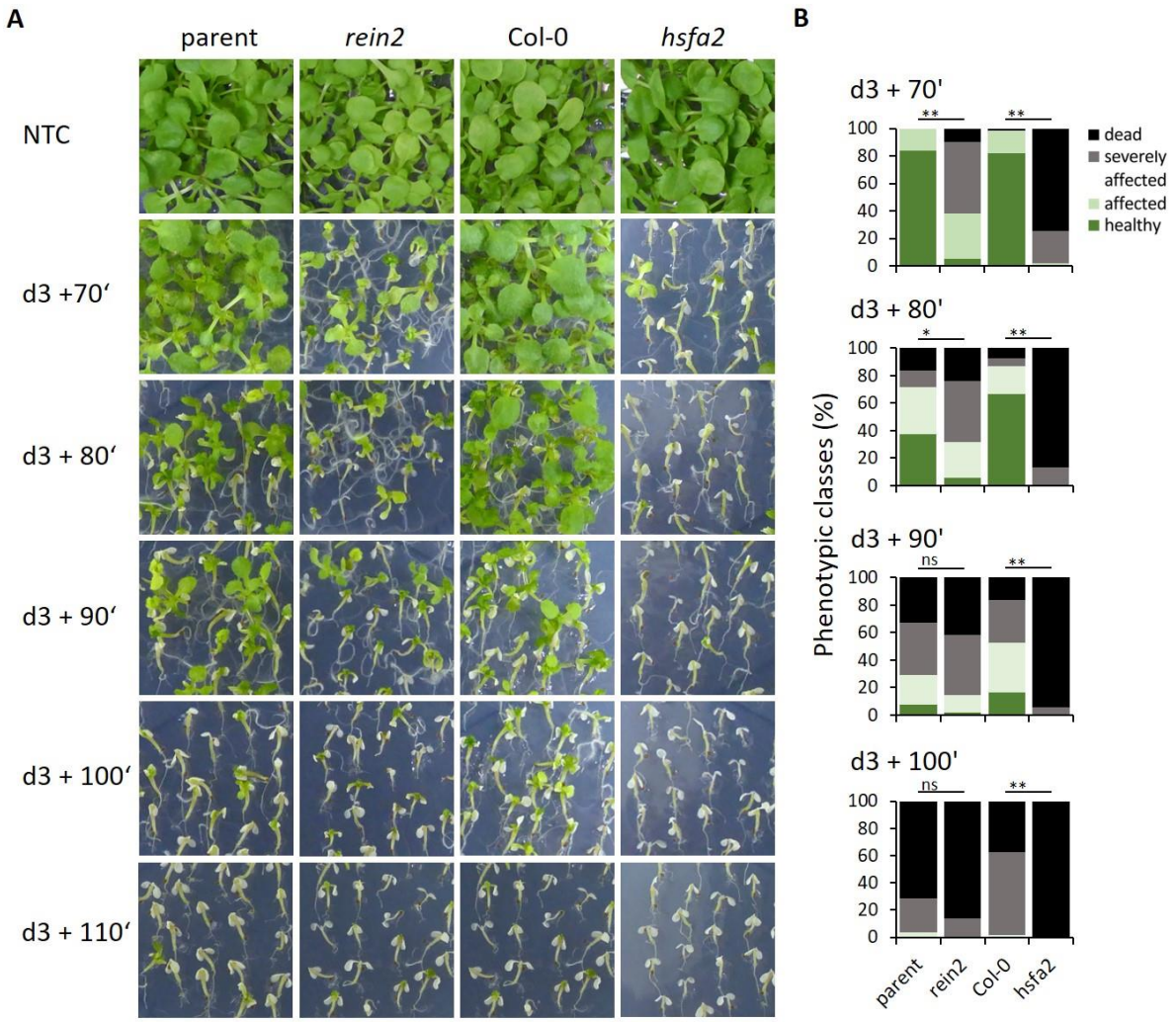

Figure 24. Reduced survival of seedlings from mutant line *rein2* in maTT tests. 4 days old seedlings were treated with a priming HS of 37°C for 60 min, recovered for 90 min and further subjected to a HS of 44°C for 45 min. The plants HS memory was tested by performing a tester HS of 70 - 110 min length and evaluating the seedlings survival after 14 days of recovery. The experiment was independently performed two times with similar results. **A** One experiment is shown as representative. **B** Classification into four phenotypic classes (dead, severely affected, affected and healthy) two weeks post HS treatment. Data was averaged over two independent assays; Fisher exact test, *, $p < 0.05$, **, $p < 0.01$, $n = 2$

The effect of the *REIN2* mutation on survival in the maTT assays was already visible after treatment with the shortest tester HS of 70 min (Figure 24 A). Over 80 % seedlings from the Col-0 were healthy at this sampling timepoint, while only 5 % of the *rein2* seedlings were unaffected and a great portion was severely affected already (Figure 24 B). Impairment in HS memory was not as strong as in *hsfa2* mutants, which exhibited a lethality of almost 80% after a 70 min tester HS.

Next, it was tested whether the defects in *rein2* seedlings were specific for maTT or if the establishment of it was impaired as well.

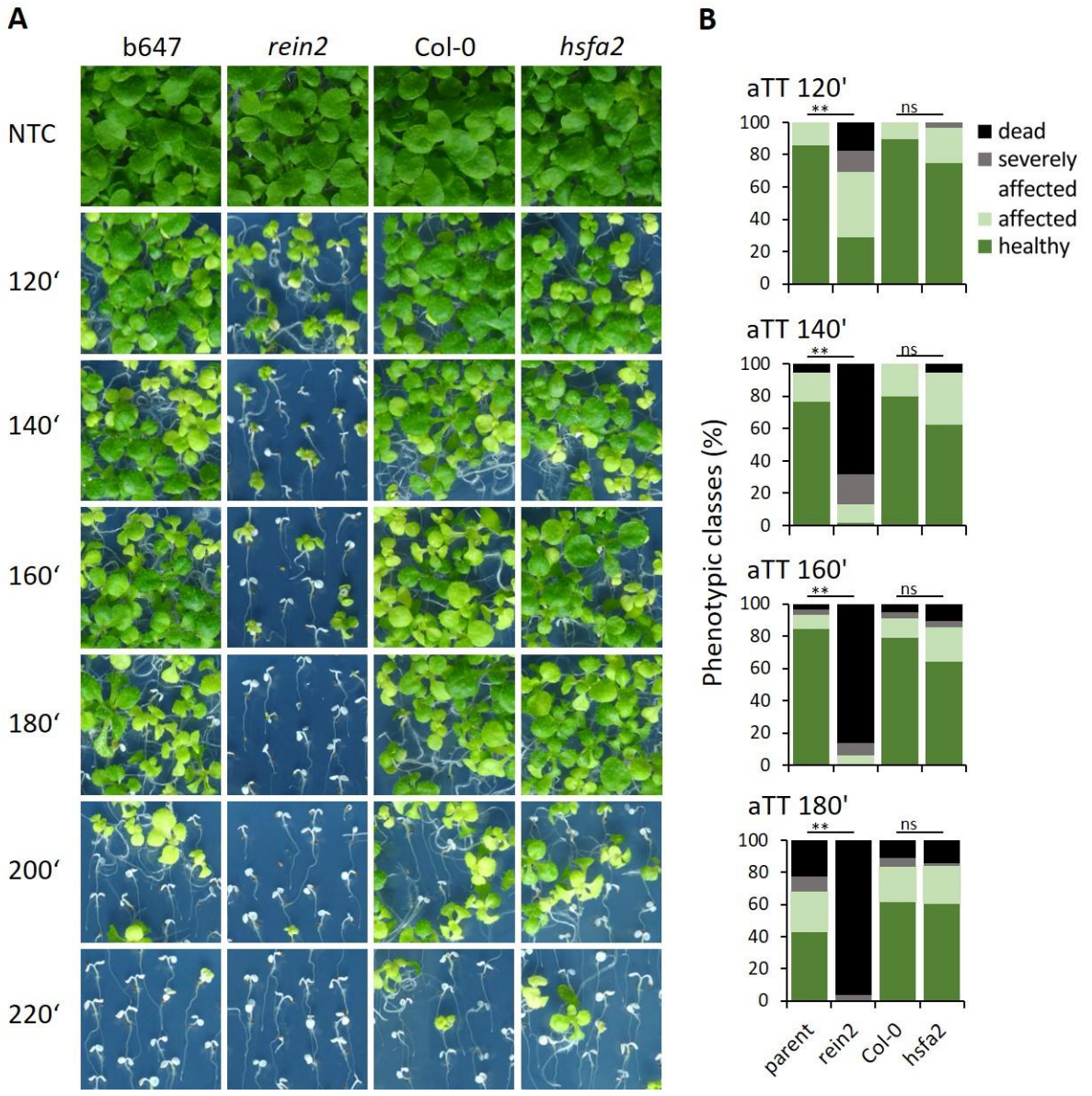

Figure 25. Performance of *rein2* mutants in aTT assays reveals defects in acquisition of thermotolerance. 4 days old seedlings were treated with a priming HS of 37°C for 60 min, recovered for 90 min and further subjected to a HS of 44°C for 120 - 220 min. **A** Photographs of one representative aTT assay are shown. **B** Classification of observed phenotypes (healthy, affected, severely affected and dead) was performed after 2 weeks of recovery. Data was averaged over two independent assays; Fisher exact test, *, $p<0.05$, **, $p<0.01$, n =2

Impairment in acquiring thermotolerance was observed after subjecting *rein2* mutants to a 44°C HS for 120 min after a 1 h HS at 37°C. A tester HS treatment of 140 min already resulted in death of 70 % of seedlings (Figure 25 B). This indicated that the priming ability in the *rein2* mutant line from a moderate HS of 37°C was impaired, leading to less thermotolerance to high temperatures.

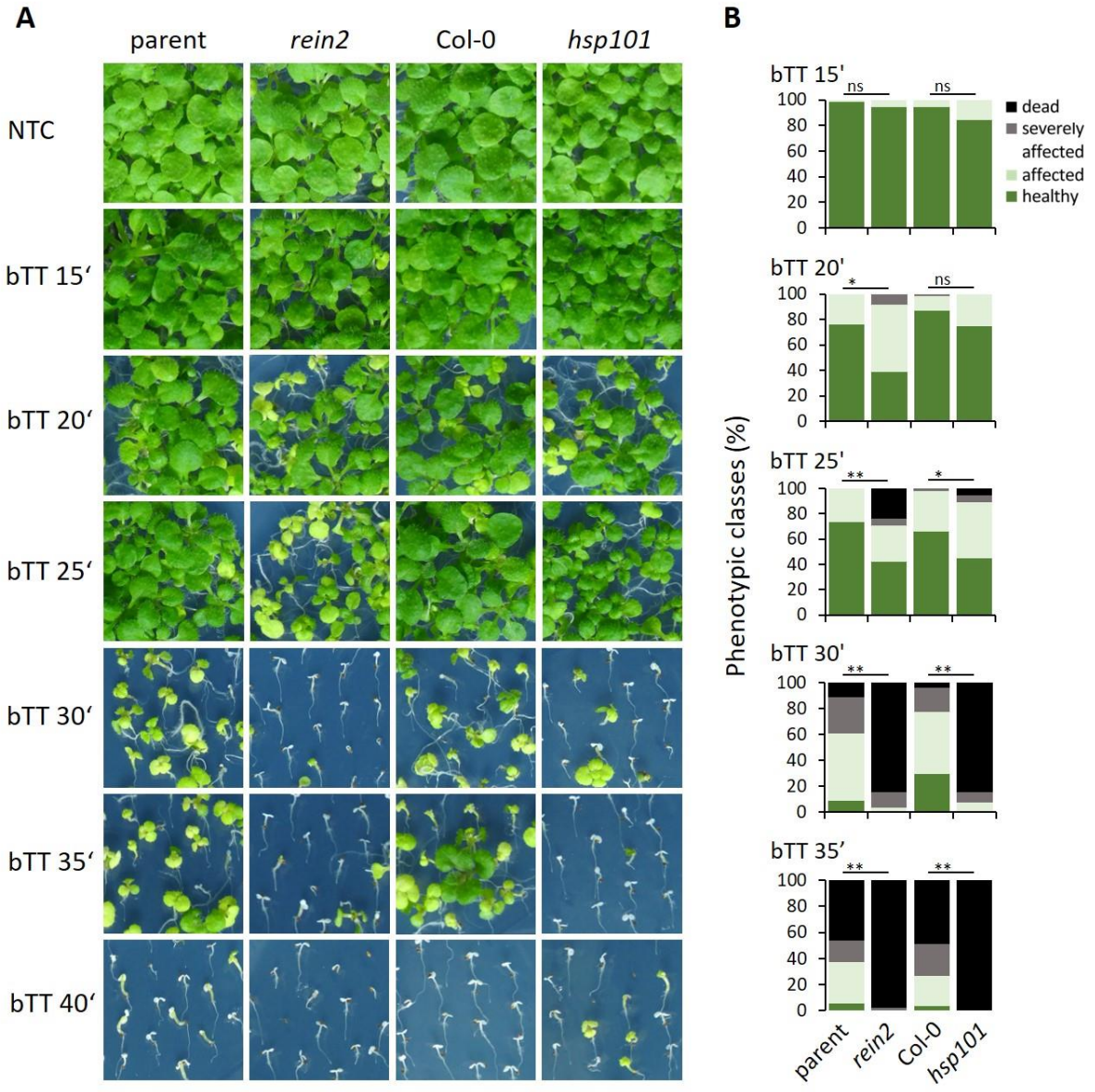

Figure 26. Mutant line *rein2* has reduced basal thermotolerance. 4 days old seedlings subjected to a severe HS of 44°C for 15 - 40 min. The experiment was independently performed twice. **A** Photographs of one representative bTT assay are shown. **B** Classification of the observed phenotypes (healthy, affected, severely affected and dead) was performed after 2 weeks of recovery. Data are averaged over two independent assays; Fisher exact test, *, $p<0.05$, **, $p<0.01$, n = 2.

Additionally, bTT was affected in *rein2* mutants. The *rein2* line had a significant reduction of survival rates already after exposure to an immediate 44°C HS treatment with a duration of 20 min (Figure 26 A). The difference in survival between Col-0 and the bTT mutant *hsp101* was only significant after 25 min of exposure to 44°C HS (Figure 26 B). It can be concluded that the mutation in the *rein2* line has a strong impact on the plant's ability to withstand high temperatures.

5.3.3 The transcriptional expression of selected HS-responsive genes is impaired in *rein2*

The *REIN2* mutation resulted in a strong defect of seedlings to acquire and maintain thermotolerance. While testing for the initial response to heat, the survival phenotype of *rein2*, was similarly affected as the *hsp101* mutant. This suggested that the *rein2* mutant might be defective in the expression of regulators that are essential for the early response to heat. To test for correlation between the physiological phenotype of *rein2* and expression of HS responsive genes, transcript levels after single and repeated HS treatments were measured by RT-qPCR. Initial induction and transcriptional memory behavior of HS memory genes *APX2*, *MIPS2*, *CYP81D11* and *HSP18.2* as well as two HS-responsive but non-memory genes *HSP101* and *HSFA2* were analyzed (Figure 27).

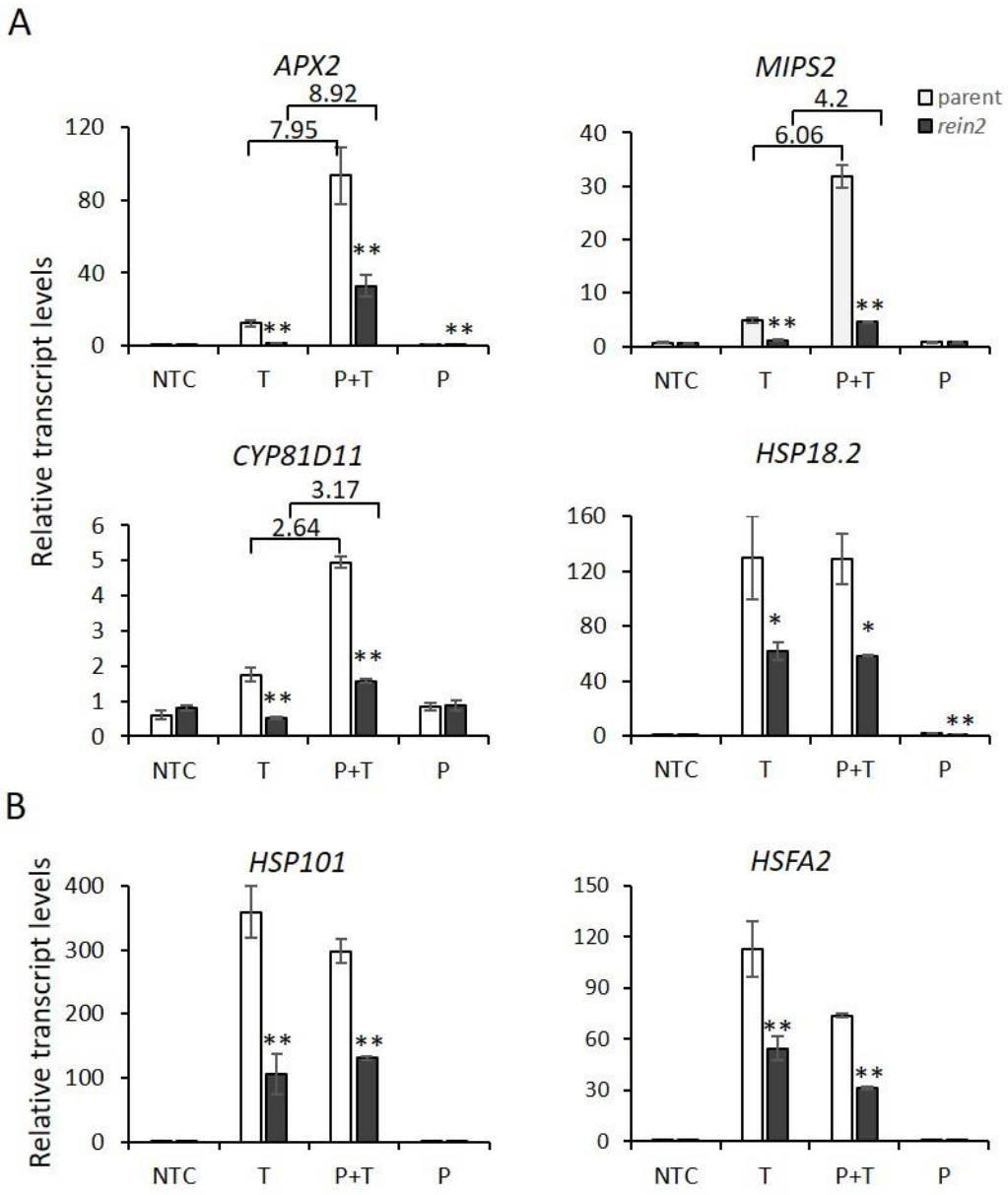

Figure 27. Expression analysis of HS-responsive genes in *rein2* mutants after different HS treatments. **A** Transcript levels of *APX2*, *MIPS2*, *CYP81D11*, *HSP18.2* and **B** the HS responsive, non-memory-genes *HSP101* and *HSFA2* relative to *At4g26410* in non-treated (NTC), triggered (T), primed and triggered (P+T) and primed (P), samples analyzed by RT-qPCR. HS was applied by subjecting seedlings to a 37°C HS for 60 min. Error bars show standard deviation of three biological replicates. Asterisks mark significant differences to the parental line (*, $p < 0.05$, ** $p < 0.01$, unpaired two-sided t-test).

The gene expression of all tested HS memory genes upon a single or recurring HS exposure was significantly reduced in *rein2* mutants (Figure 27 A). Additionally, transcript levels of HS-responsive non-memory genes *HSP101* and *HSFA2* were lower compared to the parental line (Figure 27 B).

Considering the effect of *REIN2* on gene expression combined with the strong defect in all tested types of thermotolerance, the results suggested that the candidate protein mutated in *rein2* has a broader role in the HS response.

5.3.4 The candidate mutation in *rein2* is located towards the end of chromosome 1

After backcrossing to the parental line and genome sequencing of bulk segregants, analysis of allele frequencies in *rein2* pointed out that the end of chromosome 1 was under selection, as local allele frequencies appeared higher in comparison to other regions in the genome (Figure 28). An interval between 21 Mb and 30 Mb was defined and investigated for mutations in potential candidate genes causing the mutant phenotype.

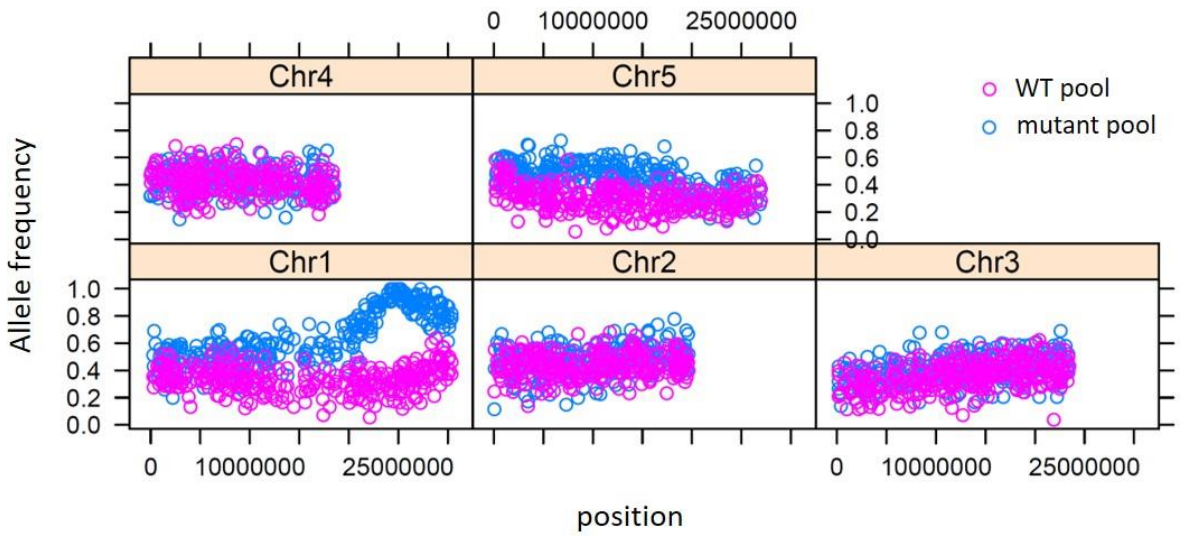

Figure 28. **Distortion of allele frequency distributions at the lower arm of chromosome 1 in *rein2* mutants.** Allele frequencies of filtered mutant vs wild type markers for BC1F2 mutant (purple) and F2 wild type (blue) pools are shown as dots (analysis done by Dr. Christian Kappel) with their position (in base pairs) on the five chromosomes. NGS revealed a region of distorted segregation of allele frequencies in the mutant pool on the lower arm of chromosome 1, with the strongest signal at around 25 Mb.

The location and consequently severity of the mutation affecting the protein function (splice acceptor, splice donor, start lost, stop gained, or missense variant), was considered for choosing the candidates in Table 2. Additionally, biological function of the affected proteins and sequencing read quality as well as quantity provided in form of the number of reads for the wild

type and the alternative allele, respectively, in both sequencing pools were taken into account for selecting candidates.

Table 2. Overview of selected genes with mutations in the *rein2* mutant line identified as candidates in the region between 21,7 Mb and 28,8 Mb on chromosome 1. The position of candidate genes on chromosome 1 (in Mb) and the SNPs are listed.

Position (Mb) on Chr1	Change	Gene ID	Target	Description	Mutation effect
21,76	C → T	AT1G58602	RECOGNITION OF PERONOSPORA PARASITICA 7 (RPP7)	LRR and NB-ARC domains-containing disease resistance protein	Stop gained Arg859*
23,33	C → T	AT1G62980	EXPANSIN 18 (EXPA18)	Member of Expansin A gene family	Stop gained Gln85*
24,24	C → T	AT1G65280	AT1G65280	DNAJ heat shock N-terminal domain-containing protein	3' UTR variant
24,30	G → A	AT1G65440	ARABIDOPSIS SUPPRESSOR OF TY INSERTION 6-LIKE (SPT6L)	Transcription elongation factor SPT6 homolog	Missense variant Cys871Tyr
24,54	G → A	At1g65950	ACTIVITY OF BC1 COMPLEX KINASE 14 (ABC1K14)	Protein kinase superfamily protein	Missense variant Ala477Thr
25,88	C → T	AT1G68840	RELATED TO ABI3/VP1 2 (RAV2)	AP2/ERF and B3 domain-containing transcription repressor DNA-binding transcription factor	Missense variant Arg242Cys
27,93	C → T	AT1G74310	HEAT SHOCK PROTEIN 101 (HSP101)	member of Casein lytic proteinase/heat shock protein 100 family	Missense variant Gly183Asp
28,79	G → A	AT1G76710	SET DOMAIN GROUP 26 (SDG26)	member of SET domain group 26	Missense variant Ser151Phe

Two high-impact mutations were detected at the start of the interval, resulting in premature STOP codons in the disease resistance gene *RECOGNITION OF PERONOSPORA PARASITICA 7* (*RPP7*) and the expansin gene *EXPANISIN 18* (*EXPA18*). The list further contained a 3' untranslated region (UTR) variant in a DNAJ heat shock containing protein with unknown function (*AT1G65280*). Though the impact of a point mutation in the 3'UTR sequences is not considered to be a high-impact mutation, mutations in 3'UTRs can generally lead to alterations of expression profiles of proteins (Srivastava *et al.*, 2018).

5.3.5 The *REIN2* mutation was fine mapped to a 1 Mb interval on chromosome 1

To narrow down the region that contained the candidate mutation causing the *rein2* mutant phenotype, derived Cleaved Amplified Polymorphic Sequences (dCAPS-) assays were performed. Nucleotide polymorphisms in *rein2* that were detected by bulk sequencing in the candidate mutant interval were used as markers and dCAPS primers were generated accordingly. EMS-induced nucleotide changes located in relatively close physical proximity to the causal mutation in the mutant of interest are statistically less likely to be included in a recombination event compared to those that happened further away on the chromosome. For dCAPS assays, segregating BC1F2 seedlings were subjected to repeated HS (P+T conditions) to distinguish mutant from wild type phenotype.

30 plants which exhibited the mutant phenotype and were determined as individuals bearing the homozygous *rein2* allele were selected for genetic mapping with dCAPS markers (Table 3). The fine mapping results showed a high proportion of mutant alleles within the tested interval in BC1F2 individuals that expressed low luciferase signal after repeated HS and therby confirmed the bulk sequencing results indicating that the position of the causal mutation is located around 25 Mb on chromosome I.

Additionally, approximately 50 plants with wild type luciferase signal (comparable to parental line) were genotyped and only plants that had a chromosomal recombination event within the interval, flanked by markers *RPP7* and *SDG26*, were selected for further analysis (Table 4). Their progeny was subsequently analyzed for segregation of luciferase signal upon repeated HS. F2 plants exhibiting a heterozygous genotype for the candidate interval were expected to result in an F3 generation with luciferase signal segregating 3:1, while no segregation was expected with two wild type alleles.

Table 3. Fine mapping of the locus containing the causal mutation responsible for the defective HS-response phenotype in the EMS mutant line *rein2* using BC1F2 plants with mutant luciferase phenotype. In total 30 F2 plants were phenotyped in luciferase assays after repeated HS. Genotypes of F2 lines at indicated dCAPS-marker positions on chromosome I (in bp) were determined. hom: homozygous mutant allele, het: heterozygous, WT: homozygous parental allele; nd: not determined. With the marker ABC1K14, it was not possible to distinguish between WT and het genotype.

		position/markers on chromosome I						
F2 Plant #	LUC signal	21 762 923 RPP7	22 511 182 AT1g61100	23 332 232 EXPA18	24 309 749 SPT6L	24 547 251 ABC1K14	27 939 315 HSP101	28 791 309 SDG26
1	low	hom	hom	hom	hom	hom	hom	hom
2	low	het	het	hom	hom	hom	hom	hom
3	low	m	m	hom	hom	hom	hom	hom
4	low	hom	hom	hom	hom	hom	het	het
5	low	hom	hom	hom	hom	hom	het	het
6	low	hom	hom	hom	hom	WT/het	het	het
7	low	hom	hom	hom	hom	hom	het	het
8	low	hom	hom	hom	hom	hom	het	het
9	low	hom	hom	hom	hom	hom	hom	hom
10	low	het	het	hom	hom	hom	het	het
11	low	hom	hom	hom	hom	hom	hom	hom
12	low	het	het	hom	hom	hom	het	het
13	low	hom	hom	hom	hom	hom	het	het
14	low	hom	hom	hom	hom	hom	het	het
15	low	hom	hom	hom	hom	hom	hom	hom
16	low	hom	hom	hom	hom	hom	het	het
17	low	hom	hom	hom	hom	hom	het	het
18	low	hom	hom	hom	hom	hom	hom	hom
19	low	hom	hom	hom	hom	hom	hom	hom
20	low	het	het	het	het	WT/het	het	het
21	low	het	het	hom	hom	hom	hom	hom
22	low	mut	mut	hom	hom	hom	hom	hom
23	low	hom	hom	hom	hom	hom	het	het
24	low	hom	hom	hom	hom	hom	het	het
25	low	nd	hom	hom	hom	hom	hom	WT
26	low	hom	hom	hom	hom	hom	het	het
27	low	het	het	WT	WT	WT/het	WT	WT
28	low	hom	hom	hom	hom	hom	hom	hom
29	low	hom	hom	hom	hom	hom	hom	hom
30	low	het	het	hom	hom	hom	hom	hom

Two F2 plants (#20 and #27) were picked for having a mutant phenotype but not showing homozygosity for either marker in the tested interval (Table 3). Those likely “mispicks” possibly resulted from variability of luciferase expression depending on the seedlings individual

fitness and position on the screening plate. Consequently, those plants were excluded from evaluation.

Since two mutant alleles are necessary to cause the *rein2* recessive mutant phenotype, loci that are heterozygous resulting from a chromosomal recombination event were unlikely to contain the causal mutation. Five plants (#2, #10, #12, #21, #30) exhibited a recombination event at the position of the marker At1g61100 (22,511,182 bp). On the right site of the interval, towards the end of chromosome I, recombination occurred in 14 out of 30 plants at the marker *HSP101* (27,939,315 bp). Consequently, candidate genes *RPP7*, *HSP101* and *SDG26* were excluded from the list of potential candidates for causing the *rein2* mutant phenotype. On top of that, one plant (plant #6) displayed a WT or heterozygous genotype for the marker at position 24,547,251 bp (*ABC1K14*). Taken together, these results helped to narrow down the interval containing the causal mutation to a region between 22,5 Mb and 24,54 Mb on chromosome 1.

Table 4. Fine mapping results of the locus containing the causal mutation in the *rein2* line using BC1F2 plants with wild type-like luciferase phenotype. 46 F2 plants with high luciferase signal after repeated HS were selected and genotypes at the indicated dCAPS-marker positions on chromosome I were determined. Only F2 plants with recombination events in the selected interval are shown. hom: homozygous mutant allele; het: heterozygous; WT: homozygous parental allele; nd: not determined. With the marker ABC1K14, it was not possible to distinguish between WT and het genotypes. The luciferase phenotype segregation in BC1F3 lines, derived from the BC1F2 plants with recombination events, was analyzed in luciferase assays (right column BC1F3 seg).

F2 Plant #	LUC signal	Position/markers on chromosome 1							BC1F3 seg
		21 762 923 RPP7	22 511 182 AT1g61100	23 332 232 EXPA18	24 309 749 SPT6L	24 547 251 ABC1K14	27 939 315 HSP101	28 791 309 SDG26	
4	high	WT	WT	WT	WT	WT/het	het	het	no
5	high	WT	WT	WT	het	WT/het	mut	mut	yes
15	high	het	het	het	WT	WT/het	WT	WT	no
20	high	het	het	het	het	WT/het	WT	WT	nd
28	high	het	het	het	het	mut	mut	mut	yes
35	high	het	het	WT	WT	WT/het	het	het	no
41	high	WT	WT	WT	WT	WT/het	het	het	no
42	high	het	het	het	het	WT/het	WT	het	yes
46	high	het	het	het	WT	WT/het	WT	WT	no

BC1F2 plants displaying high luciferase levels after HS treatments were assumed to either be heterozygous or homozygous for the WT allele at the locus causing the *rein2* mutant phenotype. BC1F2 plants exhibiting a heterozygous genotype for markers close to the causal mutation were expected to produce a BC1F3 generation that segregated 3:1. The combined results of genotyping and luciferase phenotype segregation analysis of three plants (#5, #15 and #45) led to exclusion of *EXP18* as a potential candidate (Table 3). One plant (#28) exhibited homozygosity for the mutant allele at position 24,547,251 bp (*ABC1K14*) and displayed segregation in the BC1F3 generation. This finding supported the results from genotyping plant #6 in Table 1.

In summary, the fine mapping results indicated the mutation causing the *rein2* mutant phenotype to be located between 23,33 Mb and 24,54 Mb on chromosome 1, where only 12 SNPs were located (Supplemental Table S6).

5.3.6 SPT6L is a plausible candidate gene mutated in the *rein2* mutant

The interval from 23,33 Mb to 24,54 Mb contained one gene from the candidate list (Table 2): *At1g65440*, which featured a missense mutation in a conserved amino acid in the YqgF domain of elongation factor SPT6L (Figure 29).

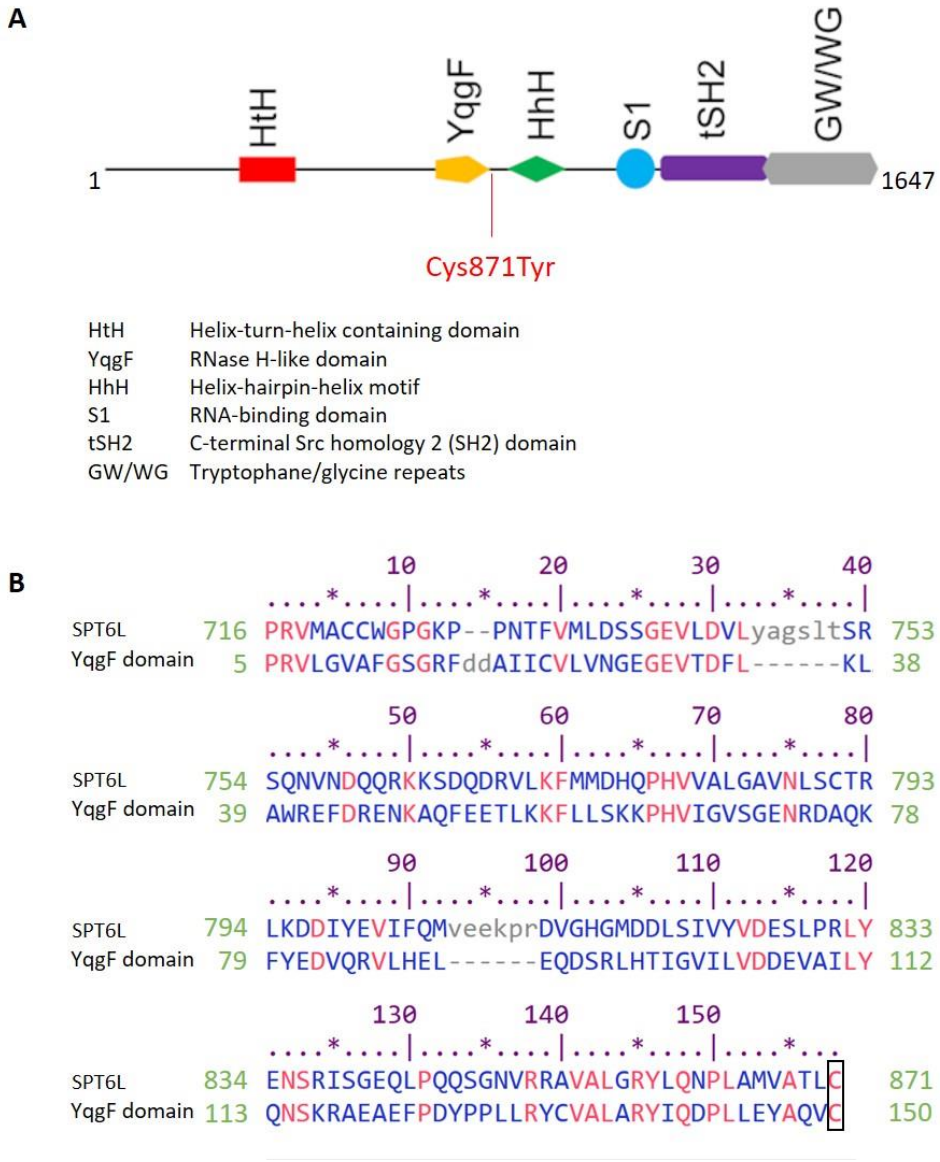

Figure 29. The YqgF domain of *SPT6L* is mutated in the *rein2* mutant. A Protein domains of *Arabidopsis SPT6L* (Chen et al., 2019). The *rein2* mutant carries a missense mutation in SPT6L, causing a cysteine to tyrosine change (Cys871Tyr) in the YqgF domain. **B** Alignment of the SPT6L YqgF domain to the Conserved Protein Domain Family YqgF (pfam 14639) using the Pfam database. Red indicates highly conserved and blue indicates less conserved amino acids, while grey are unaligned (lower case) residues. The last cysteine in the alignment (marked with a black box), which is mutated in the *rein2* mutant, is a conserved residue.

SPT6L is known for its role in transcription elongation. SPT6L interacts with Pol II and mainly targets genes marked with histone modifications like H3K4me3 (Chen *et al.*, 2019). Besides its role in transcription elongation, SPT6L is part of transcription initiation and recruitment of Pol II to the TSS and gene body of target genes. The YqgF domain was shown to be essential for these processes.

These functions of SPT6L and the observed, negative effect on transcriptional expression of HS-responsive genes in *rein2* mutants (Figure 29) aligning, highlight the amino acid change in the YqgF domain of SPT6L as a plausible causal mutation behind the *rein2* mutant-phenotype.

6. Discussion

6.1 Screening of *pAPX2::LUC* EMS mutants identified of multiple potential *rein* mutants

A previous forward genetic screen which aimed to identify regulators of the sustained type I transcriptional memory led to the identification of multiple FGT genes. The screen extensively contributed to our current knowledge of the plants ability to maintain aTT over several days (Brzezinka *et al*., 2016; Friedrich *et al.*, 2021; Urrea Castellanos *et al.*, 2020). It revealed that in addition to histone modifications, which are dependent on HSFA2, nucleosome occupancy and membrane lipid dynamics or lipid signaling are essential for HS memory. This study aimed to extend the knowledge of mechanisms driving transcriptional HS memory in plants and shed more light on the mechanisms underlying type II transcriptional memory.

The promoter fragment in the *pAPX2::LUC* reporter line contains *cis*-regulatory elements that are necessary and sufficient for transcriptional activation and memory for the subsequent coding sequence (Liu *et al.*, 2018; Schramm *et al.*, 2006). Luciferase assays with *pAPX2::LUC* transgenic seedlings following repeated HS conditions demonstrated that the enhanced re-induction of *APX2* after repeated HS was successfully mimicked by high accumulation of luciferase signals. The initial induction of *APX2* immediately after HS, however, could only be examined with a time delay to accumulate sufficient luciferase signal for detection with the NightOwl Imaging system. A recovery time of 5 hours minimum was necessary after a priming HS (1 hour of 37°C) to assure stably detectable luciferase levels in seedlings of the parental line. Early dynamics regarding *APX2* expression after a single HS could not be visualized with the luciferase imaging system due to sensitivity limits. Conclusively, a slower transcriptional induction in potential hyper-induction mutants that reaches wild type levels with delay, was not detectable in the initial luciferase screen. Likewise, a normal induction but faster decline within the first hours after HS could lead to a misinterpretation of the transcriptional behavior. A striking example for this was the luciferase phenotype of the *hsfa2* mutant crossed to the *pAPX2::LUC* genetic background that was observable after a single HS. Although it was reported that *APX2* induction is not affected by the absence of HSFA2, luciferase expression after a single HS with 5 h recovery was strongly reduced and below the detection threshold. This can be explained with the observation that *APX2* transcripts accumulate normally in the *hsfa2* mutant but get suppressed during early recovery (Charng *et al.*, 2007). Consequently, HSFA2 function is specifically required for maTT and has no physiological defect in the bTT

and aTT. Based on this observation, both mutant categories A (deficiency only in enhanced re-induction of *APX2*) and B (deficiency in enhanced re-induction and initial induction of *APX2*), that were found during the screen were evaluated to have the potential to contain HS memory-specific factors.

From a total of 1077 screened individual M2 mutant lines, 200 lines showed decreased luciferase activity after repeated HS. In 41 of a total of 58 re-tested M3 lines, the *rein* phenotype was confirmed and 25 of these lines additionally exhibited defects in the initial induction after a single HS. Regarding the limited numbers of M1 lines screened in the scope of this thesis, a remarkably high number of potential transcriptional regulators of *APX2* were found. These initial findings could indicate that *APX2* expression following heat exposure is regulated by multiple factors. However, backcrosses, which reduce the background mutation load, as well as further characterization of the luciferase phenotype and analysis of *APX2* transcript levels need to be performed to further substantiate this hypothesis.

6.2 *REIN1* encodes *CDK8*

NGS analysis of the *rein1* mutant identified a candidate mutation in the gene *AT5G63610*, encoding for *CDK8*, also named *HUA ENHANCER 3 (HEN3)* when it was first identified to control floral determinacy (Wang and Chen, 2004). A decade later, CDK8 was found in a luciferase-based genetic screen designed to identify components of mitochondrial retrograde signaling (Ng *et al.*, 2013). In search for upstream regulators of the *ALTERNATIVE OXIDASE 1* (*AOX1)*, a marker for mitochondrial retrograde response, multiple *regulators of alternative oxidase* (*rao1)* mutant alleles were discovered which contained mutations in the *CDK8* coding sequence. The study not only revealed that CDK8 is essential for integration of mitochondrial retrograde signals but also has a broader role in regulating the plants response to cellular stresses, like oxidative stress (H_2O_2) and cold stress. This involvement in stress responses favored CDK8 as a plausible candidate gene for the HS memory phenotype.

To verify that the premature termination codon in *CDK8* was causing the luciferase phenotype in *rein1* mutants, *rein1* was crossed with a previously described *cdk8* T-DNA mutant allele (Ng *et al.*, 2013) to perform a complementation test (Figure 15). The F1 generation exhibited a *rein1* mutant phenotype, confirming that that *rein1* and *cdk8* contain allelic mutations in the *CDK8* gene. The evidence for the involvement of CDK8 and other subunits of the Mediator complex in abiotic stress responses in plants is strongly increasing throughout the recent years (Crawford *et al.*, 2020; Lee *et al.*, 2021; Guo *et al.*, 2021). Studies analyzing the loss of function mutants

of individual mediator subunits result in different phenotypic changes in abiotic stress responses, suggesting subunit-specific roles in different stress conditions possibly by regulating gene-specific transcription factors. Only recently, a role for mediator in HS was reported, showing that MED14 and MED17 are positive regulators of bTT and aTT (Ohama *et al.*, 2021).

This is the first identification of a members of the CKM with a function in HS memory. The role of the CKM is complex, making a unified model for its regulatory role difficult. It has often been described as a transcriptional repressor involving different mechanisms including phosphorylation of Pol II CTD or transcription factors and steric inhibition by blocking Pol II-Mediator interaction. However, it also functions as a transcriptional activator presumably by its kinase activity that can lead to both, activation or repression depending on the given substrate. Studies in yeast and mammals, have shown that the CKM specifically functions in transcriptional memory (D'Urso *et al.*, 2016; Dimitrova *et al.*, 2022). It appears that CKM Mediator associates with gene promotors after a priming cue and supports future gene induction by enabling core Mediator or PIC binding, respectively. In plants, no role for CDK8 in transcriptional memory has been reported yet. It is tempting to speculate that the identification of CDK8 in regulating transcriptional memory behavior of HS memory genes indicates a conserved function as a regulator of transcriptional memory in plants.

6.3 CDK8 regulates thermotolerance and HS memory gene expression

Expression analysis of the HS memory genes *APX2*, *MIPS2*, *XTR6* and *CYP91D11* in the *rein1* and *cdk8* mutants revealed a deficiency in enhanced re-induction after repeated HS. Although to a lesser extent, transcript levels after a single HS were also reduced in both mutants. Interestingly, *HSP101* expression after HS treatment was also reduced in the mutants. The bTT, which is strongly impaired in *hsp101* mutants, was not affected. The reduction of *HSP101* transcripts observed in *rein1*/*cdk8* mutants was approximately 40 – 50 % which might suffice to express bTT comparable to WT in the chosen assay conditions at the physiological level. It is also possible that other mechanisms compensate for the reduced *HSP101* transcript levels in absence of CDK8. The gene expression analysis is only a snap-shot of the transcriptional levels of a few selected loci at the end of a 1 h HS treatment and therefore cannot give a comprehensive picture of transcriptional alterations. HS induces transcription within minutes and it was previously shown that enhanced transcriptional activation of HS memory genes in previously primed plants is already detectable after 15 min of HS and further increases during the recovery time after HS (Liu *et al.*, 2018). A time course expression analysis during a triggering HS (with

and without a previous priming HS) and for an extended period of time after the treatment could give insight into the kinetics of transcriptional induction of HS-induced genes in *rein1*/*cdk8* and also clarify whether faster or stronger re-induction is altered in the mutants. Considering the involvement of CDK8 in various abiotic stress responses via multiple mechanisms, it is also possible that CDK8 is not exclusively involved in transcriptional memory but also in other aspects of the HS response. To clarify this aspect, transcriptome analyses (RNA-seq) could be performed to obtain extensive insights into the expression profiles of *cdk8* mutants after single and repeated HS, compared to non-stressed conditions (C, P, T and P+T). This would provide more global information about the involvement of CDK8 in the HS response and potential defects in HS-inducible versus HS memory genes previously identified in genome-wide transcriptome profiling (Liu *et al.*, 2018).

The creation of stable tagged CDK8 lines with *rein1* mutant background was not completed as part of this thesis. However, this is necessary to support the complementation test results confirming CDK8 as REIN1. Additionally, the creation of such a transgenic line enables to perform Chromatin Immunoprecipitation coupled with quantitative PCR (ChIP-qPCR) experiments to analyze the binding of CDK8 to genomic loci. It would give insight on whether there is a direct association of CDK8 with HS memory genes that were differentially expressed in the *rein1* mutant. Further, ChIP followed by sequencing (ChIP-seq) in combination with RNA-seq might reveal global direct targets of CDK8 and also identify potential DNA promoter binding motifs and binding patterns with the benefit to interpret those data in context of gene expression.

Type II-transcriptional memory is associated with histone H3K4 hyper-methylation at HS memory gene loci (Lämke *et al.*, 2016). The accumulation of H3K4me2/3 is dependent on HSFA2 activity. The binding of HSFA2, however, occurs transiently during the first few hours after HS and dissociates from the loci, followed by high histone methylation marks that slowly decline during the memory phase. This led to the hypothesis that chromatin-modifying factors including H3K4 methyltransferases are recruited to memory loci to mediate transcriptional memory and that factors other than HSFA2 also contribute to the establishment and maintenance of the chromatin signature. Regarding the reduced re-activation of HS memory genes in *rein1*/*cdk8* mutants, the involvement of CDK8 in the process of H3K4me2/3 enrichment at memory loci should be investigated by assessing the accumulation of chromatin marks in *cdk8* mutants. Performing ChIP experiments with a tagged CDK8 line in the *hsfa2* background could provide additional information on whether CDK8 is recruited by HSFA2.

During transcriptional memory after drought stress a second memory mark, besides high levels of H3K4me3 levels, was found to be present at trainable (memory) genes (Ding *et al.*, 2012). This mark involves the phosphorylation status of Pol II. Ser5P of Pol II CTD is present at promoter-proximal regions of memory genes that get hyper-induced upon repeated stress treatment. The phosphorylation of Pol II CTD can happen at multiple sites within heptat repeats with a consensus sequence YSPTSPS and can highly influence the transcription process. Phosphorylation at different serine residues is associated with modified Pol II behaviors. While unphosphorylated Pol II is found during initial promoter binding, Ser5P is important for Pol II promoter escape and a mark that is recognized by proteins that mediate modification of promoter proximal histones (Nechaev and Adelman, 2011; Ng *et al.*, 2003). Phosphorylation of serine 2 of Pol II (Ser2P) is found at elongating Pol II with levels increasing towards the 3'end of a gene. Using techniques developed to monitor Pol II dynamics, the phosphorylation at different stages of transcription were shown to be generally conserved in *Arabidopsis* (Zhu *et al.*, 2018).

In yeast and mammalian cells, CDK8 is involved in transcriptional elongation, mediating phosphorylation of Pol II (Galbraith *et al.*, 2013; Osman *et al.*, 2021). Based on these observations in other organisms, the effect of CDK8 on Pol II accumulation and CTD phosphorylation in plants is an interesting question that should be addressed. Specific antibodies against RNA Pol II, CTD Ser2P and Ser5P are available and could be used for ChIP experiments, targeting the different forms of Pol II in samples deriving from wild type and *cdk8* mutant plants subjected to HS treatments.

6.4 CDK8 interacts with memory HSFs

Both, HSFA2 and HSFA3 were previously identified as essential components of HS memory in *Arabidopsis*. They form heteromeric complexes following HS which contribute to the accumulation of H3K4 hyper-methylation and transcriptional memory at HS memory loci (Lämke *et al.*, 2016; Friedrich *et al.*, 2021). In various organisms, including yeast and metazoans, HSFs are found in trimers or hexamers (Clos *et al.*, 1990; Peteranderl and Nelson, 1992; Chan-Schaminet *et al.*, 2009). This raises the possibility of memory HSFs, HSFA2 and HSFA3, to associate with other components contributing to specificity or strength of transcriptional activation. Considering the observed effect of CDK8 on transcriptional activation of HS memory genes and its conserved role as a coactivator it was investigated whether CDK8 interacts with the memory HSFs.

The interaction was tested using SLCA within *N. benthamiana* with split RLUC ß-Estradiol-inducible vectors (Schatlowski *et al.*, 2010). Following induced expression of either N-RLUC-HSFA2 or N-RLUC-HSFA3 together with the C-RLUC-CDK8 fusion protein, luminescence was detected due to the activity of reconstituted RLUC. In contrast to the high luminescence that was detected widespread on the infiltrated tobacco leaf in the study from Schatlowski *et al.*, 2010, luminescence from the tested interactions in scope of this thesis was mostly observable around the infiltration spots. However, the co-expression of the above-named R-Luc and C-Luc fusion protein with a respective empty vector or WRKY36, used as negative controls (Chen *et al.*, 2019), did not result in accumulation of any luciferase signal, indicating specificity of the interaction between CDK8 and the HSFs. The co-expression of HSFA2 and HSAF3 resulted in the accumulation of higher luciferase signals compared to the combination of CDK8 with the HSF, which might reflect the strength of interaction between the two. In addition to the SLCA, the interaction between the memory HSFs and CDK8 was analyzed using an *in vitro* pulldown assay. In line with the results from the SLCA, HSFA3 and to a lesser extend HSFA2 were co-immunoprecipitated with Halo-CDK8 and not with the Halo-tag alone, which was used as a negative control. Taken together, data indicate that CDK8 associates with HSFA2 and HSFA3. The HSFs possibly form active heteromeric trimers with CDK8 to act as memory complexes. Co-IP of stably tagged CDK8 and HSF lines in the mutant background could be created to confirm the obtained results. Additional information might be obtained by subjecting those stable lines to HS and analyzing the strength of the interaction between CDK8 and the memory HSFs at different timepoints, for instance during treatment, during memory phase and after repeated HS. To further analyze whether CDK8 and HSFs also interact

genetically, the creation of *cdk8 hsf* double mutants could provide information on an epistatic or additional effect on the HS phenotype.

The interaction between CDK8 and the memory HSFs raises the question of whether the latter are potential targets of the kinase activity of CDK8. In immunity to fungal pathogens, CDK8 functions in both, a kinase-dependent and independent manner, which was revealed using a transgenic line expressing kinase-dead CDK8. The kinase-dead CDK8 line was generated by substituting a conserved aspartic acid critical for the kinase activity with alanine. Expression of kinase-dead CDK8 in the *cdk8* mutant background restored only part of the transcriptional response to pathogens (Zhu *et al.*, 2014). A kinase-dead version of CDK8 would be a useful tool to evaluate the importance of the kinase function in HS response. First, it would be interesting to analyze whether loss of kinase activity impairs HS memory on a physiological level in survival assays and additionally expression of type-II HS memory genes. In addition, the identification of phosphorylation sites and their mutations in the memory HSFs would be a way to study the functional role of phosphorylation of HSFAs in the HS response.

6.5 The mutation in *rein2* affects thermotolerance and transcription of HS-responsive genes

The *rein2* mutant was chosen for initial characterization and NGS sequencing to identify the causative mutation due to its clear hyper-induction phenotype in luciferase assays. Originally, five out of 27 germinated seedlings within the M2 generation were spotted with strongly reduced luciferase expression after repeated HS (Supplemental Figure S4). This indicated that *rein2* harbored a recessive mutation, nearly segregating in the expected ratio of 7:1 in the M2 population (Page and Grossniklaus, 2002). In the M3 generation, the memory phenotype judged by luciferase activity in P+T conditions was validated. The *rein2* mutant was grouped into category A based on its memory-specific phenotype (Figure 23). However, while initial induction of *pAPX2::LUC* after HS appeared not affected in M3 seedlings, a defect in the priming response was detected after the first backcross. In the BC1F3 generation, a reduction in luciferase became apparent in individual seedlings in P as well as T conditions. A reason for this altered luciferase expression pattern might be that the backcross removed additional mutations which influenced the expression of the transgene.

In physiological HS assays, *rein2* showed a drastic impairment in thermotolerance. In maTT and aTT assays, the mutant showed worse survival rates already in the earliest tested timepoints, while the defect in bTT was comparable or even more pronounced than in *hsp101* mutants,

which has one of the most distinct reported HS phenotypes (Figure 26) (Hong and Vierling, 2000; Tiwari *et al.*, 2021). These observations indicate that the mutation in the *rein2* mutant interferes with processes confering HS tolerance. The data obtained from the gene expression analysis by RT-qPCR complemented the results from the HS assays, showing that *rein2* mutants have reduced transcript levels of HS-responsive genes following a single or repeated HS treatment (Figure 27). Together, these findings indicate that the candidate gene in *rein2* encodes a factor that positively impacts gene expression upon HS and that this positive effect might not be specific to HS memory on the transcriptional and physiological level.

6.6 SPT6L is a plausible candidate gene mutated in the *rein2* mutant

To identify the candidate mutation underlying the thermotolerance phenotype of *rein2*, genome sequencing of bulk segregants was performed and led to the detection of a region of distorted segregation in the mutant pool on chromosome 1. The high numbers of SNPs found in the *rein2* mutant compared to the parental line indicated that the EMS mutagenesis treatment resulted in a high mutation rate, which led to high numbers of potential candidate genes. Consequently, fine mapping was necessary to narrow down the interval. This revealed an allele frequency peak at ~ 25 Mb on chromosome 1. A broad interval between 21 and 30 Mb was searched for plausible candidates causing the HS phenotype in *rein2* (Table 2). At the beginning of the interval, two high impact mutations were detected, one in *RPP7* and *EXPA18*. While RPP7 is known for its role in the plant immune response, the cell wall protein EXPA18 reportedly influences root hair initiation and growth (Cho and Cosgrove, 2002; McDowell *et al.*, 2000). Like other expansins from several plant species, EXPA18 functions in stress tolerance, shown by a study analyzing the resistance to drought stress in *AtEXP18* expressing transgenic tobacco plants (Abbasi *et al*., 2021; Marowa *et al*., 2016). Expansins are incorporated into cell walls and cause cell wall relaxation and extension (Cosgrove, 2000). Because HS can cause cell wall damage, the expansin gene was considered a plausible potential candidate gene in *rein2*. Three genes from the list of candidates, *SDG26*, *HSP101* and *SPT6L*, drew attention due to their reported involvement in response to elevated temperatures. While the histone methyltransferase SDG26 has a role in the regulation of ambient temperature-induced splicing, HSP101 is essential for thermotolerance (Hong and Vierling, 2000; Pajoro *et al.*, 2017). The defect in bTT resembled the phenotype of the *hsp101* mutants. However, the SNPs in *SDG26* and *HSP101* were located at the end of the interval and fine mapping results excluded both candidates as causal variants (Table 3, Table 4).

Using dCAPS markers, the *REIN2* locus was mapped to an interval of 1.2 Mb (Supplemental Table S6). This narrowed region contained the candidate gene *SPT6L*. Two copies of the conserved SPT6 gene, SPT6 and SPT6L, exist in *Arabidopsis.* Unlike SPT6, SPT6L is commonly expressed and possesses additionally plant-specific C-terminal GW/WG repeats (Antosz *et al.*, 2017; Gu *et al.*, 2012). While the GW/WG repeats are thought to be involved in the small RNA pathway to regulate embryo development, the SH2 domain binds to the CTD of Pol II. Further, the YqgF and HtH domains are required for the enrichment of SPT6L around the TSS of target genes and its distribution along the gene body (Chen *et al.*, 2019a). SPT6L is recruited to HS responsive genes, like *HSFA7A* and *HSP70* in less than 10 min and this recruitment is impaired by deletion of the HtH/YqgF domains. The mutation in *rein2* leads to a change in the last and conserved amino acid in the YqgF domain of SPT6L and therefore can be considered to have a major impact on stress induced SPT6L expression.

Several *spt6l* mutants cause embryonic lethality or severe growth defects (Gu *et al.*, 2012). The *rein2* mutant, however, is phenotypically not altered in the seedling stage under normal growth conditions. Consequently, if the amino acid change in the YqgF domain of SPT6L is responsible for the *rein2* HS phenotype, the mutation does not lead to a total loss of SPT6L function but possibly alters the interaction with Pol II or other factors that assemble during transcription initiation or elongation. To finally validate *SPT6L* as the causal gene mutated in *rein2*, complementation assays need to be performed.

6.7 Integration of the CKM and SPT6L into the current knowledge about HS memory

The identification of CDK8 as a positive regulator of HS memory is an exciting finding in regard to recent studies revealing a specific role for the CKM in controlling HS induced gene expression (Srivastava *et al.*, 2021). In mammalian cells, MED12 and CDK8 promote HSP70 expression in response to heat shock, mediated through CDK8 kinase activity. MED12 interacts with HSF1, facilitating the recruitment of the CKM. CDK8 does not only phosphorylate components of mediator and GTFs, but also Serine 326 (S326) of HSF1 which influences HSF1 activity. In yeast, the CKM sterically represses transcription, but upon CDK8 kinase activity induced by HS, this repression is released. Active CDK8 kinase facilitates the release of the CKM from mediator, freeing its PolII-interacting surface for gene activation (Osman *et al.*, 2021). If CDK8 has a conserved function in plants and functions as an important integrator for HS, further analyses of its phosphorylation activity and targets, influence on other HS memory factors, PolII activity and PIC assembly will elucidate its role in processes that contribute to HS memory.

The second potential player involved in HS induced transcription, SPT6L, is one of two homologs of the conserved histone chaperone SPT6 (Bortvin and Winston, 1996). Besides its role in transcription elongation, SPT6 controls genome integrity through direct interaction with histones, primarily histone H3. In plants, SPT6L interacts with SWItch/Sucrose Non-Fermentable (SWI/SNF)-type chromatin remodelers SYD and BRM, which influence nucleosome occupancy (Shu et al., 2022). It would be interesting to examine whether SPT6L is also interacting with HS memory factor FGT1, which was found to associate with chromatin remodelers of the SWI/SNF and Imitation SWItch (ISWI) families to contribute to maintaining an open chromatin state at HS memory genes (Brzezinka *et al.*, 2016). Given that yeast SPT6 together with the FACT complex regulate nucleosome occupancy during transcription (Duina, 2011), a possible role for SPT6L in the HS memory could lie in integrating or maintaining nucleosomes and H3K4 methylated histones during the memory phase (Figure 30).

Further work on factors arising from the screen for modifiers of type II transcriptional memory will extend the current knowledge on thermomemory in plants and has the potential to link already identified HS memory regulators. This would help to understand the network underlying HS memory. With regard to climate change which poses a serious threat to crop yield and food security, it is crucial to investigate the molecular mechanisms that enable plants to cope with recurring episodes of heat. This knowledge may provide new insights for improving HS resilience of crop plants in the future.

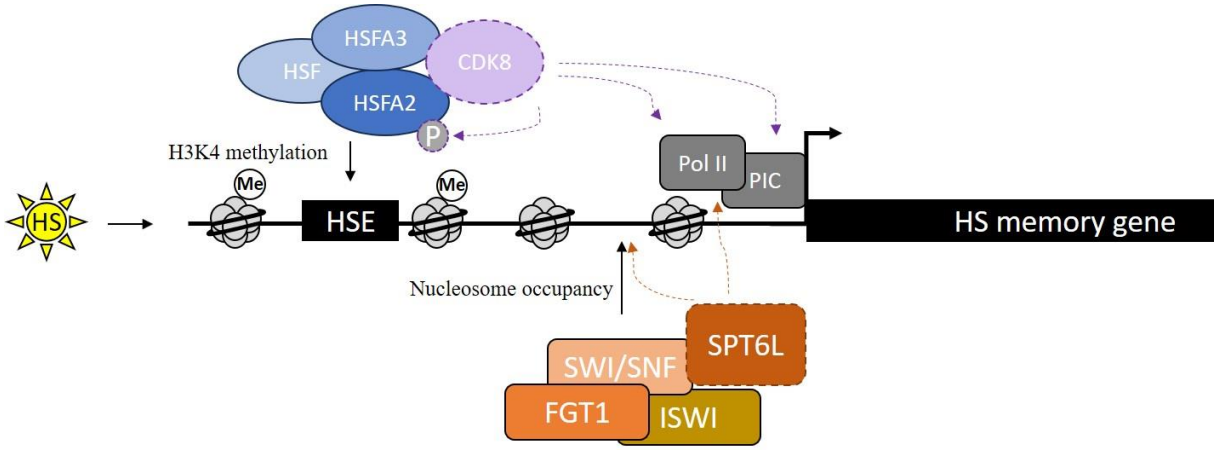

Figure 30. Integration of CDK8 and SPT6L into the current picture of HS memory processes. Upon HS, HSFA2 in complex with other HSFs binds to the Heat Shock Elements (HSEs) in promotors of HS memory genes and triggers accumulation of H3k4me3 (Me) which lasts throughout the memory phase. CDK8 may regulate HSF activity or other targets through its kinase activity. CDK8 may also promote enhanced gene expression at the HS memory loci by affecting Pol II dynamics or the PIC assembly during the memory phase or after repeated HS. Additionally, FGT1, in a complex with chromatin remodelers from the SWI/SNF and ISWI families, binds around the +1 nucleosome after HS to maintain low nucleosome occupancy, which keeps the chromatin in an open state, thus facilitating sustained active transcription of HS memory genes. SPT6L associates with Pol II and facilitates its occupancy across transcribed genes, having an influence on the transcription initiation and elongation process. SPT6L was recently shown to interact with chromatin remodelers of the SWI/SNF family as well and might support to keep the chromatin in an active state. Dashed lines represent proposed concepts based on this thesis; solid lines represent proven concepts.

7. Material and Methods

7.1 Plant growth conditions and plant material

Arabidopsis thaliana seeds were plated on plates with growth medium (GM) containing 1 % (w/v) Glucose after sterilization with 6 % NaClO for 5 min. The seeds were stratified at 4°C for 3-4 days in the dark before the plates were moved to a growth chamber set to long day conditions (16 h light at 23°C / 8 h dark at 21°C) with 120-140 µmol/(s·m²) PAR. For propagation and crossing, 7-10 days old seedlings were moved from plate to soil containing pots in a glasshouse with long day lightning.

Nicotiana benthamiana plants used for transient expression were grown in a greenhouse under long day conditions and used after 4-5 weeks of growth for infiltration.

Table 5. Genotypes of *A. thaliana* used in this study.

Genotype	**Ecotype**	**Type**	**Reference**
WT	Col-0	Wildtype	
hsfa2	Col-0	T-DNA insertion line, SALK_008978	Charng *et al.*, 2007
hsp101	Col-0	T-DNA insertion line, SALK_066374	Alonso *et al.*, 2003
cdk8	Col-0	T-DNA insertion line, GABI564F11	Ng *et al.*, 2013
pAPX2::LUC	Col-0	Transgenic plant line	Liu *et al.*, 2018
rein1	*pAPX2::LUC*	EMS mutant line	Bäurle lab
rein2	*pAPX2::LUC*	EMS mutant line	Bäurle lab

7.2 DNA extraction

DNA was extracted by grinding tissue from A. *thaliana* with a Teflon pestle for a few seconds in a 1.5 mL microcentrifuge tube. After addition of 0.4 mL of extraction buffer (200 mM Tris HCl pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8, 0.5 (w/v) % SDS), solids were removed by centrifugation for 10 min at 13000 rpm. The supernatant was transferred to a fresh microcentrifuge tube and nucleic acids were precipitated by addition of 350 µL Isopropanol. The tube was inverted several times and a subsequent centrifugation step of 20 min at 13000 rpm pelleted the DNA. The pellet was washed with 300 µL 70 % Ethanol and 5 min centrifugation at 13000 rpm. The pellet was allowed to dry at RT and DNA was dissolved in 50 µL TE (10 mM Tris HCl pH 7.5, 1 mM EDTA pH 8) by incubation on a shaker at 37°C for 30 min. For PCR reactions, 2 - 5 µL were used in a 20 – 25 µL reaction.

DNA was extracted with the DNeasy Plant Maxi Kit (Qiagen) for high throughput sequencing (Next Generation Sequencing, NGS) to identify causative EMS mutations. The protocol for Purification of Total DNA from Plant Tissue (Maxi Protocol) was followed using the TissueLyser procedure and elution was performed with the minimal volume possible (500 µL).

7.3 Cloning PCR and dCAPS assays

A reaction mixture protocol and cycling conditions for a routine cloning PCR using Phusion DNA-Polymerase (Thermo Fisher Scientific) are described below. Reaction conditions were individually optimized for different primer pairs by variation of annealing temperature and cycle number.

Component	25 µl reaction
5X HF Phusion Buffer	5 µl
10 mM dNTPs	0.5 µl
10 mM Forward Primer	0.7 µl
10 mM Reverse Primer	0.7 µl
DMSO (100 %)	0.75 µL
Phusion DNA Polymerase	0.25 µl
Template DNA (cDNA)	variable (1-3 µL)
Nuclease-free water	to 25 µL

Cycle step	Temperature (°C)	Time (min)	Cycles
Initial Denaturation	95	00:30	1
Denaturation	95	00:30	
Annealing	45-68	00:30	30-35
Extension	68	01:00 per kb	
Final Extension	72	05:00	1
Hold	4	∞	

PCR products were separated in a 1-2 % agarose gel using gel electrophoresis. DNA was visualized with EtBr under ultraviolet (UV) light on GelDocTM XR+ instrument (Bio-Rad) and for further processing of DNA, the desired PCR band was extracted by using NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel).

DCAPS (Derived Cleaved Amplified Polymorphic Sequence) assays, which allow the detection of single-nucleotide polymorphisms (SNPs), were used for genetic mapping of EMS mutants. The reaction mixture protocol and cycling conditions for a routine dCAPS PCR and subsequent restriction digest, are described below. dCAPs PCR primers were designed using the program dCAPS Finder 2.0 (Neff et al., 2002). The primer pairs were used to amplify an approximately 300 bp PCR fragment using Dream Taq Polymerase (Thermo Fisher Scientific). Reaction conditions were individually optimized for different primer pairs by variation of annealing temperature and cycle number.

Component	25 µl reaction
10X DreamTaq Buffer	4 µl
10 mM dNTPs	0.4 µl
10 mM Forward Primer	0.8 µl
10 mM Reverse Primer	0.8 µl
25 mM MgCl2	1,3 µL
DreamTaq DNA Polymerase	0.07 µl
Template DNA (cDNA)	variable (1-3 µL)
Nuclease-free water	to 25 µL

Cycle step	Temperature (°C)	Time (min)	Cycles
Initial Denaturation	95	03:00	1
Denaturation	95	00:30	
Annealing	45-65	00:30	30-35
Extension	68	01:00 per kb	
Final Extension	72	05:00	1
Hold	4	∞	

The PCR product was subjected to restriction enzyme digestion (12.8 µL PCR product, 2µL buffer, 0.2 µL enzyme, add water to 30 µL) overnight to ensure complete digestion. The presence or absence of the SNP was determined by the resulting restriction pattern on a 1.5% agarose gel containing EtBr.

7.4 RNA extraction

Total RNA was extracted using TRI Reagent (Sigma) following a modified version of the RNA extraction TRIZOL protocol (Bäurle lab). Approximately 100 mg seedlings, grown on GM plate were harvested and collected in a 2 mL tube and directly frozen in liquid nitrogen. The plant material was ground to fine powder with a Swing Mill (Retsch). While the material was still frozen, 1 mL of TRI Reagent was added and kept slowly shaking at RT for 5 min. The homogenate was centrifuged for 11000 rpm for 10 min. The clear supernatant was transferred to a new tube and 300µL of chloroform was added. After vortexing for 30 sec, phases were separated by centrifugation at 11000 rpm for 10 min. The upper aqueous phase containing the RNA was carefully transferred to a 1.5 mL microcentrifuge tube. For precipitation of the RNA, 0.5 volumes isopropanol was added, followed by an incubation at -20°C overnight. On the next day, a centrifugation step of 30 min at 4°C and 11000 rpm was performed to pellet the RNA. The pellet was washed twice with 80% EtOH (11000 rpm, 10 min, 4°C). EtOH residues were carefully removed without disrupting the pellet and the pellet was dried at RT for 20 min. RNA was dissolved in 30 µL RNase free water.

7.5 cDNA synthesis

For cDNA synthesis, 5 µg of RNA was treated with reagents from the TURBO DNA-free Kit (Invitrogen) to remove DNA. The DNA-free RNA was reverse transcribed with SuperScript III RT (Invitrogen) using oligo(dT) as primer and in presence of RNAsin (Promega) for RNase inhibition.

7.6 Gene expression analysis

To analyze gene expression, cDNA was diluted 1:20 and RT-qPCR was performed in a LightCycler480 (Roche). A protocol and cycling conditions for a routine RT-qPCR are shown below.

Component	13µL reaction
GoTaq qPCRMasterMix	6,67 µL
Primers (1µM)	3,33 µL
cDNA template (1:20)	3 µL

Cycle step	Temperature (°C)	Time (min)	Cycles
Initial Denaturation	94	03:00	1
Denaturation	95	00:15	
Annealing	57	00:20	35
Extension	72	00:40	
Melting Curve	from 65 to 97	2.5°C/sec interval	1

To obtain relative transcript levels of selected genes, the obtained Ct values of three technical and biological replicates were normalized to the Ct values of a reference gene (*AT4G26410* or *TUB6*) using the comparative Ct method.

7.7 Library preparation for NGS sequencing

For library preparation, DNA from 150-200 bulked 7 days old seedlings was extracted using the DNeasy Plant Maxi Kit. The DNA quality and integrity was analyzed with an Agilent TapeStation instrument using the Genomic DNA ScreenTape. To obtain DNA fragments of approximately 350 bp length, DNA was sheared with a Covaris S220 Focused-ultrasonicator. To confirm successful fragmentation, the sheared DNA was analyzed by TapeStation with a D1000 ScreenTape. The fragmented DNA served as input material for library preparation with

the NEBNext Ultra II DNA Library Kit for Illumina. The instructions and recommended conditions for an insert size of 300-400 bp were followed. For library enrichment, 6 PCR cycles were performed. Finally, the library was tested with a D1000 ScreenTape on the TapeStation to confirm the size distribution. The quantification was done with NEBNext Library Quant Kit for Illumina to generate the sequencing pool for the sequencing with NEB Next Seq 500/550 Mid Output Kit (Illumina). Mapping of paired end reads against the *A. thaliana* reference genome (TAIR10) and further analysis of sequencing data was carried out by Dr. Christian Kappel (Genetics Research Group, University of Potsdam).

7.8 Gateway cloning

Two recombination reactions were used for Gateway cloning (Thermo Fisher Scientific). A PCR fragment was amplified using forward and reverse primers incorporating attB and attB2 sites. The purified PCR fragment was then used in a BP reaction with the vector pDONR221 to generate an attL-flanked entry clone. The entry clone was send for sanger sequencing by LGC Genomics.

The desired expression clone was generated by performing an LR recombination reaction between the entry clone and a Gateway destination vector. Detailed protocols for recombination reactions can be found in the User Guide: Gateway Technology (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/gatewayman.pdf).

7.9 Transformation of chemically competent *E.coli*

50 µL of chemically competent *E. coli* (*DH5α* strain) were thawed on ice for 10 min. After adding 2-5 µL of the plasmid or DNA insert, the cells were incubated on ice for 30 min. Subsequently, a heat shock of 42°C for 45 sec was performed and the Eppendorf tube was put on ice immediately for 2 min. After adding 950 µL of LB medium, the cells were incubated at 37°C for one hour with continuous shaking. The cells were plated on the corresponding selection agar plates and bacteria were incubated at 37°C overnight.

7.10 Plasmid isolation

For preparation of liquid cultures, 4 ml of LB media containing the appropriate antibiotics was inoculated with a single colony of *E. coli* Bacteria were grown over night at 37°C with continuous shaking. 2 mL of the culture were centrifuged at 13000 rpm for 1 min. The supernatant was discarded and the pellet resuspended in 150 µL E1 buffer (50 mM Tris-HCl ph 8, 10 mM EDTA). Subsequently, 150 µL of E2 buffer (200 mM NaOH, 1 % (w/v) SDS) was added. After inverting the tube several times, 300 µL E3 buffer (3.1 M Potassium acetate pH 5.5, adjusted with acetic acid) was added. Again, the sample was inverted several times to precipitate the plasmid DNA out of the solution. The sample was centrifuged for 10 min at 13000 rpm and the 500 µL supernatant was transferred to an equal amount of isopropanol and mixed by vortexing. To pellet the DNA, the sample was centrifuged for 15 min at 13000 rpm. The pellet was washed with 500 µL ethanol (70%) for 5 min on ice and centrifuged for 5 min. The supernatant was carefully removed. The pellet was dried at 37°C for approximately 30 min. The DNA was dissolved in 30– 50 µL TE buffer (10 mM Tris/HCl pH 7.8, 1 mM EDTA pH 8, adjusted to pH 8) supplemented with RNase. The concentration was determined with the NanoDrop2000 (Thermo Scientific).

7.11 Heat stress assays

HS assays were performed with *A. thaliana* seedlings on petri dishes with a standardized volume of GM (25 mL for round petri dishes, 30 mL for square petri dishes). Round petri dishes were used for survivals assays and square petri dishes for luciferase activity assays.

For survival assays, 28 seeds of each genotype to be tested were sown in equal spacing to each other on GM plates. A maximum of 6 genotypes were tested on one plate and mutants were always placed next to their respective wild type background. Stratification was performed for 3 days before transfer to a plant growth chamber, where the plates were put vertically (Percival). The first HS was applied after 4-5 days of growth (depending on the tested genotypes) in a waterbath with exclusion of light after sealing the plates with water-repellent tape. After termination of the HS treatment, plates were taken out of the waterbath and kept at RT for 45 min after the sealing tape was removed. The plates were transferred back to the Percival and kept horizontally until evaluation.

In case of a second HS to be applied, the plates were kept vertically in the recovery phase between the two treatments. Seedling growth was analyzed and scored into four categories 14 days after application of the last HS (Figure 31). To avoid diurnal effects, HS treatments were performed 6 h after dawn.

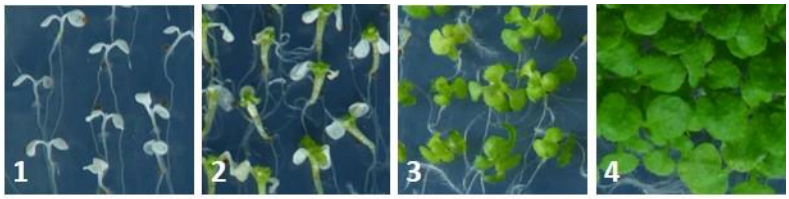

Figure 31. Representation of the four phenotypic classes used to score the survival of HS tested seedlings. (1) Dead, (2) severely affected - cotyledons are largely bleached out, but the meristem is intact and true leaves are formed, (3) slightly affected – cotyledons are not affected, but seedlings are smaller and/or yellowish and (4) healthy seedlings, which display a phenotype compared to non-treated seedlings.

7.12 Basal thermotolerance (bTT)

bTT is the plants ability to survive the direct exposure to extreme high temperatures for a short time period. To evaluate bTT of seedlings, 4-5 days old plants were subjected to a 44°C HS for 15 to 40 min. After 45 min recovery at RT, the plates were transferred back to the growth chamber and evaluation of growth was done 14 days after the HS by categorizing the seedlings according to four classes (Figure 32).

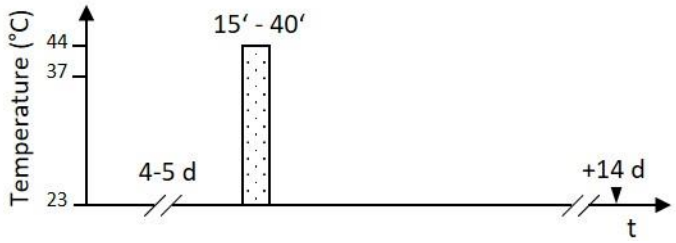

Figure 32. HS treatment scheme for the bTT assays performed in this study. The triangle (14 days after HS treatment) symbolizes the day of evaluation.

7.13 Acquired thermotolerance (aTT)

The ability to endure lethal high temperatures for a longer time span following acclimation at a sublethal high temperature is referred to as aTT. 4 - 5 days old seedlings were acclimatized with a mild HS at 37°C for 60 min. After a recovery time of 90 min at RT during which the water proof tape was removed, the plates were sealed again and subjected to a 44°C HS for 120-200 min. The plates were put back to the growth chamber after resting for 45 min at RT and analyzed 14 days after the 44°C treatment (Figure 33).

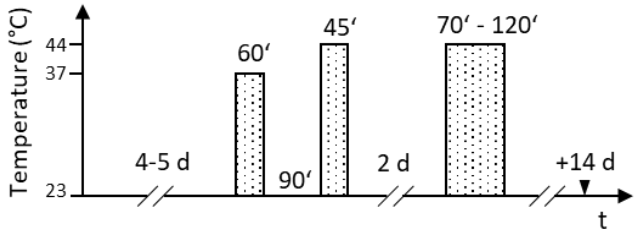

Figure 33. HS treatment scheme for the aTT assays performed in this study. The triangle (14 days after HS treatment) symbolizes the day of evaluation.

7.14 Maintenance of acquired thermotolerance (maTT)

The protection against lethal high temperatures acquired from a previously experienced sublethal HS can be maintained by plants over several days. The maTT of the genotypes to be tested was assessed by subjecting 4-5 days old seedlings to a full ACC. This ACC was done according to an aTT assay, see above, while the 44°C HS was set to 45 min. After the ACC treatment, the plates were transferred back to the growth chamber and kept vertically for 2 days. The tester HS of 44°C was done for 70 - 120 min in 10 min intervals. The evaluation of survival was performed 14 days after the tester HS (Figure 34).

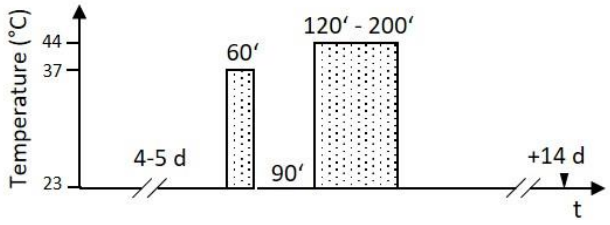

Figure 34. HS treatment scheme for the maTT assays performed in this study. The triangle (14 days after the latter HS treatment) symbolizes the day of evaluation.

7.15 Luciferase assays

Luciferase reporter gene activity was measured in seedlings grown on square petri dishes with GM medium using a NightOWL LB 983 in vivo imaging system (Berthold Technologies). Seeds were sown with equal distance to each other, stratified for 3 days and transferred to a growth chamber. Three different HS regimes were performed in this study to investigate *pAPX2*::LUC activity after initial induction (T), sustained induction (P) and hyper-induction following repeated HS (P+T) :

T: 4-5 days old seedlings were treated with a 60 min HS at 37°C. The plates were incubated at RT for five hours before imaging.

P: 4-5 days old seedlings were treated with a 60 min HS at 37°C. The plates were transferred back to the growth chamber for 48 h before imaging.

P+T: 4-5 days old seedlings were treated with a 60 min HS at 37°C. After recovery at RT for 60 min, the plates were transferred back to the growth chamber. A second HS was performed 2 days later for 60 min at 37°C and imaged after 60 min of recovery at RT.

For luciferase reporter activity detection, seedlings were sprayed with a 1 mM solution of D-luciferin (Promega). The plates were incubated in the dark for 12 min before detection. Using the IndiGO software, images were taken with an exposure time of 200 s. The picture was subsequently edited by adjusting the threshold of luciferase signals manually depending on the experimental setup.

7.16 Split Luciferase Complementation Assays (SLCA)

To study protein-protein interactions in SCLA, genes of interest were fused to N- and C-terminal fragments of *Renilla* luciferase (RLUC) and transiently expressed in tobacco leaves. The physical interaction of the proteins reconstitutes luciferase activity and results in light emission in the presence of the luciferase substrate. The ß-estradiol-inducible destination vectors pYS40 (RLUC-N) and pYS39 (RLUC-C) were obtained from Prof. Dr. Daniel Schubert (Schatlowski *et al.*, 2010). Using Gateway Cloning Technique (section 7.8), entry clones in pENTR221 which contained the cDNA of the gene of interest without stop codon were recombined by LR reaction with pYS40 and PYS39 to obtain expression vectors containing C-terminal fusion to N-RLUC and C-RLUC, respectively. The expression vectors were used for transient expression in *N. benthamiana*.

7.17 Transformation of *Agrobacterium tumefaciens*

50 µL aliquots of electrocompetent *A. tumefaciens* cells (GV3101) were thawed on ice. After 2µL of plasmid DNA was added, the cells were incubated 5 min on ice before they were gently pipetted into a cuvette, thereby avoiding bubbles. Electroporation was performed by using a Gene Pulser II (BioRad) with 1.8 kV, 25 µFD and 200 Ω to transfer the plasmid DNA into the bacteria by increasing the permeability of the cell membrane. The cells were allowed to recover by adding 950 µL of LB medium, transfer to a new 2 mL Eppendorf tube and incubation at 28°C in a shaker for 2 h at 200 rpm. 100 µL cells were plated on a LB agar plates which contained the required antibiotics. The plates were incubated for 2-3 days at 28°C for bacterial colony growth.

7.18 Transient expression of fluorescent fusion proteins in tobacco plants

For the transient expression of fusion proteins in tobacco leaves, a 5 mL *A. tumefaciens* liquid culture was prepared by inoculation of LB medium with the required antibiotics with a single *A. tumefaciens* colony. The liquid culture was incubated overnight at 28°C with 200 rpm. 2 mL of culture were pelleted at 1000 g for 10 min at RT. After the supernatant was removed, the cells were resuspended in infiltration medium (0.5 % D-Glucose, 50 mM MES, 2 mM Na_3PO_4*12H2O, 100 µM acetosyringone). The cells were centrifuged again for 10 min at RT and resuspended in 1 mL of infiltration medium to remove traces of antibiotics. The absorbance at 600 nm (OD_{600}) of a 1 in 10 dilution was measured. The required dilutions of bacterial suspensions to be coexpressed with a final $OD6_{000}$ of 0.5 were prepared together in a 2 mL Eppendorf tube. To avoid gene silencing, an Agrobacterium culture containing a T-DNA with the gene for the silencing suppressor p19 (Dunoyer *et al.*, 2004) was mixed in a 1:1:1 ratio with the Agrobacterium cultures. For the infiltration into tobacco epidermal cells, 4 weeks old *N. benthamiana* plants were removed from the growth chamber. Healthy leaves were infiltrated on the abaxial side with bacteria suspension by using a 1 mL syringe to gently press the liquid inside. The infiltrated area was marked with a marker pen and the tobacco plants were placed back in the growth chamber, where direct light was avoided.

7.19 β-Estradiol induction and luciferase detection

The expression of the N-RLUC/ C-RLUC fusion proteins in tobacco was induced by brushing the infiltrated leaves with a ß-Estradiol solution (100 µM ß-Estradiol (Sigma) and 0.1 % Tween in water). The plants were kept for 24 h on a tray covered with a lid. For luminescence detection, the leaves which had been induced with ß-Estradiol were infiltrated with 10 µM ViviRen live cell substrate (Promega). After incubation for 10 min in darkness, luminescence was detected with a NightOWL LB 983 in vivo imaging system for 30 min.

7.20 *In vitro* expression of recombinant proteins

For *in vitro* translation of YFP-Tag and Halo-Tag fusion proteins, the TnT Coupled Wheat Germ Extract System (Promega), where transcription is incorporated in the translation mix, was used. The reaction components below were thawed on ice and column-purified plasmid DNA was used as template for the reaction described below.

Component	50 µL reaction
TnT Wheat Germ Extract	25 µL
TnT Reaction Buffer	2 µL
TnT RNA Polymerase	1 µL
Amino Acid Mixture minus Methionine, 1mM	0.5 µL
Amino Acid Mix minus Leucine, 1mM	0.5µL
RNasin Ribonuclease Inhibitor 40U/µL	1 µL
Template DNA, plasmid 1	5 µL
Template DNA; plasmid 2	5µL
Nuclease- free water	10 µL

The reaction was incubated for 2 h at 30°C. Before continuing with the pulldown of the synthesized Halo-Tag protein, 5 µL was removed and frozen at -20°C from the 50 µL reaction and served as input.

7.21 Protein Pull-Down from a Cell-Free Expression System

Protein pull-downs were performed using Magne-Halotag Beads (Promega). Before use, the beads were resuspended thoroughly by inverting the bottle. Before the beads could settle down, 10 µL were transferred to a 1.5 mL microcentrifuge tube with 50 µL of equilibration buffer, consisting of Phosphate-buffered saline (137 mM NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, 8.1mM Na_2HPO_4, pH 7.4) with 0.005 % NP 40. The beads were washed four times by repeating the following steps. The tube was placed in a magnetic stand for 30 sec to capture the beads and the supernatant was discarded. The tube was removed from the magnetic stand and 50 µL equilibration buffer was added. The beads were gently mixed by flicking the bottom of the tube before putting back to the magnetic stand. After removing the supernatant from the final washing step, an equal volume (45 µL) of equilibrium buffer and cell-free protein expression reaction which were pipetted together, were immediately added to the beads. The protein binding was performed overnight by placing the tubes on a tube rotator at 4°C. The protein-bead solution was placed in a magnetic stand for 30 sec before the supernatant was removed. 50 µL of equilibrium was added and mixed by flicking the tube for 5 min. The last two steps were repeated twice for a total of three washes. After the supernatant was removed from the last wash, the tube was removed from the magnetic stand and 40 µL of 5X Laemmli buffer (300 mM Tris-HCl, 50 % Glycerol, 5 % SDS, 0.025 % Bromphenol blue, 5 % ß-mercaptoethanol freshly added) was added and mixed for 5 min. Finally, the beads were attached and the supernatant containing the proteins were transferred to a new 1.5 mL Eppendorf tube.

7.22 SDS-Page and Immunoblot analysis

To separate proteins by electrophoresis, samples (9 µL Input, 18 µL protein sample) were run on a 12 % SDS-PAGE (Bio-RAD) next to a PAGEruler plus marker (Thermo Scientific) for protein size estimation. Subsequently, the proteins were transferred onto a nitrocellulose membrane by using a wet electroblotting system with the Mini Trans Blot cell (Bio-Rad). Blotting was performed for 1 h at 100 V in presence of a cooling unit. The membrane was blocked overnight with TBST (200 mM Tris-HCl pH 8, 150 mM NaCl, 0.1 % Tween20) supplemented with 5 % milk powder with gentle shaking at 4°C. On the next day, the blocking solution was removed and the membrane was incubated with the primary antibody anti-FLAG (M2, F1804, Sigma, 1:2500) in 10 mL blocking solution for 1 h. Subsequently, the membrane

was washed three times by repeating the incubation of the membrane in 10 mL TBST for 10 min and gentle shaking. For detection of the tagged fusion proteins, the membrane was incubated with the secondary antibody (Goat-anti-Mouse IRDye 800 CW 1:10000) in TBST for 30 min. After three washes with TBST, the nitrocellulose membrane was developed using an Odyssey® infrared imaging system (Li-COR Bioscience) at 800 nm.

7.23 EMS mutagenesis

Approximately 1 g of seeds were imbibed in water on miracloth at 4˚C for 4 days and then dried on filter paper at 25˚C for 24 h. The working area in the fume hood and a magnetic stirrer were covered with plastic foil and paper. 50 mL of tap water was transferred to a 100 mL bottle. 0.15 mL of EMS (Sigma, M0880) were carefully injected into the water using a syringe. The EMS solution was placed on a magnetic stirrer and gently mixed for 5 min. Seeds were added to the EMS solution and incubated for 8 hours with gentle stirring. Everything that was in contact with EMS was placed into a decontaminating solution (160 g NaOH, 50 ml thioglycolic acid (Sigma, 528056), 3950 mL H_2O). After the incubation, the EMS solution was carefully decanted into the decontamination solution. 50 ml of water was added to the seeds bottle. After seeds sank down, the water was decanted into decontamination solution. This washing step was repeated one time. After adding 50 mL of water, the seeds were transferred to a filter bag and the seeds were washed for 15 times with fresh water. Finally, the seeds were dispersed in pots in the glasshouse to grow the M1 generation. The decontamination solution was left in the fume hood overnight and was neutralized with HCl (345 ml HCl (37 %) + 655 ml water) the next day.

8. Conclusion

Through a forward genetic screen multiple potential *rein* mutants were identified with modified type II transcriptional memory of a *pAPX2::LUC* reporter. In the scope of this thesis, two *rein* mutants were investigated regarding their HS memory phenotype and causative mutations.

In the *rein1* mutant, LUC activity was induced normally after a single HS, however, following a repeated HS, LUC activity was strongly reduced compared to the parental line. After backcrossing to the parental line and genome re-sequencing of bulk segregants, the candidate mutation in *rein-1* was identified. A SNP in exon 2 of *AT5G63610* introduced a premature stop codon at Gly425 in the CDK8 gene, which is a subunit of the Mediator kinase module.

Consistent with the reduced LUC activity after repeated HS, several type II memory genes including the endogenous *APX2* exhibited reduced re-activation in *rein-1* and *cdk8* mutants. The defect in transcriptional memory correlated with reduced HS memory at the physiological level. Survival assays revealed significant defects in maTT and indicated additionally a slightly reduced capacity for aTT in the absence of CDK8. These findings suggest that CDK8 as part of the CKM has a role in mediating transcriptional memory after HS.

Using *in vitro* Co-Immunoprecipitation and Split Luciferase Complementation Assays, CDK8 was found to interact with the memory HSFs HSFA2 and HSFA3. This association suggests that the memory HSFs are potential CDK8 kinase targets or that the Mediator complex might be recruited via HSFA2/HSFA3 for regulation of the PIC or Pol II activity.

The *rein2* mutant was found with impaired hyper-induction of *pAPX2::LUC* activity after repeated HS. After backcrossing to the parental line, a defect in the induction of *pAPX2::LUC* after the priming HS was detected. In line with this observation, the *rein2* mutant exhibited an overall reduced thermotolerance and reduced HS-induced gene expression. NGS and subsequent fine mapping indicate that a missense mutation in the histone chaperone SPT6L is the causal mutation in *rein2*. A potential function of SPT6L during HS induced gene expression may involve the integration or preservation of nucleosomes and histones with H3K4 methylation.

9. Bibliography

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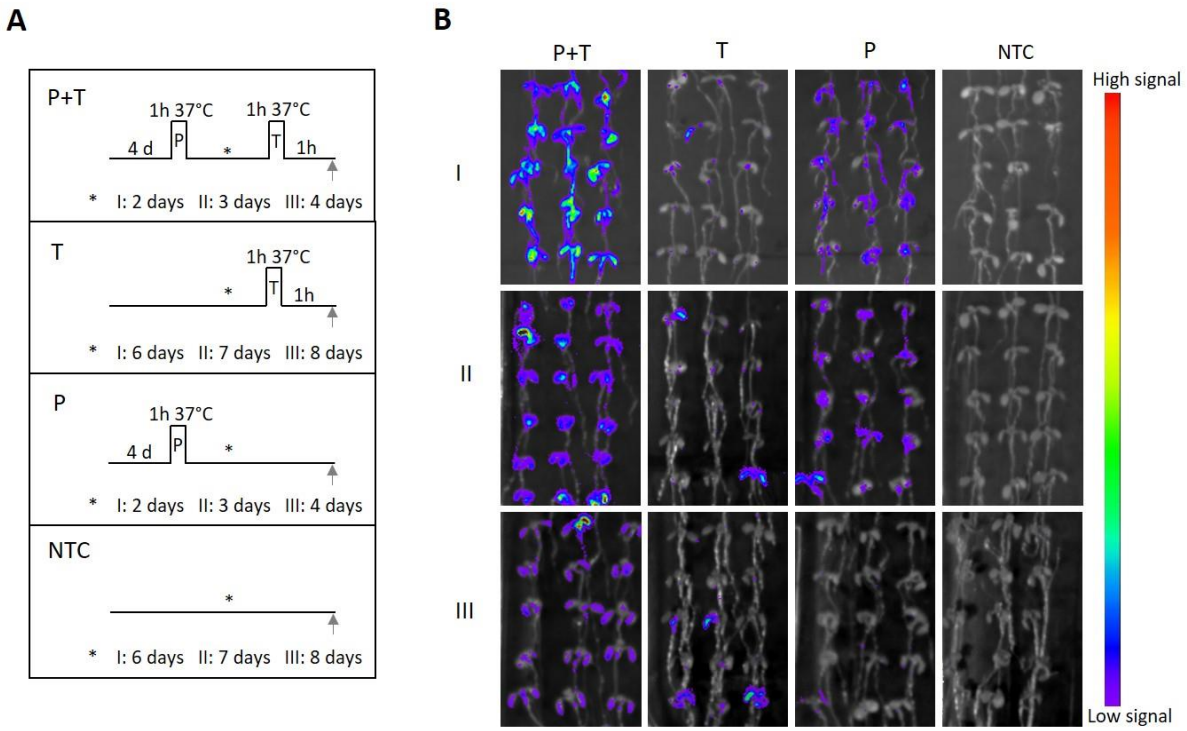
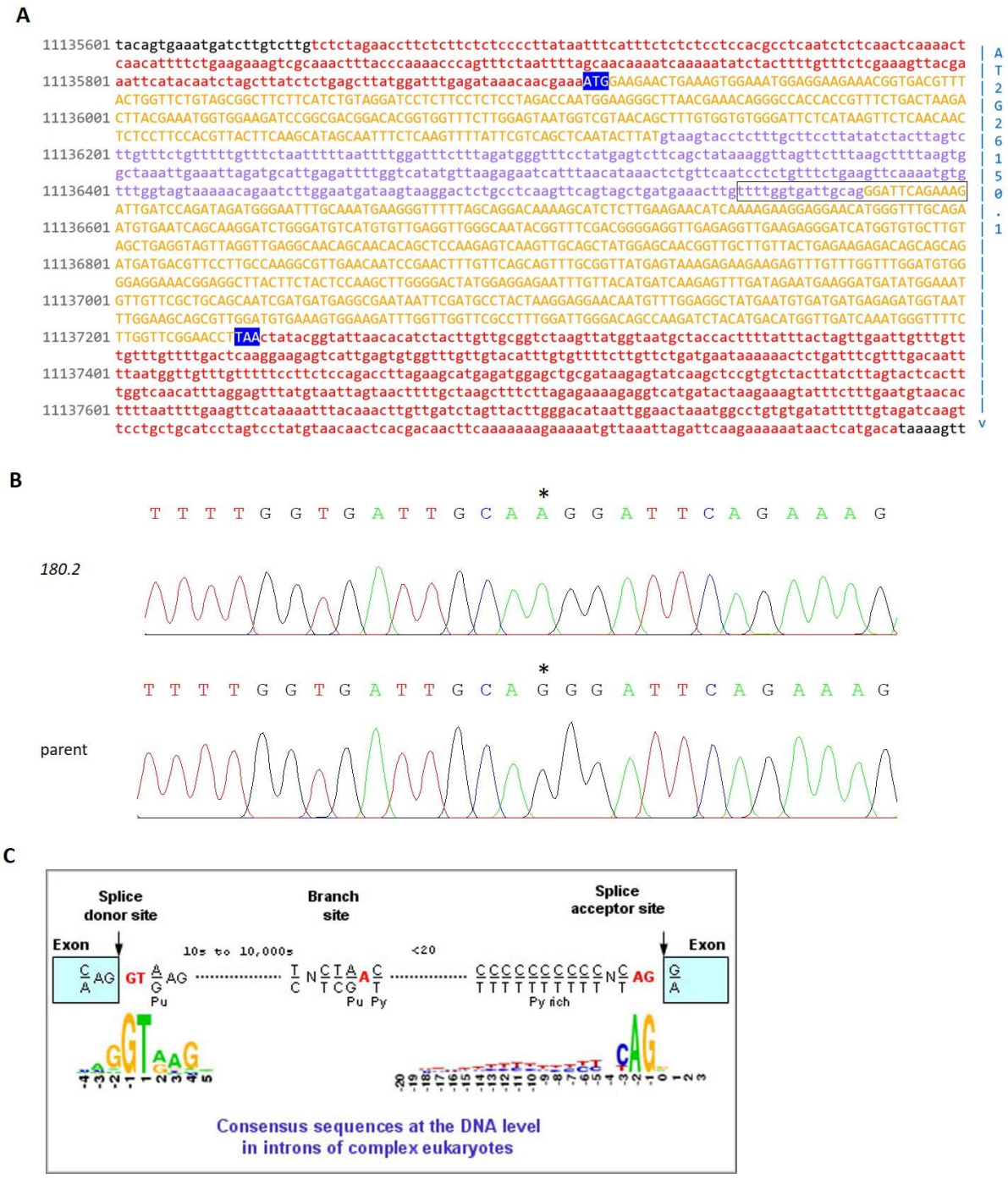
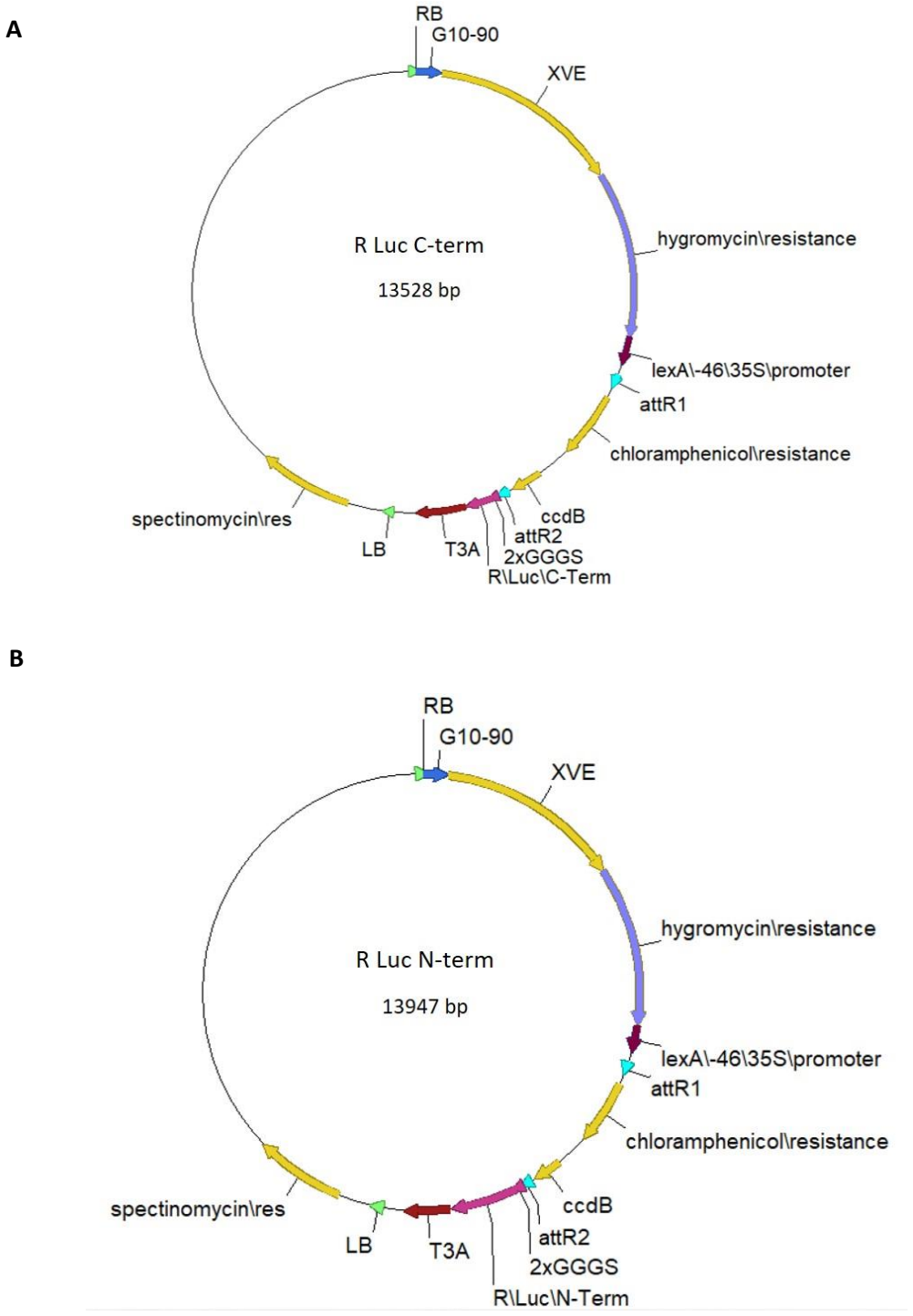
10. Supplemental material



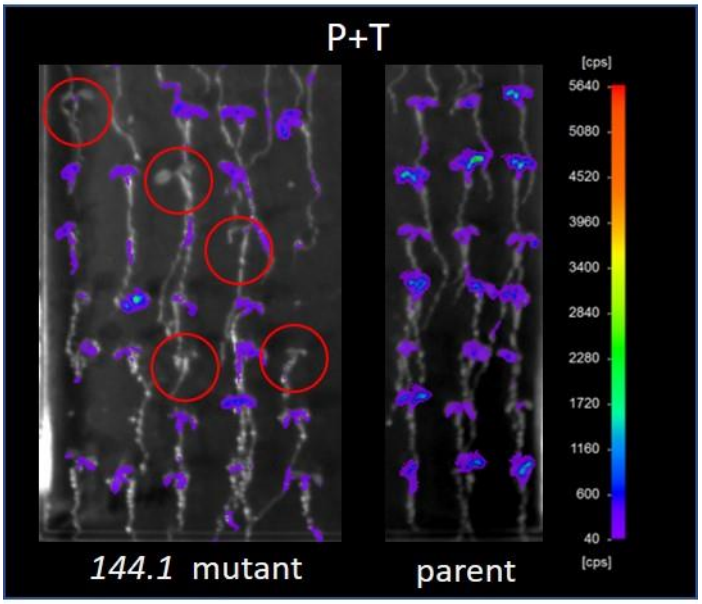
Supplemental Figure S1. Gradual decrease of transcriptional memory in the *pAPX2::LUC* reporter line. A Treatment schemes for assaying transcriptional memory. **B** Transgenic seedlings carrying *pAPX2::LUC* were heat-treated at the indicated time points (shown in B) and imaged for luciferase activity at the indicated timepoints (arrows in B). The false-color scale of LUC activity is shown on the right. The recovery phase after a priming HS (P) or in between two treatments (P+T) was extended from 2 to 3 and 4 days and reflected the gradual decrease of the *APX2* transcriptional memory.



Supplemental Figure S2. Mutation in the EMS mutant line 180.2 affects the splice acceptor site of HSFA2. A Genomic sequence of At2g26150 (HSFA2) (source Tair). The exon intron boundary that contains the SNP is marked with a black box. **B** Sequencing of the HSFA2 gene in the parental line *pAPX2::LUC* and the *180.2* mutant revealed a nucleotide change from A to G in the splice acceptor site of the intron. **C** Consensus splice junction sequences in introns of complex eucaryotes (source picture http://www.geneinfinity.org/sp/sp_coding.html).



Supplemental Figure S3. RLUC vectors used for SCLA. A The destination vector RLUC-C contains the C-terminal region of *Renilla reniformis* luciferase (R\Luc\C-Term). **B** RLUC-N contains the N-terminal region (R/Luc/N-Term). The vectors contains the β-Estradiol-regulated transactivator XVE (Curtis and Grossniklaus, 2003), whose expression is controlled by the strong constitutive promoter G10-90. XVE activates the LexA\35S promoter for expression of the fusion protein with a flexible linker sequence 2xGGGGS (Linker) in between.



Supplemental Figure S4. Reduced hyper-induction of *pAPX2::LUC* expression following P+T conditions in *rein2* mutants from M2 generation. Luciferase activity was reduced in multiple individual seedlings (red circles) of the *rein2* mutant line after repeated HS.

Supplemental Table S5. List of the SNPs in *rein1* in the region between 23.5 Mb and 26.5 Mb of chromosome (chr) 5. The number of the reference (ref) and alternative allele (alt) in the mutant (mt.) or wildtype (WT.) pool is listed. The info column contains details about the type of mutation, the respective gene ID and name, if present, as well as the nucleotide (and aa) change in the target sequence.

chr	position	ref	alt	qual	mt. ref	WT. ref	mt. alt	WT. alt	info
5	23727391	G	A	447	1	0	32	34	intron_variant|MODIFIER||AT5G58740|transcript| AT5G58740.1|protein_coding|c.50-13C>T|
5	24076141	G	A	438	6	28	33	17	synonymous_variant|LOW||AT5G59760|transcript| AT5G59760.1| protein_coding|c.186C>T|p.Asp62Asp
5	24079595	G	A	455	2	24	29	26	missense_variant|MODERATE||AT5G59770|transcript| AT5G59770.1|protein_coding|c.745C>T|p.Leu249Phe
5	24154066	G	A	455	4	25	40	22	intergenic_region|AT5G59990-AT5G60000||| n.24154066G>A
5	24258686	G	A	348	2	20	35	11	missense_variant|MODERATE|LECRK17|AT5G60270| transcript|AT5G60270.1| protein_coding|c.926G>A| p.Arg309Lys
5	24279191	G	A	458	3	26	35	20	intergenic_region|AT5G60350-AT5G60360||| n.24279191G>A
5	24477582	G	A	426	3	29	30	16	intergenic_region|AT5G60840-AT5G60850||| n.24477582G>A
5	24737095	G	A	328	2	28	49	10	missense_variant|MODERATE||AT5G61510|transcript| AT5G61510.1| protein_coding|c.1210C>T|p.Leu404Phe
5	24952351	G	A	408	1	24	36	16	intron_variant|MODIFIER||AT5G62130|transcript| AT5G62130.1| protein_coding|c.98+470C>T
5	25080506	G	A	397	1	24	34	13	intron_variant|MODIFIER|MYB96|AT5G62470|transcript| AT5G62470.2| protein_coding|c.134-60C>T
5	25126705	G	A	449	1	35	31	18	synonymous_variant|LOW|AT5G62600||transcript| AT5G62600.1| protein_coding|c.1471C>T|p.Leu491Leu
5	25463785	G	A	397	2	29	38	15	stop_gained|HIGH|CDKE-1|AT5G63610|transcript| AT5G63610.1| protein_coding|c.1273C>T|p.Gln425*
5	25541663	G	A	362	0	33	31	11	intron_variant|MODIFIER|AT5G63820|AT5G63820| transcript|AT5G63820.1| protein_coding|c.72+16G>A
5	25868249	G	A	379	1	31	41	14	intergenic_region|AT5G64700-AT5G64710|||n.25868249G>A
5	26549390	G	A	349	2	20	26	10	synonymous_variant|LOW|PCMP-E38|AT5G66500| transcript|AT5G66500.1| protein_coding|c.285C>T| p.Ser95Ser
5	26580729	G	A	449	5	30	46	20	intergenic_region|AT5G66600-AT5G66607||| n.26580729G>A

Supplemental Table S6. List of the SNPs in the *rein2* mutant in the region between 23,3 Mb and 24,5 Mb of chromosome (chr) 1. For information about the columns see Supplemental table S5.

chr	pos	ref	alt	qual	mt.ref	WT.ref	mt.alt	WT.alt	info
1	23332232	C	T	436	3	20	25	12	stop_gained|HIGH|EXPA18|AT1G62980|transcript|AT1G62980.1|protein_coding|c.253C>T|p.Gln85*
1	23763226	C	T	330	3	20	32	8	synonymous_variant|LOW|AT1G64050|AT1G64050|transcript| AT1G64050.1|protein_coding|c.462C>T|p.Thr154Thr
1	23942502	C	T	261	2	23	28	5	synonymous_variant|LOW|AT1G64450|AT1G64450|transcript| AT1G64450.1|protein_coding|c.285G>A|p.Ser95Ser
1	23956613	C	T	396	1	15	31	9	intron_variant|MODIFIER|RPN12A|AT1G64520|transcript|AT1G64520.1|protein_coding|c.105+50C>T
1	24220515	C	T	384	0	26	33	12	missense_variant|MODERATE|AT1G65200|AT1G65200|transcript| AT1G65200.1|protein_coding|c.2107G>A|p.Glu703Lys
1	24248589	C	T	362	4	33	38	11	3_prime_UTR_variant|MODIFIER|AT1G65280|AT1G65280|transcript| AT1G65280.1|protein_coding|c.*68C>T
1	24276442	C	T	186	1	7	11	2	synonymous_variant|LOW|UBQ13|AT1G65350|transcript|AT1G65350.1|protein_coding|c.834G>A|p.Glu278Glu
1	24309749	C	T	325	0	21	33	10	missense_variant|MODERATE|GTB1|AT1G65440|transcript|AT1G65440.1|protein_coding|c.2612G>A|p.Cys871Tyr
1	24475190	C	T	354	0	19	35	10	synonymous_variant|LOW|SD16|AT1G65800|transcript|AT1G65800.1|protein_coding|c.1563C>T|p.Asn521Asn
1	24499939	C	T	357	1	26	28	12	missense_variant|MODERATE|FMOGS-OX1| AT1G65860|transcript| |protein_coding|c.949G>A|p.Ala317Thr
1	24538336	C	T	272	0	24	24	5	intergenic_region|AT1G65920-AT1G65930||| n.24538336C>T
1	24547251	C	T	365	2	27	29	9	missense_variant|MODERATE|AT1G65950|AT1G65950|transcript| AT1G65950.1|protein_coding|c.1429G>A|p.Ala477Thr

Supplemental Table S7. Oligonucleotides used for RT-qPCR and dCAPS analysis.

Target	Primer name	Sequence	
LUC	3032/pAPX2::LUC_ATG_+60_F	ACCGCTGGAGAGCAACTGC	RT-qPCR
LUC	3031/pAPX2::LUC_ATG+60_R	CTGCCAACCGAACGGACAT	RT-qPCR
APX2	634/AT3G09640 FOR	CTTGATGATCCTCTCTTTCTCCCA	RT-qPCR
APX2	633/AT3G09640 REV	ACTCCTTGTCAGCAAACCCGAG	RT-qPCR
MIPS2	2938/At2g22240-1f	GTAGCTAGTAATGGCATCCTCTTTGA	RT-qPCR
MIPS2	2939/At2g22240-1r	ATCCGCAACATATGGCACATAC	RT-qPCR
HSP101	267/HSP101F	ATGACCCGGTGTATGGTGCTAG	RT-qPCR
HSP101	268/HSP101R	CGCCTGCATCTATGTAAACAGTG	RT-qPCR
HSFA2	281/HsfA2LP	TCGTCAGCTCAATACTTATGGATTC	RT-qPCR
HSFA2	282/HsfA2RP	CACATGACATCCCAGATCCTTGC	RT-qPCR
CYP81D11	4152/CYP81D11-F	CAGCCTGATTACTACACGGATGTG	RT-qPCR
CYP81D11	4153/CYP81D11-R	AGTCCCGGCAAGTATCATAACAAG	RT-qPCR
SPT6L	4696/qF-SPT6L	TAACGACCAGCAACGTAAAA	RT-qPCR
SPT6L	4697/qR-SPT6L	CCACCATCTGGAATATGACC	RT-qPCR
HSP18	359/AT5G59720_qPCR_F	ACAAACGCAAGAGTGGATTGGA	RT-qPCR
HSP18	360/AT5G59720_qPCR_R	GCTCCTCTCTCCGCTAATCTGC	RT-qPCR
XTR6	2953/XTR6_intron_spanning_F	TGTCACCGCTTACTATTTGAAATCC	RT-qPCR
XTR6	2954/XTR6_R	GTTTGAATTGTTGTTCTCTATCGCC	RT-qPCR
LACS9	4150/LACS9-F	CACGAAAGAGCAAGCCGTGAAAG	RT-qPCR
LACS9	4151/LACS9-R	ATCGTGATTGTTTAGCCGCCTTC	RT-qPCR
LUC	3031/pAPX2::LUC_ATG+60_R	CTGCCAACCGAACGGACAT	RT-qPCR
LUC	3032/pAPX2::LUC_ATG_+60_F	ACCGCTGGAGAGCAACTGC	RT-qPCR
At4g26410	1547/F-AT4G26410	GAGCTGAAGTGGCTTCAATGAC	RT-qPCR
At4g26410	1548/R-AT4G26410	GGTCCGACATACCCATGATCC	RT-qPCR
TUB6	161/TUB6 RT F	GGTGAAGGAATGGACGAGAT	RT-qPCR
TUB6	160/TUB6 RT R	GTCATCTGCAGTTGCGTCTT	RT-qPCR
RPP7	4532/*144.1*_RPP7_fw_2.17	CACTTCTTCTTACTCTCAATATCTTTGATTTT	dCAPS

RPP7	4533/*144.1*_RPP7_rev_2.17	TAGAGTATGAAGCCGTGGCAT	dCAPS
At1g61100	4611 /AT1G61100_fw_22.5	TTACATACCTTAACCTTGAAGCCCC	dCAPS
At1g61100	4612 /AT1G61100_rev_22.5	AATCTGAGAATGGCAATGAGGTGGGAGGA	dCAPS
EXP18	4538/*144.1*_EXPA18_fw_2.33	CGATTGTTTGTTTGCATTTCTATGTTTTA	dCAPS
EXP18	4539/*144.1*_EXPA18_rev_2.33	AGCAGTTAGGCGACGACACACACTTTATCT	dCAPS
HSP101	4536/*144.1*_HSP101_fw_2.79	AGCAAGCAGGGAAGCTTGATCCTGTGACTG	dCAPS
HSP101	4537/*144.1*_HSP101_rev_2.79	TCACTTTGCCTTCAGCGTCCT	dCAPS
SPT6L	4656/SPT6L_AccI_fw	GCATTATCAGGTCCTTGCGGAC	dCAPS
SPT6L	4657/SPT6L_AccI_rev	CAGAATCCACTAGCAATGGTGGCAACTGTAT	dCAPS
SDG26	4534/*144.1*_SDG26_fw_2.87	AGGATGCATACATTATATCTCTCAATCCAT	dCAPS
SDG26	4535/*144.1*_SDG26_rev_2.87	GCACCACAGAGACAACGC	dCAPS
ABC1K14	4613 /AT1G65950_fw_24.5	AAAGAGTTTGAAGTTTACACACAAGAC	dCAPS
ABC1K14	4797/At1g65950_NspI_rev	CGGGTGAGACTACTCGCTTATGCGAAACAT	dCAPS

11. Abbreviations

°C	Degree Celsius
$^{\bullet}OH$	Hydroxyl radical
A	Adenine
AACT1	AGMATINE COUMAROYLTRANSFERASE
ABA	Abscisic acid
ABC1K14	ACTIVITY OF BC1 COMPLEX KINASE 14
ACC	Acclimation
Ala	Alanine
AOX1	ALTERNATIVE OXIDASE 1
AP2/ERF	APETALA2/ETHYLENE RESPONSIVE FACTOR
APX	ASCORBATE PEROXIDASE
Arg	Arginine
Asp	Aspartic acid
At	*Arabidopsis thaliana*
aTT	Acquired thermotolerance
BC1F2	Backcross-derived F2 population
BC1F3	Backcross-derived homozygous F3 population
bHLH	Basic helix–loop–helix
BRM	BRAHMA
bTT	Basal thermotolerance
bZIP	Basic leucine zipper
C	Cytosine
Ca^{2+}	Calcium ion
Calmodulin	Calcium-modulated protein
CaM	CALCIUM-MODULATED PROTEIN
CBF	C-repeat binding factor
CBL	CALCINEURIN B LIKE PROTEIN
CBP1	CCG-binding protein 1
CDK8	CYCLIN DEPENDENT KINASE 8
CDKA1	A-TYPE CYCLIN-DEPENDENT KINASE
cDNA	complementary DNA
CDPK	CALCIUM-DEPENDENT PROTEIN KINASE
Chr	Chromosome
CHR11	CHROMATIN-REMODELING PROTEIN 11
CHR17	CHROMATIN-REMODELING PROTEIN 17
CKM	CDK8 kinase module
cMed	Core Mediator
CML	CALMODULIN-LIKE PROTEIN
CNGC	CYCLIC NUCLEOTIDE-GATED ION CHANNEL
Co-IP	Co-Immunoprecipitation
Col-0	Columbia-0
COR	Cold-regulated
CRK1	CDPK-RELATED KINASE 1
CRT/DRE	C repeat/dehydration-responsive element
CTD	Carboxy-terminal domain
CYCC	CYCLIN C
CYP81D11	CYTOCHROME P450 81D11
Cys	Cysteine
DBD	DNA-binding domain
dCAPS	Derived Cleaved Amplified Polymorphic Sequences
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DREB2	DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN 2
EDTA	Ethylenediaminetetraacetic acid
ELF3	EARLY FLOWERING 3
EMS	ethyl methanesulfonate
ER	Endoplasmic reticulum
EtBr	Ethidium bromide
EtOH	Ethanol
EXPA18	EXPANSIN 18
F	Filial generation
FGT	FORGETTER
FKBP	FK506 BINDING PROTEIN
FLC	FLOWERING LOCUS C
G	Guanine
Gln	Glutamine
Gly	Glycine
GM	Growth Medium
H2A	Histone 2A
H_2O_2	hydrogen peroxide
H3K4	Histone H3 lysine 4
H3K36me3	Tri-methylation of Lysine 36 at histone 3
H3K4me2	Di-methylation of Lysine 4 at histone 3
H3K4me3	Tri-methylation of Lysine 4 at histone 3
HAC1	HISTONE ACETYLTRANSFERASES OF THE CBP FAMILY 1
HAC5	HISTONE ACETYLTRANSFERASES OF THE CBP FAMILY 5
HEN3	HUA ENHANCER 3
het	Heterozygous
hom	Homozygous
HS	Heat stress
HSA32	HEAT-STRESS-ASSOCIATED 32-KD PROTEIN
HSE	Heat shock element
HSF	HEAT SHOCK FACTOR
HSFA	CLASS A HEAT SHOCK FACTOR
HSFB	CLASS B HEAT SHOCK FACTOR
HSFC	CLASS C HEAT SHOCK FACTOR
HSP	HEAT SHOCK PROTEIN
HSP70	70 kDa Heat shock protein
HSP90	90 kDa Heat shock protein
HSR	Heat shock response
HTT5	HEAT-INDUCED TAS1 TARGET
HUB1	HISTONE MONOUBIQUITINATION1
IFN-y	Gamma interferon
INO1	INOSITOL-1-PHOSPHATE SYNTHASE
IRE1	INCREASED ORGAN REGENERATION 1
ISWI	Imitation SWItch
JA	Jasmonic acid
kDa	Kilodaltons
KIN10	SNF1 KINASE HOMOLOG 10
LACS9	LONG CHAIN ACYL-COA SYNTHETASE 9
LB	Lysogeny broth
LECRK-1.7	L-TYPE LECTIN RECEPTOR KINASE I.7
LUC	Luciferase
MAPK	MITOGEN-ACTIVATED PROTEIN KINASE
maTT	Maintenance of acquired thermotolerance

Mb	Megabase pairs
MBF1	MULTIPROTEIN BRIDGING FACTOR 1
MDA	Malondialdehyde
Me	Methylation
MED	MEDIATOR
MES	2-(N-morpholino) ethanesulfonic acid
min	minute
MIPS2	MYO-INOSITOL-1-PHOSPHATE SYNTHASE 2
ml	millilitre
mmol	millimolar
miRNA	MicroRNA
mRNA	Messenger RNA
N. benthamiana	*Nicotiana benthamiana*
NAC	NAM-ATAF1,2-CUC2
NADPH	NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE
NCBI	National Center for Biotechnology Information
NGS	Next generation sequencing
NPR1	NONEXPRESSER OF PR GENES 1
NTC	Non-treated control
NTL4	NAC WITH TRANSMEMBRANE MOTIF 1-LIKE 4
O_2^-	Superoxide
Os	*Oryza sativa*
PAMP	Pathogen-associated molecular pattern
PAR	Photosynthetically active radiation
PDF1	PLANT DEFENSIN 1.2
PFT 1	PHYTOCHROME AND FLOWERING TIME1
Phe	Phenylalanine
phyB	Phytochrome B
PIC	Preinitiation complex
PIF	PHYTOCHROME INTERACTING FACTOR
Pol II	RNA polymerase II
PP2C	Type 2C protein phosphatases
PP7	PROTEIN PHOSPHATASE 7
P	Primed
PCR	Polymerase chain reaction
Pst DC3000	*Pseudomonas syringae* pv. *tomato* DC3000
PTR3	PEPTIDE TRANSPORTER 3
RAP2.6	RELATED TO AP2 6
RAV2	RELATED TO ABI3/VP1 2
RBOH	RESPIRATORY BURST OXIDASE HOMOLOGUE
RD29B	RESPONSIVE TO DESICCATION 29B
RE	Restriction endonuclease
REF	RELATIVE OF EARLY FLOWERING
REIN1	RE-INDUCTION 1
RLUC	*Renilla* luciferase
RNA	Ribonucleic acid
ROF1	ROTAMASE FK506 BINDING PROTEIN
ROS	Reactive oxygen species
rpm	Revolutions per minute
RPP7	RECOGNITION OF PERONOSPORA PARASITICA 7
RT	Room temperature
SA	Salicylic acid
SAR	Systemic acquired resistance
SLCA	Split Luciferase Complementation Assay
SDG26	SET DOMAIN GROUP 26

SDS	Sodium dodecyl sulfate
Ser	Serine
Ser2P	Phosphorylation of serine 2 of Pol II
Ser5P	Phosphorylation of serine 5 of Pol II
SFR6	SENSITIVE TO FREEZING 6
SGIP1	SGS3-INTERACTING PROTEIN 1
SGS3	SUPPRESSOR OF GENE SILENCING 3
siRNA	small interfering RNA
Sno	Strawberry notch
SNP	Single Nucleotide Polymorphism
SnRK1	SUCROSE NONFERMENTING 1-RELATED PROTEIN KINASE 1
SnRK2.6	SUCROSE NONFERMENTING 1-RELATED PROTEIN KINASE 2-6
SPL	SQUAMOSA-PROMOTER BINDING LIKE
SPT6L	ARABIDOPSIS SUPPRESSOR OF TY INSERTION 6-LIKE
SWI/SNF	SWItch/Sucrose Non-Fermentable
tasiRNA	trans-acting siRNA
T	Triggered
TFIIH	TRANSCRIPTION FACTOR IIH
Thr	Threonine
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
Tyr	Tyrosine
UTR	Untranslated region
UV	Ultraviolet
WT	Wildtype
XTR6	XYLOGLUCAN ENDOTRANSGLYCOSYLASE 6
Y2H	Yeast two-hybrid
YY1	YIN YANG 1
ZFHD1	ZINC FINGER HOMEODOMAIN 1
µL	microlitre

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13. Statement of Authorship

I hereby declare that this thesis, “Identification of regulators in heat stress memory in *Arabidopsis thaliana*”, has not been previously submitted for another degree at any university, is the result of my own work and used only the cited references and resources.

Hiermit erkläre ich, dass die vorliegende Arbeit mit dem Titel “ Identification of regulators in heat stress memory in *Arabidopsis thaliana* ” an keiner anderen Hochschule eingereicht, von mir selbständig und nur unter Verwendung der angegebenen Quellen und Hilfsmittel angefertigt wurde.