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BRUSHY1/TONSOKU/MGOUN3 is required for heat stress memory

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Abstract

Plants encounter biotic and abiotic stresses many times during their life cycle and this limits their productivity. Moderate heat stress (HS) primes a plant to survive higher temperatures that are lethal in the naïve state. Once temperature stress subsides, the memory of the priming event is actively retained for several days preparing the plant to better cope with recurring HS. Recently, chromatin regulation at different levels has been implicated in HS memory. Here we report that the chromatin protein BRUSHY1/TONSOKU/MGOUN3 (BRU1/TSK/MGO3) plays a role in the HS memory in *Arabidopsis thaliana*. *BRU1* is also involved in transcriptional gene silencing and DNA damage repair. This corresponds with the functions of its mammalian orthologue TONSL/NFKBIL2. During HS memory, BRU1 is required to maintain sustained induction of HS memory-associated genes, whereas it is dispensable for the acquisition of thermotolerance. In summary, we report that BRU1 is required for HS memory in *A. thaliana*, and propose a model where BRU1 mediates the epigenetic inheritance of chromatin states across DNA replication and cell division.

Summary statement: Plants can be primed by a heat stress exposure to deal more efficiently with a future heat stress incident, that occurs after a lag phase at normal growth temperatures. Studying the molecular basis of priming and memory in response to heat stress, we show here that the chromatin protein BRUSHY1 is required for heat stress memory and that it acts through sustaining the activation of heat stress-memory related gene expression during the lag phase. Our findings suggest a model where heat stress memory is mediated through the epigenetic inheritance of chromatin states across cell divisions.

Introduction

As sessile organisms, plants are frequently exposed to environmental conditions that are stressful in the sense that they interfere with their optimal growth and development. Such extreme environmental conditions are likely to increase in frequency and severity with climate change (Battisti and Naylor, 2009, Lobell *et al.*, 2011). In recent years, it has become increasingly clear that plants can be primed by stress exposure and that this enhances their response to a second stress exposure, which may be qualitatively the same or different, after a stress-free period (Bruce *et al.*, 2007, Conrath *et al.*, 2015, Hilker *et al.*, 2016). The molecular basis of priming and memory is still not well understood (Lämke and Bäurle, 2017). However, evidence is emerging that priming of stress-induced gene expression is a crucial component of stress priming that is at least in part mediated by epigenetic regulation. Indeed, several studies indicate that chromatin organization and modifications distinguish genes in the primed state from those in the naïve state (Ding *et al.*, 2012, Mozgova *et al.*, 2015, Brzezinka *et al.*, 2016, Lämke *et al.*, 2016). In dividing tissues, this process involves the inheritance of the primed state across DNA replication and cell division. How this is mediated remains unclear.

Due to the high economic relevance and recurring nature of heat stress (HS), both acute responses as well as longer-term responses including priming and memory have been investigated (Bäurle, 2016, Lämke and Bäurle, 2017, Ohama *et al.*, 2017). The immediate responses to HS have been intensively studied in in all kingdoms; a conserved core module, collectively referred to as the heat shock response (HSR) (Richter *et al.*, 2010), comprises the rapid activation of heat shock transcription factors (HSFs) that in turn induce the expression of heat shock proteins (HSPs), which act as chaperones and prevent or repair protein damage (Akerfelt *et al.*, 2010, Anckar and Sistonen, 2011). In plants the HSR results in the acquisition of thermotolerance and is mediated by a subset of the strongly radiated HSF

family (Scharf *et al.*, 2012, Yeh *et al.*, 2012, Ohama *et al.*, 2017). In *A. thaliana*, seven of the 21 HSF genes have been implicated in the acquisition of thermotolerance, among them three HSFA1 isoforms that act as master regulators of the HSR (Mishra *et al.*, 2002, Schramm *et al.*, 2008, Ikeda *et al.*, 2011, Liu *et al.*, 2011, Yoshida *et al.*, 2011, Scharf *et al.*, 2012, Yeh *et al.*, 2012).

Notably, at the physiological level, HS primes a plant to subsequently withstand a stronger HS even after a lag phase of 3 days at normal growth temperatures (Charng *et al.*, 2006, Charng *et al.*, 2007). This process is called maintenance of acquired thermotolerance or HS memory and is an active process that is genetically separable from the acquisition of thermotolerance, as evidenced by a (growing) list of mutants that are specifically defective in the maintenance rather than the acquisition of thermotolerance (Charng *et al.*, 2006, Charng *et al.*, 2007, Stief *et al.*, 2014, Brzezinka *et al.*, 2016).

HS memory requires *HSFA2*, which is so far the only *HSF* gene in *A. thaliana* that functions specifically in HS memory (Charng *et al.*, 2007). *HSFA2* expression is induced by HSFA1 isoforms, and HSFA2 prolongs and amplifies the HS-induction at a subset of HSFA1-target genes (Charng *et al.*, 2007, Liu *et al.*, 2011, Nishizawa-Yokoi *et al.*, 2011, Lämke *et al.*, 2016). The target genes of HSFA2 are enriched in genes that show sustained activation of gene expression after HS; after a short HS pulse, these genes remain induced for at two to three days, and while their initial induction is not changed in *hsfa2* mutants their transcriptional activity declines faster in *hsfa2* mutants (Stief *et al.*, 2014, Lämke *et al.*, 2016). Interestingly, HSFA2 binds transiently to memory-gene loci and presumably recruits sustained chromatin modifications, in particular the hyper-methylation of histone H3K4 (Lämke *et al.*, 2016). This hyper-methylation correlates closely with the duration of the memory period. A second link between HS memory and chromatin organization has been reported through the finding that the *FORGETTER1* (*FGT1*) gene is required for sustained

induction of *HSA32* and other memory genes after HS (Brzezinka *et al.*, 2016). FGT1 is a highly conserved helicase-domain protein that maintains low nucleosome occupancy throughout the memory phase through the interaction with chromatin remodelers of the SWI/SNF and ISWI classes (Farrona *et al.*, 2004, Li *et al.*, 2014, Brzezinka *et al.*, 2016). Taken together, the current model is that the sustained induction of HS memory-associated genes is mediated through chromatin modifications and involves both nucleosome occupancy and posttranslational modification of nucleosomes. As the sustained induction of gene expression is maintained for several days, an interesting question is whether and how the primed chromatin states are inherited through DNA replication and cell division.

Chromatin regulation also plays an important role in the regulation of somatic stress memory in response to other biotic and abiotic stresses (Bruce *et al.*, 2007, Sani *et al.*, 2013, Hilker *et al.*, 2016, Vriet *et al.*, 2015, Lämke and Bäurle, 2017). In particular, histone H3K4 hyper-methylation has been implicated in stress memory after dehydration and pathogen infection (Jaskiewicz *et al.*, 2011, Ding *et al.*, 2012, Kim *et al.*, 2012, Singh *et al.*, 2014, Feng *et al.*, 2016). The regulation of nucleosome occupancy was implicated in the priming of defence genes (Mozgova *et al.*, 2015).

The *BRUSHY1* (*BRU1*)/ *TONSOKU* (*TSK*)/ *MGOUN3* (*MGO3*) gene was originally identified based on its fasciated stems and loss of transcriptional silencing (Guyomarc'h *et al.*, 2004, Suzuki *et al.*, 2004, Takeda *et al.*, 2004). It encodes a nuclear protein with tetratricopeptide-repeat (TPR) and leucine-rich repeats (LRR) protein interaction domains. *Bru1* mutant plants have disorganized meristems due to altered *WUSCHEL* expression, and the stems tend to enlarge and separate into multiple stems (fasciation) (Guyomarc'h *et al.*, 2004, Suzuki *et al.*, 2004). As *bru1* is hypersensitive to DNA damage, it was also suggested that *BRU1* is involved in DNA damage repair pathways (Takeda *et al.*, 2004). Since mutants in Chromatin assembly factor (CAF-1) components (*FASCIATA1* (*FAS1*) and *FAS2*) have

similar phenotypes with respect to both developmental aspects and DNA damage hyper-sensitivity, it was suggested that *BRU1* has a similar function (Takeda *et al.*, 2004). CAF-1 deposits H3 and H4 into newly assembling nucleosomes after DNA replication and repair (Smith and Stillman, 1989, Probst *et al.*, 2009). Thus, *BRU1* was proposed to function in the epigenetic inheritance of chromatin states. This is in line with the observation that in synchronized cell cultures *BRU1* expression peaks during S-phase (Suzuki *et al.*, 2005).

More recently, a BRU1 orthologue was identified in mammals and named TONSOKU-LIKE (TONSL)/NFKBIL (Duro *et al.*, 2010, O'Connell *et al.*, 2010, O'Donnell *et al.*, 2010). TONSL interacts with MMS2L and is involved in DNA repair, where the complex binds to ssDNA and facilitates loading of RAD51 (Huang *et al.*, 2018). TONSL also regulates DNA replication and acts as a H3-H4 histone chaperone (Piwko *et al.*, 2010, Campos *et al.*, 2015). Interestingly, a connection to the epigenetic inheritance of chromatin modifications remains elusive, although the Ankyrin repeat domain of TONSL acts as a histone reader domain (Saredi *et al.*, 2016).

Here, we report that *BRU1* is required for the memory of HS. We show that *BRU1* is required for sustained activation of HS memory-associated genes and that this occurs at the transcriptional level. The moderate HS used in this study induced only very low amounts of cell death and did not induce the expression of DNA damage marker genes *RAD51* and *PARP2*. Neither cell death nor DNA damage marker gene expression is increased in *bru1*, indicating that the DNA damage hyper-sensitivity is unlikely to be responsible for the HS memory defects. In addition, the acquisition of thermotolerance is not affected in *bru1*, and mutants in CAF-1 do not display a HS memory defect. The effect on memory-gene expression is confirmed by our global transcriptome analysis. In summary, we find that *BRU1* is specifically required for HS memory and propose a model where *BRU1* acts in the

inheritance of chromatin-based memory components through chromatin reassembly that is necessitated by nucleosome turnover and DNA replication.

Materials and Methods

Plant Materials and Growth Conditions

Seedlings of the Col-0 or Wassilewskaja (Ws) backgrounds were germinated on GM medium (1 % (w/v) glucose) under a 16/8 h light/dark cycle at 23/21°C. *hsa32* and *hsp101* were described previously (Stief *et al.*, 2014). *bru1-1* (Ws) and *BRU1::BRU1-GFP* in *bru1-1* background were obtained from S. Takeda (Ohno *et al.*, 2011). *bru1-2, bru1-4* (N534207, (Takeda *et al.*, 2004)), *fas1-4* (SAIL662_D10) and *fas2-4* (N533228, both (Exner *et al.*, 2006)) were obtained from the Nottingham Arabidopsis Stock Center.

Thermotolerance assays

Heat treatments were performed on 4 d-old seedlings. Seedlings were treated with an acclimatizing HS (ACC) of 37°C, followed by 90 min at room temperature, and by 45 min at 44°C, starting 8 h after light onset. As tester HS a 44°C treatment for the indicated duration was applied at the indicated number of days after ACC. After HS, plants were returned to normal growth conditions until analysis. Acquisition of thermotolerance and basal thermotolerance were assayed as described (Stief *et al.*, 2014). For all thermotolerance assays, all genotypes of one treatment were grown on the same plate.

Gene expression analysis

Transcript levels were quantified by qRT-PCR analysis as described previously (Stief *et al.*, 2014, Brzezinka *et al.*, 2016). In brief, total RNA from flash-frozen seedlings was extracted using the Hot Phenol method, residual DNA was removed using Turbo DNAse (Ambion) and

the RNA was reverse transcribed using oligo-dT primers and SuperScript III (Invitrogen). Quantitative PCR was performed on 1:20 diluted cDNA using GoTaq SybrGreen qPCRMasterMix (Promega). Oligonucleotide sequences are listed in Supplemental Table S1. Transcript levels were quantified using the ΔΔCt method. Transcript levels were normalized to the reference genes *TUB6* or *At4g26410* (Czechowski *et al.*, 2005).

Trypan blue staining for cell death

Trypan Blue staining was performed 96 h after ACC treatment as described (Inagaki *et al.*, 2009). In brief, seedlings were incubated in 0.5 mg/ml trypan blue, dissolved in phenol/glycerol/lactic acid/water/ethanol (1:1:1:1:8), in a boiling water bath for 1 min. The tissues were left in staining solution at room temperature for 1 h, cleared in chloral hydrate solution, and examined with a Leica stereomicroscope.

Microscopy

GFP fluorescence was imaged using a Zeiss LSM710 confocal microscope.

Microarray Analysis

For microarray hybridization, 4 d-old seedlings of *Col-0* or *bru1-2* were either treated with ACC (see above) and harvested 4 h or 52 h after the end of the treatment (three biological replicates). As control, No-HS samples (three biological replicates) were harvested together with the 4 h ACC samples. The *bru1-2* samples described here were part of a larger experiment reported previously, in which the Col-0 samples were already described (Stief *et al.*, 2014). RNA extraction, probe preparation and hybridization of Affymetrix GeneChip ATH1 microarrays and their analysis was described previously (Stief *et al.*, 2014). The clustered heat map analysis was performed using Heatmapper (Babicki *et al.*, 2016) with

settings value, average linkeage, and Euklidean distance. The microarray data are deposited as GSE83136 and GSE112161.

Results

BRU1 is specifically required for HS memory

From a reverse genetic screen we identified *bru1-2* as a mutant with impaired HS memory. 4 d-old seedlings were treated with a two-step acclimation treatment (consisting of 1 h at 37°C and then 45 min at 44°C, see Methods for details), which was followed by a 2 d period at standard growth temperatures, and then a tester HS that is lethal to a plant that has not been acclimated (Figure 1a, (Stief *et al.*, 2014)). As a control we included the *hsa32* mutant that was previously shown to have a defective HS memory (Charng *et al.*, 2006). *Bru1-2* mutants displayed reduced growth and survival after the second HS relative to the wild type Col-0 control (Figure 1a). We next confirmed that the basal thermotolerance (i. e. the level of HS at 44°C that a not-acclimatized plant can survive) and the acquired thermotolerance (i. e. the level of HS at 44°C that a plant can survive after acclimation for 1 h at 37°C) were not affected in *bru1-2* mutants (Supplementary Figure S1). Together, this indicates that *bru1-2* mutants are specifically defective in the long-term, but not the acute HS responses.

BRU1 mediates sustained transcriptional induction of *HSA32* after HS

To begin to investigate the molecular basis of this loss-of-memory phenotype, we next analyzed the expression of *HSA32* in *bru1-2* mutants. *HSA32* is essential for HS memory in *A. thaliana* (Charng *et al.*, 2006) and is highly induced by HS. Interestingly, induction of *HSA32* is sustained over 3 d following the ACC treatment (Charng *et al.*, 2006, Stief *et al.*, 2014) and thus the expression pattern correlates well with HS memory. We profiled transcript

levels of acclimated plants at the end of the ACC treatment and during a 3 d recovery phase. Similar to the Col-0 wild type, *bru1-2* mutants displayed high induction of *HSA32* and sustained high expression levels during the first day of recovery (Figure 1b). However, *bru1-2* thereafter displayed a stronger decline of *HSA32* expression compared to wild type, consistent with a role in HS memory.

The sustained induction of *HSA32* after ACC occurs at the transcriptional level, as was previously found by the quantification of unspliced transcripts (Brzezinka *et al.*, 2016). Since splicing occurs in close proximity to transcription, quantifying unspliced transcript levels is used as a proxy for transcriptional activity (Bäurle *et al.*, 2007, Stief *et al.*, 2014). Unspliced *HSA32* transcript levels in *bru1-2* were similar to Col-0 until 24h after ACC, but thereafter declined faster (Figure 1c), thus confirming the results for the spliced *HSA32* transcripts and indicating that BRU1 acts to sustain induction of *HSA32* at the transcriptional level.

bru1 mutants are not generally hyper-sensitive to HS

BRU1 has been previously implicated in DNA damage repair and *bru1* mutants are hyper-sensitive to DNA damage, resulting in increased cell death (Takeda *et al.*, 2004). Thus, we tested whether the HS memory defect in *bru1-2* could be ascribed to this hyper-sensitivity. To this end, we first tested whether ACC increased cell death in *bru1-2* cotyledons using Trypan Blue staining. This staining visualizes individual dead cells within tissues. Under no-HS conditions, we did not observe any lesions in Col-0 and only very few in *bru1-2* (Figure 2). After ACC the proportion of wild type seedlings that showed individual dead cells on their cotyledons increase to 39 % (Figure 2). In contrast, only 18 % of *bru1-2* seedlings displayed lesions on their cotyledons, indicating that the ACC treatment does not induce increased cell death in *bru1-2* compared to Col-0.

BRU1 has also been implicated in cell cycle control; *BRU1* is expressed during the S-phase of the cell cycle and its loss delays cell cycle progression (Suzuki *et al.*, 2005). To estimate cell division dynamics after ACC, we assayed the expression levels of histone H4, whose expression is limited to the S-phase. In wild type, histone H4 transcript levels were roughly halved after ACC and remained slightly reduced during the 3 d recovery period that was assayed. In *bru1-2* mutants, histone H4 transcript levels in non-stressed seedlings were roughly halved compared to Col-0 (Figure 3), confirming a general reduction in cell cycle progression (Suzuki *et al.*, 2005). In contrast to wild type, H4 transcript levels in *bru1-2* were not further reduced after HS (Figure 3).

Consistent with a role in DNA damage repair, it was previously reported that the DNA damage marker *PARP2* was hyper-induced in *bru1-2* mutants (Takeda *et al.*, 2004). Thus, we were interested to test whether ACC (further) induced the expression level of the DNA damage markers *RAD51* and *PARP2* (Breuer *et al.*, 2007). Interestingly, neither gene displayed increased expression levels in *bru1-2* under our no-HS conditions. After HS, *RAD51* and *PARP2* transcript levels did not change in wild type (Figure 3). The same was true for *bru1-2* mutants. Together, our findings indicate that the ACC treatment, which is a moderate HS, does not induce ectopic DNA damage or sustained repression of cell division. Moreover, they indicate that DNA damage responses that are mediated by *RAD51* and/or *PARP2* are not ectopically triggered by our NHS growth conditions or by the ACC treatment in *bru1-2*. This corroborates the idea that *BRU1* has a specific function in sustaining HS memory gene expression, and that the observed HS memory defects are not caused by a generic HS hyper-sensitivity.

FAS1 and *FAS2* are not required for HS memory

Mutants in two subunits of the histone chaperone CAF-1 in *A. thaliana*, *fas1* and *fas2*, have similar developmental phenotypes as *bru1*, such as fascinated stems (Kaya *et al.*, 2001), and the mammalian orthologues of BRU1 and CAF-1 interact functionally (Huang *et al.*, 2018). To test whether CAF-1 is also required for HS memory, we tested mutants in the *FAS1* and *FAS2* subunits for their ability to maintain acquired thermotolerance with the assay described above (Figure 4). In contrast to *bru1-2* and *bru1-4*, the loss-of-function mutants *fas1-4* and *fas2-4* displayed normal HS memory. This indicates that *BRU1* function during HS memory is independent of CAF-1 and that the common developmental aberrations of the mutants do not cause the phenotype.

BRU1 is required for sustained activation of HS memory genes

To confirm that the mutation in *BRU1* was responsible for the phenotype observed in *bru1-2*, we tested two additional alleles, *bru1-4* (in the Col-0 background, Figure 4) and *bru1-1* (in the Ws background, Figure 5a) (Suzuki *et al.*, 2004, Takeda *et al.*, 2004, Ohno *et al.*, 2011). Both alleles showed a similar phenotype as *bru1-2* in the HS memory assay. Moreover, reintroduction of a functional *BRU1* copy (*pBRU1::BRU1-GFP*) into *bru1-1* (Ohno *et al.*, 2011) complemented the HS memory defects (Figure 5a). We also confirmed nuclear expression of the *BRU1-GFP* transgene (Figure 5b, c).

Besides *HSA32* a number of other genes display sustained activation after ACC and are together referred to as HS memory genes (Charng *et al.*, 2006, Charng *et al.*, 2007, Stief *et al.*, 2014, Brzezinka *et al.*, 2016). To analyze whether BRU1 affects expression of other memory genes besides *HSA32*, we analyzed their transcript levels during a 3 d recovery phase after ACC. We investigated transcript levels of the HS memory-associated genes *APX2*, *HSA32*, *HSP22.0*, and *HSP21* (Stief *et al.*, 2014, Lämke *et al.*, 2016), as well as the

putative upstream regulator *HSFA2* (Nishizawa *et al.*, 2006, Charng *et al.*, 2007, Lämke *et al.*, 2016), and the HS-inducible non-memory gene *HSP101* (Hong and Vierling, 2000) in the Ws wild type, *bru1-1* and the complementation line. For *HSP101*, no significant difference was found in *bru1-1* relative to Ws (Figure 6). For *HSFA2*, we observed a slightly reduced induction at 0 and 4 h after ACC in *bru1-1*, but later no significant difference relative to the Ws control. In contrast, for *APX2*, *HSA32*, *HSP22.0*, and *HSP21* induction after ACC was more highly sustained in Ws wild type between 28 and 76 h after ACC relative to *HSP101* and *HSFA2*. However, in *bru1-1* the expression started to decrease already between 4 h and 28 h and declined significantly faster than in either Ws or the complemented line. This indicates that *BRU1* is widely required for sustained activation of HS memory-associates genes, but it is dispensable for the initial activation of these genes.

Global transcriptome analysis indicates that *BRU1*-dependent genes are enriched among HS memory genes

We next sought to investigate the requirement of *BRU1* for HS-dependent gene expression at the global level. To this end we performed transcriptome analysis using ATH1 microarray technology on Col-0 wild type and *bru1-2* seedlings sampled at either 4 h or 52 h after ACC, and a no-HS control that was sampled together with the 4 h time point (Stief *et al.*, 2014). We first identified ACC-responsive genes (based on $\log_2$FC > 2 or < -2 and FDR corrected p value <0.05) in Col-0 for either ACC time point relative to NHS (Stief *et al.*, 2014). Figure 7a displays a clustered heat map analysis of the $\log_2$FC changes of these genes in Col-0 and *bru1-2* relative to the NHS control. Overall, the data cluster by treatment rather than genotype indicating that treatment-specific effects outweigh genotype effects. Moreover, the genotype differences at 52 h appear more pronounced than at 4 h (see below for a more detailed analysis). We next investigated the expression correlation at either time point for the genes

that are significantly changed in Col-0. At 4 h, genes that are upregulated in Col-0 tend to be more strongly upregulated in *bru1-2* whereas downregulated genes seem to be less strongly downregulated (Figure 7b). In contrast, at 52 h genes that are upregulated in Col-0 tend to be upregulated less strongly or not at all in *bru1-2* and downregulated genes are less strongly or not at all downregulated in *bru1-2*. Thus, the response at 52 h is dampened in *bru1-2*. These findings confirm the hypothesis that *bru1-2* mutants have a reduced HS memory response. This was next quantified in Figure 7c; the three classes of genes that are differentially expressed in Col-0 at 4 h ACC or 52 h ACC relative to NHS (4 h only, 4 h and 52 h, 52 h only) are all enriched in genes that are differentially expressed in *bru1-2* versus Col-0 (ACC/NHS_{bru1-2} at least two-fold different from ACC/NHS_{Col-0}) compared to the whole genome (8-75 % versus 1 % of all other genes). More specifically, more than half of the genes that are upregulated in Col-0 ACC versus NHS at 52 h only (54 %), and more than half of the genes that are downregulated at either both time points (75 %) or 52 h only (60 %), are differentially expressed in *bru1-2* compared to the corresponding Col-0 time point. The genes that are differentially expressed in *bru1-2* under control conditions are listed in Supplemental Data Set S1. In summary, *BRU1* is required for the correct expression of HS-responsive genes at a global level. Notably, both upregulated and downregulated genes were strongly affected at 52 h and for both groups the differential expression in *bru1-2* was dampened (Figure 7b). This trend was not observed for the genes upregulated at 4 h, corroborating the idea that *BRU1* is mostly required for the memory phase rather than for acute HS responses.

Discussion

Here, we have identified a role for the chromatin-regulatory protein BRU1 during HS memory in *A. thaliana*. Interestingly, BRU1 is not required for the acquisition of thermotolerance or basal thermotolerance, and it is dispensable for early HS responses and

for the initial HS gene activation. At the molecular level, *BRU1* is required to maintain high levels of expression in HS memory-associated genes throughout the memory phase. Globally, it is also required for sustained repression after HS as revealed by the transcriptome analysis. Our observations are in line with the previously established functions of *BRU1* (see below).

BRU1 has been implicated in the epigenetic inheritance of transcriptional silencing and in the DNA damage response (Suzuki *et al.*, 2004, Takeda *et al.*, 2004). In addition, it has very similar phenotypes as the histone chaperone CAF-1 (Takeda *et al.*, 2004). Together, this has led to a model where BRU1 ensures faithful inheritance of chromatin states across DNA replication and cell division. Recent mechanistic work from the mammalian field indicates that TONSL, the BRU1 orthologue, directly binds to ssDNA during DNA damage repair and recruits RAD51 (Huang *et al.*, 2018). In addition, TONSL regulates DNA replication and binds to newly incorporated nucleosomes after replication (Saredi *et al.*, 2016). In summary, these findings are consistent with the idea that the conserved function of BRU1 may be not only in DNA damage repair, but also in the faithful inheritance of chromatin states after replication.

The requirement of the chromatin regulator *BRU1* for HS memory strengthens the observation that chromatin structure and organization are important for sustained gene expression during HS memory. Previously, hyper-methylation of histone H3K4 was implicated in this process (Lämke *et al.*, 2016). Histone H3K4 hyper-methylation was also implicated in somatic stress memory after drought stress, salt stress and pathogen infection (Jaskiewicz *et al.*, 2011, Ding *et al.*, 2012, Feng *et al.*, 2016), as well as in priming phenomena in metazoans and yeast (Ng *et al.*, 2003, Guenther *et al.*, 2007, D'Urso and Brickner, 2014, D'Urso *et al.*, 2016). It remains to be investigated in future studies, whether BRU1 is required for the maintenance of H3K4 methylation at memory genes or for the control of nucleosome occupancy at these loci. Due to the heterogeneity of whole seedlings

and technical limitations, chromatin immunoprecipitation analyses provide a relatively coarse picture and are to be interpreted with caution. Our findings extend previous reports on the function of BRU1 in the key point that BRU1 may not only be required for the inheritance of repressive chromatin states, but also for the inheritance of active chromatin states that are conducive to ongoing transcription. This suggests a mode of action where BRU1 does not bind to individual chromatin modifications that are to be copied but may act more generally in the faithful inheritance of such chromatin modifications. This may be achieved by providing a binding platform for various reader and writer enzymes or by ensuring the transmission of the epigenetic information from "old" nucleosomes onto "new" nucleosomes.

We found that *CAF-1* is not required for HS memory. This is a noticeable difference to other phenotypic defects that have been observed in *bru1*, such as stem fasciation and DNA damage repair (Kaya *et al.*, 2001, Takeda *et al.*, 2004). It suggests that although CAF-1 and BRU1 act in connected pathways, their functions do not fully overlap. Notably, CAF-1 has been implicated in the priming of plant defences, as it modulates nucleosome occupancy of primed genes (Mozgova *et al.*, 2015). Whether BRU1 is also required for defence priming or stress memory in response to other biotic or abiotic stress cues remains to be investigated.

HS memory at the physiological level was reported to last for at least three days (Stief *et al.*, 2014). At the molecular level, a memory after HS as evidenced by enhanced re-induction after a second HS has been detected for up to seven days (Liu *et al.*, 2018). The HS treatment that is used to activate the memory response (ACC or an even milder treatment) does not cause visual damage and only a minimal delay in growth (Stief *et al.*, 2014). Hence, considerable growth is taking place during the memory period that may at least partially be attributed to ongoing cell division. This is in line with our finding that ACC reduces histone H4 expression, as a marker gene for the S-phase, only transiently and moderately. Although detailed further investigations will be required to determine cell division rates in different

tissues after ACC, it appears plausible that the memory is transmitted over at least a few cell divisions. This raises the question of how memory components are inherited during DNA replication and cell division. Previous work has demonstrated that histone modifications as well as nucleosome occupancy both regulate HS memory (Brzezinka *et al.*, 2016, Lämke *et al.*, 2016). It is tempting to speculate that BRU1 may play a role in the inheritance of HS memory determinants through DNA replication and their faithful transmission to daughter cells.

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Conflict of interest

The authors declare no conflict of interest.

References

Akerfelt, M., Morimoto, R.I. and Sistonen, L. (2010) Heat shock factors: integrators of cell stress, development and lifespan. *Nature Reviews Molecular Cellular Biology*, **11**, 545-555.

Anckar, J. and Sistonen, L. (2011) Regulation of HSF1 function in the heat stress response: implications in aging and disease. *Annual Review of Biochemistry*, **80**, 1089-1115.

Babicki, S., Arndt, D., Marcu, A., Liang, Y., Grant, J.R., Maciejewski, A. and Wishart, D.S. (2016) Heatmapper: web-enabled heat mapping for all. *Nucleic Acids Research*, **44**, W147-153.

Battisti, D.S. and Naylor, R.L. (2009) Historical warnings of future food insecurity with unprecedented seasonal heat. *Science*, **323**, 240-244.

Bäurle, I. (2016) Plant Heat Adaptation: priming in response to heat stress. *F1000Research*, **5**.

Bäurle, I., Smith, L., Baulcombe, D.C. and Dean, C. (2007) Widespread role for the flowering-time regulators FCA and FPA in RNA-mediated chromatin silencing. *Science*, **318**, 109-112.

Breuer, C., Stacey, N.J., West, C.E., Zhao, Y., Chory, J., Tsukaya, H., Azumi, Y., Maxwell, A., Roberts, K. and Sugimoto-Shirasu, K. (2007) BIN4, a novel component of the plant DNA topoisomerase VI complex, is required for endoreduplication in Arabidopsis. *Plant Cell*, **19**, 3655-3668.

Bruce, T.J.A., Matthes, M.C., Napier, J.A. and Pickett, J.A. (2007) Stressful "memories" of plants: Evidence and possible mechanisms. *Plant Science*, **173**, 603-608.

Brzezinka, K., Altmann, S., Czesnick, H., Nicolas, P., Górka, M., Benke, E., Kabelitz, T., Jähne, F., Graf, A., Kappel, C. and Bäurle, I. (2016) Arabidopsis FORGETTER1 mediates stress-induced chromatin memory through nucleosome remodeling. *eLife*, **5**, e17061.

Campos, E.I., Smits, A.H., Kang, Y.H., Landry, S., Escobar, T.M., Nayak, S., Ueberheide, B.M., Durocher, D., Vermeulen, M., Hurwitz, J. and Reinberg, D. (2015) Analysis of the Histone H3.1 Interactome: A Suitable Chaperone for the Right Event. *Molecular Cell*, **60**, 697-709.

Charng, Y.Y., Liu, H.C., Liu, N.Y., Chi, W.T., Wang, C.N., Chang, S.H. and Wang, T.T. (2007) A heat-inducible transcription factor, HsfA2, is required for extension of acquired thermotolerance in Arabidopsis. *Plant Physiology*, **143**, 251-262.

Charng, Y.Y., Liu, H.C., Liu, N.Y., Hsu, F.C. and Ko, S.S. (2006) Arabidopsis Hsa32, a novel heat shock protein, is essential for acquired thermotolerance during long recovery after acclimation. *Plant Physiology*, **140**, 1297-1305.

Conrath, U., Beckers, G.J., Langenbach, C.J. and Jaskiewicz, M.R. (2015) Priming for enhanced defense. *Annual Review of Phytopathology*, **53**, 97-119.

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K. and Scheible, W.R. (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. *Plant Physiology*, **139**, 5-17.

D'Urso, A. and Brickner, J.H. (2014) Mechanisms of epigenetic memory. *Trends in Genetics*, **30**, 230-236.

D'Urso, A., Takahashi, Y.H., Xiong, B., Marone, J., Coukos, R., Randise-Hinchliff, C., Wang, J.P., Shilatifard, A. and Brickner, J.H. (2016) Set1/COMPASS and Mediator are repurposed to promote epigenetic transcriptional memory. *eLife*, **5**.

Ding, Y., Fromm, M. and Avramova, Z. (2012) Multiple exposures to drought 'train' transcriptional responses in Arabidopsis. *Nature Communications*, **3**, 740.

Duro, E., Lundin, C., Ask, K., Sanchez-Pulido, L., MacArtney, T.J., Toth, R., Ponting, C.P., Groth, A., Helleday, T. and Rouse, J. (2010) Identification of the MMS22L-TONSL complex that promotes homologous recombination. *Molecular Cell*, **40**, 632-644.

Exner, V., Taranto, P., Schonrock, N., Gruissem, W. and Hennig, L. (2006) Chromatin assembly factor CAF-1 is required for cellular differentiation during plant development. *Development*, **133**, 4163-4172.

Farrona, S., Hurtado, L., Bowman, J.L. and Reyes, J.C. (2004) The Arabidopsis thaliana SNF2 homolog AtBRM controls shoot development and flowering. *Development*, **131**, 4965-4975.

Feng, X.J., Li, J.R., Qi, S.L., Lin, Q.F., Jin, J.B. and Hua, X.J. (2016) Light affects salt stress-induced transcriptional memory of P5CS1 in Arabidopsis. *Proceedings of the National Academy of Science U S A*, **113**, E8335-E8343.

Guenther, M.G., Levine, S.S., Boyer, L.A., Jaenisch, R. and Young, R.A. (2007) A chromatin landmark and transcription initiation at most promoters in human cells. *Cell*, **130**, 77-88.

Guyomarc'h, S., Vernoux, T., Traas, J., Zhou, D.X. and Delarue, M. (2004) MGOUN3, an Arabidopsis gene with TetratricoPeptide-Repeat-related motifs, regulates meristem cellular organization. *Journal of Experimental Botany*, **55**, 673-684.

Hilker, M., Schwachtje, J., Baier, M., Balazadeh, S., Bäurle, I., Geiselhardt, S., Hincha, D.K., Kunze, R., Mueller-Roeber, B., Rillig, M.C., Rolff, J., Romeis, T., Schmülling, T., Steppuhn, A., van Dongen, J., Whitcomb, S.J., Wurst, S., Zuther, E. and Kopka, J. (2016) Priming and memory of stress responses in organisms lacking a nervous system. *Biological reviews of the Cambridge Philosophical Society*, **91**, 1118-1133.

Hong, S.W. and Vierling, E. (2000) Mutants of Arabidopsis thaliana defective in the acquisition of tolerance to high temperature stress. *Proceedings of the National Academy of Science U S A*, **97**, 4392-4397.

Huang, T.H., Fowler, F., Chen, C.C., Shen, Z.J., Sleckman, B. and Tyler, J.K. (2018) The Histone Chaperones ASF1 and CAF-1 Promote MMS22L-TONSL-Mediated Rad51 Loading onto ssDNA during Homologous Recombination in Human Cells. *Molecular Cell*, **69**, 879-892 e875.

Ikeda, M., Mitsuda, N. and Ohme-Takagi, M. (2011) Arabidopsis HsfB1 and HsfB2b act as repressors of the expression of heat-inducible Hsfs but positively regulate the acquired thermotolerance. *Plant Physiology*, **157**, 1243-1254.

Inagaki, S., Nakamura, K. and Morikami, A. (2009) A link among DNA replication, recombination, and gene expression revealed by genetic and genomic analysis of TEBICHI gene of Arabidopsis thaliana. *PLoS Genetics*, **5**, e1000613.

Jaskiewicz, M., Conrath, U. and Peterhänsel, C. (2011) Chromatin modification acts as a memory for systemic acquired resistance in the plant stress response. *EMBO Reports*, **12**, 50-55.

Kaya, H., Shibahara, K.I., Taoka, K.I., Iwabuchi, M., Stillman, B. and Araki, T. (2001) FASCIATA genes for chromatin assembly factor-1 in arabidopsis maintain the cellular organization of apical meristems. *Cell*, **104**, 131-142.

Kim, J.M., To, T.K., Ishida, J., Matsui, A., Kimura, H. and Seki, M. (2012) Transition of chromatin status during the process of recovery from drought stress in Arabidopsis thaliana. *Plant Cell Physiology*, **53**, 847-856.

Lämke, J. and Bäurle, I. (2017) Epigenetic and chromatin-based mechanisms in environmental stress adaptation and stress memory in plants. *Genome Biology*, **18**, 124.

Lämke, J., Brzezinka, K., Altmann, S. and Bäurle, I. (2016) A hit-and-run heat shock factor governs sustained histone methylation and transcriptional stress memory. *EMBO Journal*, **35**, 162-175.

Li, G., Liu, S., Wang, J., He, J., Huang, H., Zhang, Y. and Xu, L. (2014) ISWI proteins participate in the genome-wide nucleosome distribution in Arabidopsis. *Plant Journal*, **78**, 706-714.

Liu, H.-c., Lämke, J., Lin, S.-y., Hung, M.-J., Liu, Kuan-Ming, Charng, Y.-y. and Bäurle, I. (2018) Distinct heat shock factors and chromatin modifications mediate the organ-autonomous transcriptional memory of heat stress. *Plant Journal*, in press. DOI: 10.1111/tpj.13958..

Liu, H.C., Liao, H.T. and Charng, Y.Y. (2011) The role of class A1 heat shock factors (HSFA1s) in response to heat and other stresses in Arabidopsis. *Plant, Cell & Environment*, **34**, 738-751.

Lobell, D.B., Schlenker, W. and Costa-Roberts, J. (2011) Climate trends and global crop production since 1980. *Science*, **333**, 616-620.

Mishra, S.K., Tripp, J., Winkelhaus, S., Tschiersch, B., Theres, K., Nover, L. and Scharf, K.D. (2002) In the complex family of heat stress transcription factors, HsfA1 has a unique role as master regulator of thermotolerance in tomato. *Genes & Development*, **16**, 1555-1567.

Mozgova, I., Wildhaber, T., Liu, Q., Abou-Mansour, E., L'Haridon, F., Metraux, J.P., Gruissem, W., Hofius, D. and Hennig, L. (2015) Chromatin assembly factor CAF-1 represses priming of plant defence response genes. *Nature Plants*, **1**, 15127.

Ng, H.H., Robert, F., Young, R.A. and Struhl, K. (2003) Targeted recruitment of Set1 histone methylase by elongating Pol II provides a localized mark and memory of recent transcriptional activity. *Molecular Cell*, **11**, 709-719.

Nishizawa-Yokoi, A., Nosaka, R., Hayashi, H., Tainaka, H., Maruta, T., Tamoi, M., Ikeda, M., Ohme-Takagi, M., Yoshimura, K., Yabuta, Y. and Shigeoka, S. (2011) HsfA1d and HsfA1e involved in the transcriptional regulation of HsfA2 function as key regulators for the Hsf signaling network in response to environmental stress. *Plant Cell Physiology*, **52**, 933-945.

Nishizawa, A., Yabuta, Y., Yoshida, E., Maruta, T., Yoshimura, K. and Shigeoka, S. (2006) Arabidopsis heat shock transcription factor A2 as a key regulator in response to several types of environmental stress. *Plant Journal*, **48**, 535-547.

O'Connell, B.C., Adamson, B., Lydeard, J.R., Sowa, M.E., Ciccia, A., Bredemeyer, A.L., Schlabach, M., Gygi, S.P., Elledge, S.J. and Harper, J.W. (2010) A genome-wide camptothecin sensitivity screen identifies a mammalian MMS22L-NFKBIL2 complex required for genomic stability. *Molecular Cell*, **40**, 645-657.

O'Donnell, L., Panier, S., Wildenhain, J., Tkach, J.M., Al-Hakim, A., Landry, M.C., Escribano-Diaz, C., Szilard, R.K., Young, J.T., Munro, M., Canny, M.D., Kolas, N.K., Zhang, W., Harding, S.M., Ylanko, J., Mendez, M., Mullin, M., Sun, T., Habermann, B., Datti, A., Bristow, R.G., Gingras, A.C., Tyers, M.D., Brown, G.W. and Durocher, D. (2010) The MMS22L-TONSL complex mediates recovery from replication stress and homologous recombination. *Molecular Cell*, **40**, 619-631.

Ohama, N., Sato, H., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2017) Transcriptional Regulatory Network of Plant Heat Stress Response. *Trends in Plant Science*, **22**, 53-65.

Ohno, Y., Narangajavana, J., Yamamoto, A., Hattori, T., Kagaya, Y., Paszkowski, J., Gruissem, W., Hennig, L. and Takeda, S. (2011) Ectopic gene expression and organogenesis in Arabidopsis mutants missing BRU1 required for genome maintenance. *Genetics*, **189**, 83-95.

Piwko, W., Olma, M.H., Held, M., Bianco, J.N., Pedrioli, P.G., Hofmann, K., Pasero, P., Gerlich, D.W. and Peter, M. (2010) RNAi-based screening identifies the Mms22L-Nfkbil2 complex as a novel regulator of DNA replication in human cells. *EMBO Journal*, **29**, 4210-4222.

Probst, A.V., Dunleavy, E. and Almouzni, G. (2009) Epigenetic inheritance during the cell cycle. *Nature Reviews Molecular Cellular Biology*, **10**, 192-206.

Richter, K., Haslbeck, M. and Buchner, J. (2010) The heat shock response: life on the verge of death. *Molecular Cell*, **40**, 253-266.

Sani, E., Herzyk, P., Perrella, G., Colot, V. and Amtmann, A. (2013) Hyperosmotic priming of Arabidopsis seedlings establishes a long-term somatic memory accompanied by specific changes of the epigenome. *Genome Biology*, **14**, R59.

Saredi, G., Huang, H., Hammond, C.M., Alabert, C., Bekker-Jensen, S., Forne, I., Reveron-Gomez, N., Foster, B.M., Mlejnkova, L., Bartke, T., Cejka, P., Mailand, N., Imhof, A., Patel, D.J. and Groth, A. (2016) H4K20me0 marks post-replicative chromatin and recruits the TONSL-MMS22L DNA repair complex. *Nature*, **534**, 714-718.

Scharf, K.D., Berberich, T., Ebersberger, I. and Nover, L. (2012) The plant heat stress transcription factor (Hsf) family: structure, function and evolution. *Biochimica et Biophysica Acta*, **1819**, 104-119.

Schramm, F., Larkindale, J., Kiehlmann, E., Ganguli, A., Englich, G., Vierling, E. and von Koskull-Doring, P. (2008) A cascade of transcription factor DREB2A and heat stress transcription factor HsfA3 regulates the heat stress response of Arabidopsis. *Plant Journal*, **53**, 264-274.

Singh, P., Yekondi, S., Chen, P.W., Tsai, C.H., Yu, C.W., Wu, K. and Zimmerli, L. (2014) Environmental History Modulates Arabidopsis Pattern-Triggered Immunity in a HISTONE ACETYLTRANSFERASE1-Dependent Manner. *Plant Cell*, **26**, 2676-2688.

Smith, S. and Stillman, B. (1989) Purification and characterization of CAF-I, a human cell factor required for chromatin assembly during DNA replication in vitro. *Cell*, **58**, 15-25.

Stief, A., Altmann, S., Hoffmann, K., Pant, B.D., Scheible, W.-R. and Bäurle, I. (2014) Arabidopsis miR156 Regulates Tolerance to Recurring Environmental Stress through SPL Transcription Factors. *Plant Cell*, **26**, 1792-1807.

Suzuki, T., Inagaki, S., Nakajima, S., Akashi, T., Ohto, M.A., Kobayashi, M., Seki, M., Shinozaki, K., Kato, T., Tabata, S., Nakamura, K. and Morikami, A. (2004) A novel Arabidopsis gene TONSOKU is required for proper cell arrangement in root and shoot apical meristems. *Plant Journal*, **38**, 673-684.

Suzuki, T., Nakajima, S., Inagaki, S., Hirano-Nakakita, M., Matsuoka, K., Demura, T., Fukuda, H., Morikami, A. and Nakamura, K. (2005) TONSOKU is Expressed in S Phase of the Cell Cycle and its Defect Delays Cell Cycle Progression in Arabidopsis. *Plant Cell Physiology*, **46**, 736–742.

Takeda, S., Tadele, Z., Hofmann, I., Probst, A.V., Angelis, K.J., Kaya, H., Araki, T., Mengiste, T., Mittelsten Scheid, O., Shibahara, K., Scheel, D. and Paszkowski, J. (2004) BRU1, a novel link between responses to DNA damage and epigenetic gene silencing in Arabidopsis. *Genes & Development*, **18**, 782-793.

Vriet, C., Hennig, L. and Laloi, C. (2015) Stress-induced chromatin changes in plants: of memories, metabolites and crop improvement. *Cellular and molecular life sciences : CMLS*, **72**, 1261-1273.

Yeh, C.H., Kaplinsky, N.J., Hu, C. and Charng, Y.Y. (2012) Some like it hot, some like it warm: phenotyping to explore thermotolerance diversity. *Plant Science : an international journal of experimental plant biology*, **195**, 10-23.

Yoshida, T., Ohama, N., Nakajima, J., Kidokoro, S., Mizoi, J., Nakashima, K., Maruyama, K., Kim, J.M., Seki, M., Todaka, D., Osakabe, Y., Sakuma, Y., Schöffl, F., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2011) Arabidopsis HsfA1 transcription factors function as the main positive regulators in heat shock-responsive gene expression. *Mol Genetics & Genomics*, **286**, 321-332.

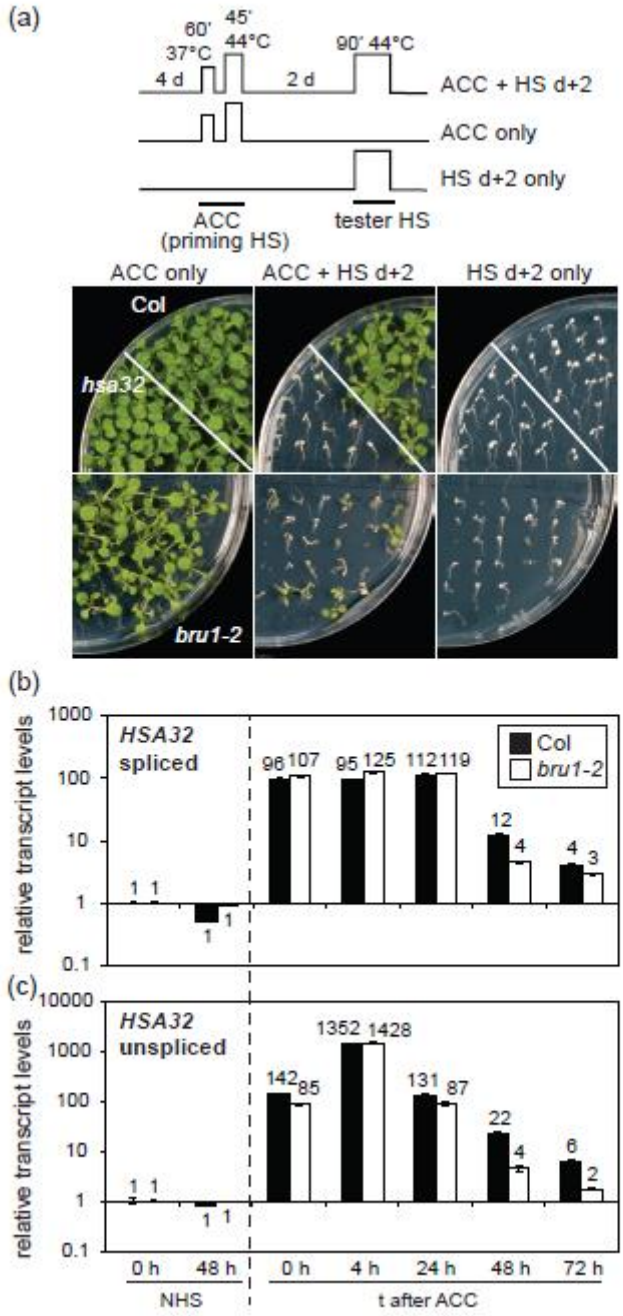

Figure 1 *BRU1* is specifically required for HS memory and sustained transcriptional induction of *HSA32* after HS

(a) *Bru1-2* mutants are impaired in HS memory at the physiological level. 4 d-old seedlings of the indicated genotypes were subjected to ACC treatment; two days later they were exposed to a tester HS at 44°C for 90 min. All genotypes were grown on the same plate.

Photographs were taken 14 d after ACC. One representative of more than three independent experiments is shown.

(b, c) Transcript levels of spliced (b) and unspliced (c) *HSA32* were analyzed by quantitative RT-PCR in Col-0 and *bru1-2* at the indicated time points after the end of ACC or in no-HS controls (NHS) harvested at corresponding time points. Expression values were normalized to *TUB6* and to 0 h NHS. Data are averages of three biological replicates ± SEM. Data are plotted on a $\log_{10}$ scale.

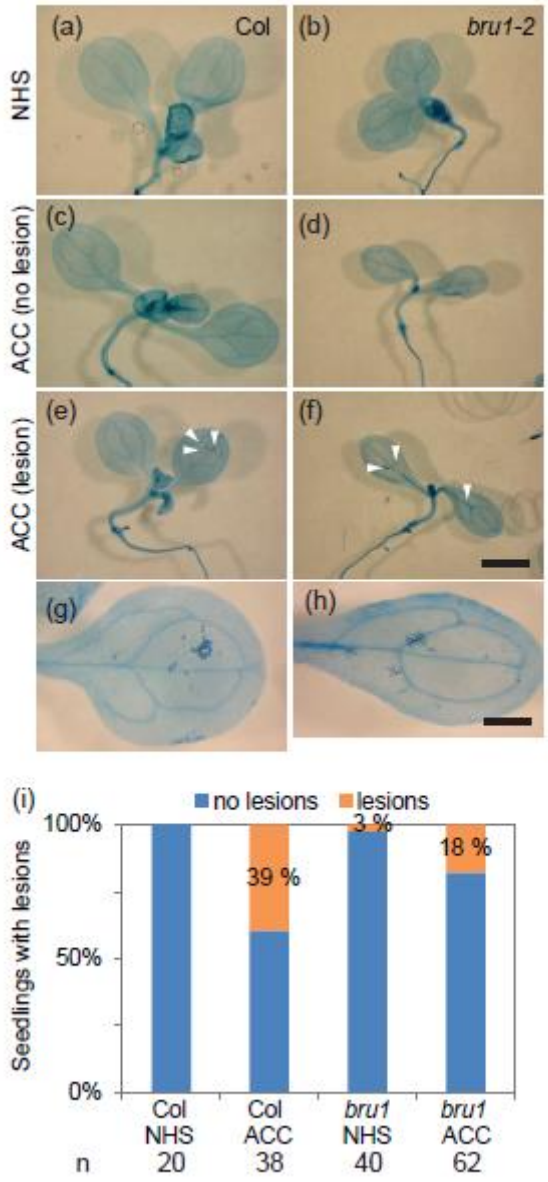

Figure 2 Cell death after ACC treatment is not increased in *bru1-2* mutants relative to Col-0 wild type

(a-h) Cell death was assessed by Trypan Blue staining of 4 d-old seedlings of Col-0 (a, c, e, g) and *bru1-2* (b, d, f, h) that were treated with ACC (cf. Figure 1a); 96 h after the end of ACC, mock- (a, b) or ACC-treated (c-h) seedlings were stained with Trypan Blue to visualize cell death and examined under a Leica Stereomicroscope. (g, h) Close-ups of representative cotyledons with lesions. Size bars: 5 mm (a-f), 1 mm (g, h).

(i) Percentage of seedlings with lesions of the indicated genotypes and treatments shown in (a-h). n indicates the number of seedlings that were scored for the analysis.

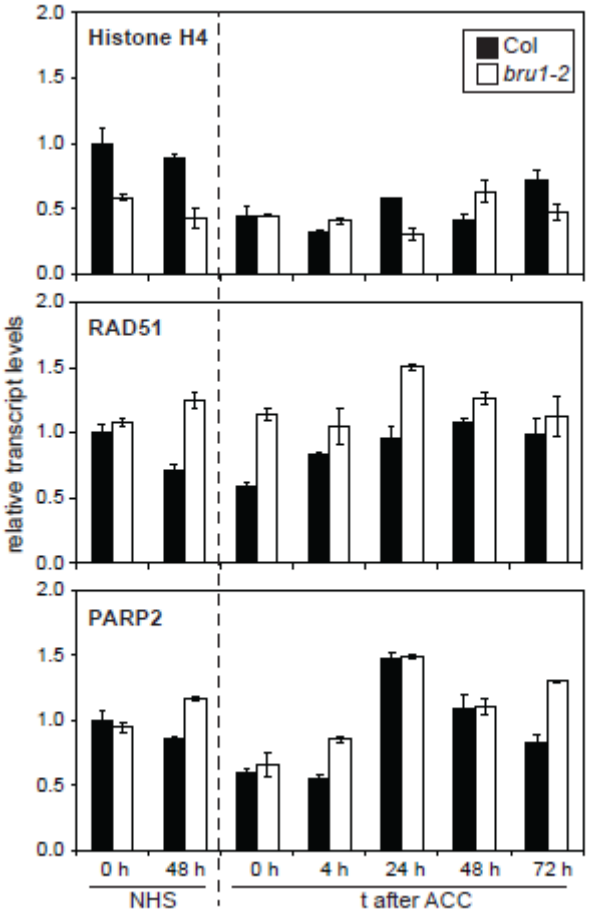

Figure 3 Transcript levels of DNA damage marker genes and histone H4 in *bru1-2*

Transcript levels of histone *H4*, *RAD51* and *PARP2* were analyzed by quantitative RT-PCR in Col-0 and *bru1-2* at the indicated time points after the end of ACC or in no-HS controls (NHS). Expression values were normalized to *TUB6* and Col-0 0 h NHS. Data are averages of three biological replicates ± SEM.

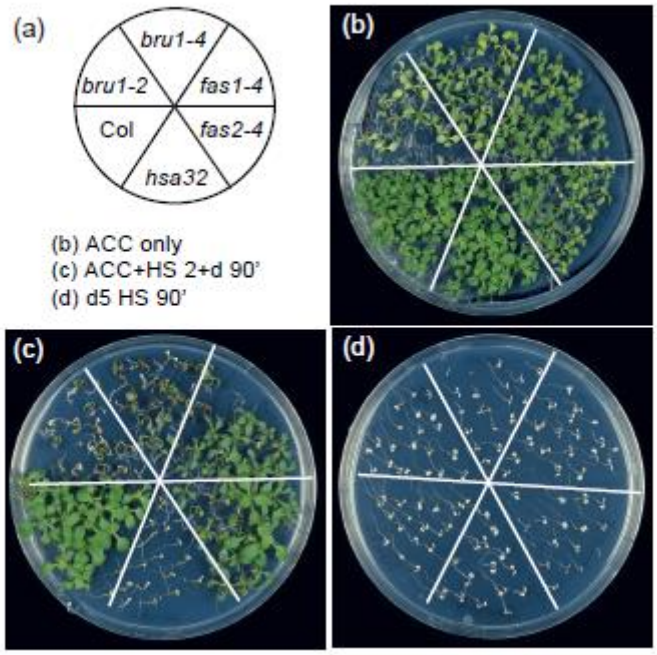

Figure 4 The *FAS1* and *FAS2* genes are dispensable for HS memory

(a - d) 4 d-old seedlings of the indicated genotypes (a) were subjected to ACC treatment; two days later they were exposed to a tester HS at 44°C for 90 min (c). The control plates were either exposed to ACC only (b) or to the tester HS only (d). For each treatment all genotypes were grown and treated on the same plate. Photographs were taken 14 d after ACC. One representative of several independent experiments is shown.

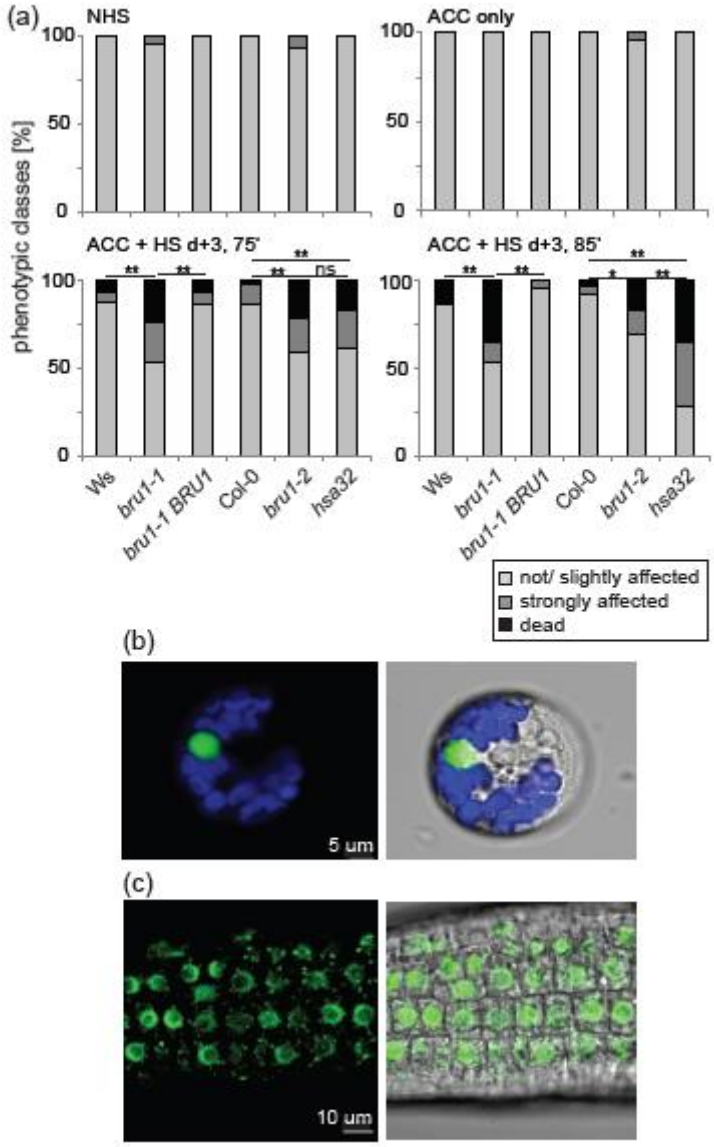

Figure 5 Complementation of *bru1-1* with a *pBRU1::BRU1-GFP* construct

(a) BRU1-GFP expressed from *pBRU1::BRU1-GFP* complements the HS memory defect of *bru1-1*. 4d-old seedlings of the indicated genotypes were subjected to ACC treatment; 3 d later they were exposed to a tester HS at 44°C for the indicated times. Photographs were taken 14 d after ACC (Supplemental Figure S2) and seedling survival was quantified as described previously (Brzezinka *et al.*, 2016). *, $p<0.05$; **, $p<0.01$, Fisher's exact test. Data shown are averages of at least two independent biological replicates each containing at least 43 individuals per genotype.

(b) BRU1-GFP expressed from *pUBC10::BRU1-GFP* is localized to the nucleus of transiently transformed *A. thaliana* protoplasts. The GFP signal is shown in green, chlorophyll autofluorescence is shown in blue.

(c) BRU1-GFP expressed from *pBRU1::BRU1-GFP* is localized to the nucleus in the roots of stably transformed *A. thaliana*. The GFP signal is shown in green.

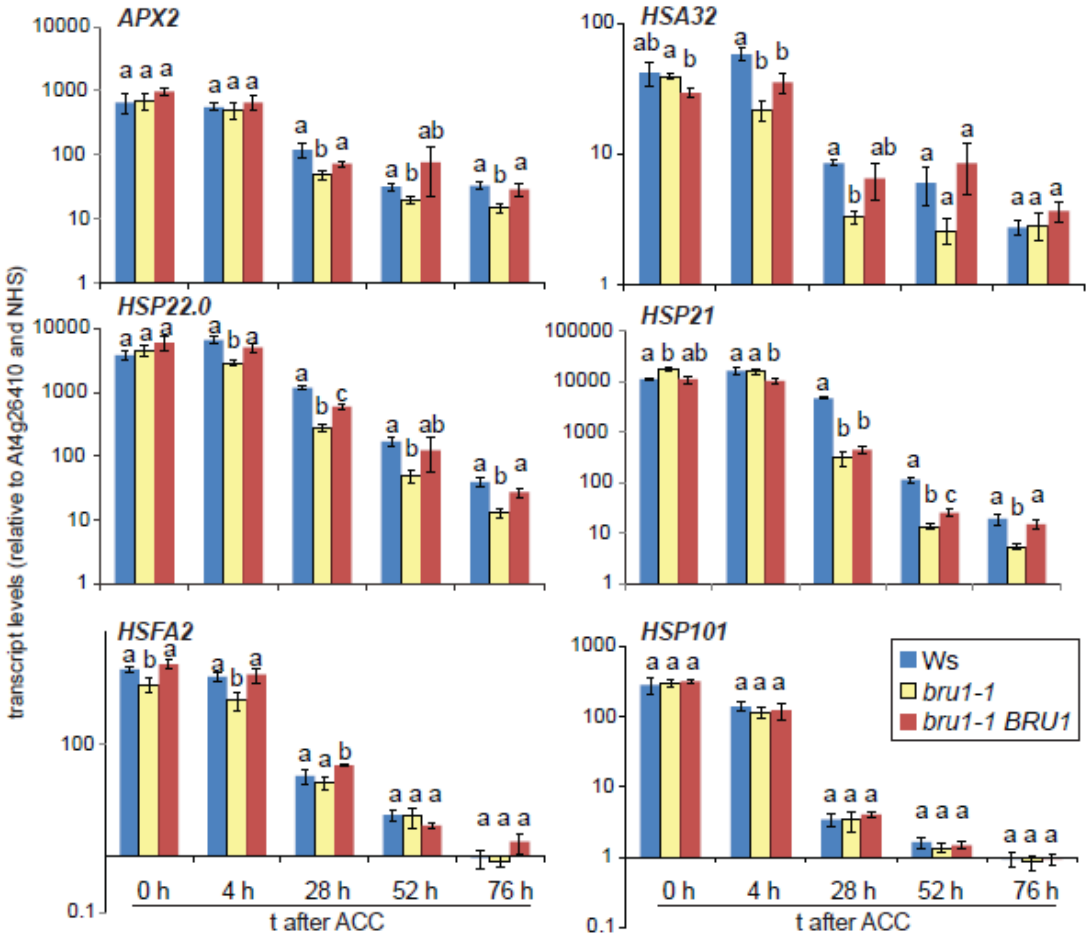

Figure 6 Sustained induction of several memory genes after ACC is impaired in *bru1-1*

Expression profiles of HS memory-associated genes (*APX2*, *HSA32*, *HSP22.0*, *HSP21*), *HSFA2*, and a HS-inducible non-memory gene (*HSP101*) after ACC in Ws wild type, *bru1-1* mutants and the complementation line. Transcript levels determined by qRT-PCR were normalized to the *At4g26410* reference gene and the respective NHS control harvested at the same time point. At each time point different letters (a, b, c) indicate significant differences between the tested genotypes ($p<0.05$, Student's t-test), whereas the same letter indicates that there was no significant difference detected. . Data represent averages of at least three biological replicates ± SEM.

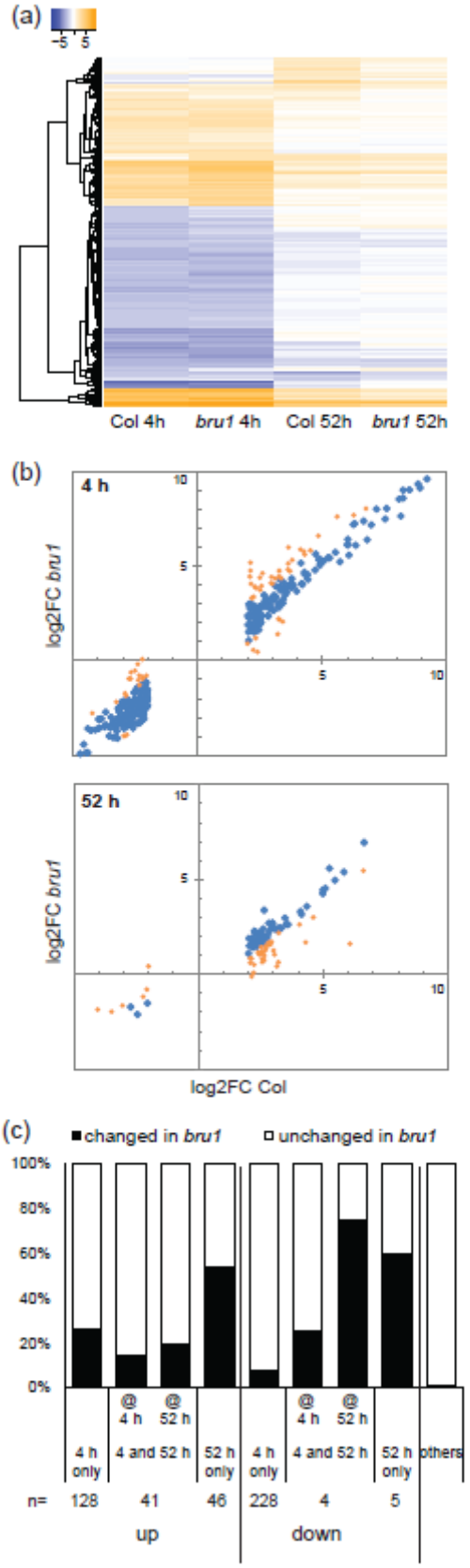

Figure 7 Global gene expression analysis after ACC in *bru1-2*

(a) Clustered heat map analysis including all 452 differentially expressed genes (DEGs) with $log_2FC > 2$ or < -2 in Col-0, including 356 "4 h-only" DEGs, 45 "4 and 52" h DEGs, and 51 "52 h-only" DEGs. Scale indicates log_2FC relative to the NHS control for Col-0 and *bru1-2*.

(b) Expression correlation analysis of DEGs ($log_2FC > 2$ or < -2) at 4 h (top panel) and 52 h (bottom panel) in Col-0 and *bru1-2*. DEGs that differ in *bru1-2* at least two-fold from the value of Col-0 are highlighted in orange.

(c) Percentage of DEGs whose induction or repression differed at least twofold in *bru1-2* compared to Col-0 in the indicated classes. The total number of DEGs in these classes is indicated with n. The number of differentially expressed genes is enriched among the HS-responsive genes compared to the rest of the genome (others).