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RESEARCH PAPER

Arabidopsis HIT4 encodes a novel chromocentre-localized protein involved in the heat reactivation of transcriptionally silent loci and is essential for heat tolerance in plants

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Abstract

The *Arabidopsis* mutant *heat-intolerant 4-1* (*hit4-1*) was isolated from an ethyl methanesulphonate-mutagenized M2 population on the basis of its inability to withstand prolonged heat stress (4 days at 37°C). Further characterization indicated that *hit4-1* was impaired specifically in terms of basal but not acquired thermotolerance. Map-based cloning revealed that the *HIT4* gene encoded a plant-specific protein for which the molecular function has yet to be studied. To investigate the cellular role of HIT4 and hence elucidate better its protective function in heat tolerance in plants, a GFP-HIT4 reporter construct was created for a protoplast transient expression assay. Results showed that fluorescently tagged HIT4 was localized to the chromocentre, a condensed heterochromatin domain that harbours repetitive elements for which transcription is normally suppressed by transcriptional gene silencing (TGS). DAPI-staining analysis and FISH with a probe that targeted centromeric repeats showed that heat-induced chromocentre decondensation was inhibited in nuclei of *hit4-1* subjected to direct heat treatment, but not in those that were allowed to acquire thermotolerance. Moreover, heat reactivation of various TGS loci, regardless of whether they were endogenous or transgenic, or existed as a single copy or as repeats, was found to be attenuated in *hit4-1*. Meanwhile, the levels of transcripts of heat shock protein genes in response to heat stress were similar in both *hit4-1* and wild-type plants. Collectively, these results demonstrated that HIT4 defines a new TGS regulator that acts at the level of heterochromatin organization and is essential for basal thermotolerance in plants.

Key words: *Arabidopsis thaliana*; chromocentre, heterochromatin organization, HIT4, heat stress, transcriptional gene silencing.

Introduction

Heat stress is one of the major environmental stresses experienced by all living organisms, because alternations to membrane fluidity and the denaturation of proteins caused by elevated temperatures can disrupt membrane-linked processes, inactivate enzymes, elicit secondary oxidative injuries, and finally lead to cell death (Wahid *et al*., 2007). Plants, being sessile organisms, have little control over their growth environments and thus have evolved specific strategies, which include morphological and metabolic changes, to cope with heat stress. Nevertheless, the protective effects exerted by these strategies require a heat-induced transcriptional response, by which a subset of genes are activated or suppressed, and this response must be finely coordinated.

The heat-induced transcriptional response can be influenced by mechanisms that involve the direct interactions of regulatory proteins with specific DNA sequences in the promoter region of target genes. These interactions either assist or hinder the access of the transcriptional machinery and consequently result in enhanced or suppressed transcription of the genes. The recognition of heat stress elements (HSEs) by heat stress factors (Hsfs) is representative of these mechanisms in the heat response of plants. In *Arabidopsis*,

Hsfs are encoded by a large gene family with 21 members (von Koskull-Döring *et al.*, 2007). Among them, differential expression analysis has shown that AtHsfA1a and AtHsfA1b together are responsible for 4% of the total change in gene expression in response to heat during the first hour of heat stress; this change involves both up- and downregulation. The AtHsfA1a/1b-dependent genes include those that encode heat shock proteins (HSPs), which can counteract the cytotoxic effects of heat-denatured proteins, and the promoter regions of these *HSP* genes contain either perfect or variant HSEs (Lohmann *et al.*, 2004; Busch *et al.*, 2005; Guo *et al.*, 2008). Meanwhile, AtHsfA2 is the dominant Hsf in thermotolerant cells and is required for the transcription of the heat-induced ascorbate peroxidase 2 gene *APX2* (Schramm *et al.*, 2006). The HSE is also found in the promoters of *APX2* and other AtHsfA2 target genes, and the binding of AtHsfA2 to these promoters has been demonstrated by DNA electrophoretic mobility and transient reporter assays (Nishizawa *et al.*, 2006; Schramm *et al.*, 2006). In contrast, transient expression studies that used tobacco protoplasts showed that AtHsfB1 is a repressor of class A Hsf activity (Czarnecka-Verner *et al.*, 2000, 2004). Nevertheless, AtHsfB1 and B2b together are necessary for acquired thermotolerance (Ikeda *et al.*, 2011).

In addition to the Hsf and HSE regulatory network, recent studies have linked the heat-induced transcriptional response to chromatin configurations. In interphase nuclei, chromatin is arranged into regions of either microfibrils, called euchromatin, or condensed masses, termed heterochromatin. In *Arabidopsis*, heterochromatin can cluster further into discernible nuclear domains that are known as chromocentres. The main genetic components of chromocentres are tandemly repeated DNA and dispersed transposons whose activities are suppressed normally by transcriptional gene silencing (TGS) (Fransz *et al.*, 2003). Several independent studies have shown concurrently that heat stress can decondense chromocentres and reactivate silenced genes within them (Lang-Mladek *et al.*, 2010; Pecinka *et al.*, 2010; Tittel-Elmer *et al.*, 2010). The heterochromatic TGS loci are often modified epigenetically, which mainly involves dense methylation at cytosine residues (^{m}C) and deacetylation of molecules of histone H3 that are methylated at lysine 9 (Habu *et al.*, 2001; Probst *et al.*, 2004). This implies that heat reactivation of heterochromatic TGS loci might result from alteration of the repressive epigenetic modifications. However, analysis of DNA revealed that the reactivation of TGS by heat occurred without significant changes in the level of either DNA demethylation or histone modifications. Furthermore, the observed heat-induced release of TGS could be affected by neither direct treatment with 5-azacytidine, an inhibitor of DNA methylation, nor mutations that compromised epigenetic regulation, including those involved in the maintenance of DNA methylation, RNA-mediated gene silencing, and histone deacetylation (Tittel-Elmer *et al.*, 2010; Ito *et al.*, 2011). On the other hand, given that the decondensation of chromocentres could potentially increase the accessibility of DNA to various transcription regulators and because heat-induced decondensation was only found in differentiated but not meristematic nuclei (Pecinka *et al.*, 2010), it is possible that the reorganization of chromatin under conditions of heat stress is a controlled process that plays an active role in the observed alleviation of TGS. However, the molecules that are involved in this control process and the extent to which the process affects the release of TGS and heat tolerance in plants have yet to be elucidated. It was suggested that a forward genetics approach is required to shed new light on these issues (Tittel-Elmer *et al.*, 2010).

To identify novel genetic determinants that are essential for heat tolerance in plants, this study group has screened for *h*eat-*in*tolerant (*hit*) mutants of *Arabidopsis* after ethyl methanesulphonate (EMS) mutagenesis (Wu *et al.*, 2000, 2010; Lee *et al.*, 2006; Wang *et al.*, 2008, 2011). Here is reported the isolation of *hit4-1*, for which growth was inhibited under conditions of sustained high temperature. Incubation at 37 °C for 4 days was lethal for *hit4-1*, but not for wild-type plants. The mutated locus was mapped to At5g10010, which encodes a plant-specific protein, for which the molecular and biological functions were unknown previously. By tagging with a fluorescent protein, HIT4 was shown to localize to chromocentres. Furthermore, heat-mediated chromocentre decondensation and release of TGS were found to be attenuated in *hit4-1*. These results revealed HIT4 to be a novel regulator of stress-triggered chromatin reorganization and demonstrated the essential role of this regulation for heat tolerance in plants.

Materials and methods

Plant materials and growth conditions

Wild-type *Arabidopsis thaliana* seeds of the ecotypes Columbia-0 (Col-0) and Landsberg *erecta* (L*er*-0) and EMS-mutagenized M2 seeds of Columbia-5 (Col-5) were purchased from Lehle Seeds (Round Rock, TX, USA). The T-DNA insertion lines SALK_047656 (*hit4-2*), SALK_081564C (*hit4-3*), SAIL_610_G01 (*mom1-2*), and SALK_066374 (*hsp101*) were obtained from the Arabidopsis Biological Resources Center (ABRC, Columbus, OH, USA). The *hit1-1* and *hit2* mutants and the transgenic line L5 were as described previously (Morel *et al.*, 2000; Wu *et al.*, 2000, 2010; Lee *et al.*, 2006). Detailed preparation of growth medium, procedures for mutant screening, and heat stress assays were as described by Wu *et al.* (2010). For seed propagation, plants were grown in soil under a 16/8 h light/dark cycle. The light intensity for all growth conditions was 100 μmol m^{-2} s^{-1}.

Genetic analysis and gene mapping of the hit4-1 *locus*

For genetic analysis, the *hit4-1* mutant was backcrossed with the wild-type Col-0. F_1 and selfed F_2 seedlings were tested in a heat survivability assay by incubating 7-day-old seedlings at 37°C for 4 days. These conditions were determined to be lethal for *hit4-1* but not for the wild-type seedlings. For gene mapping, *hit4-1* plants in a Col-5 background were crossed with wild-type L*er*-0 to create an F_2 mapping population. F_2 plants were selfed to produce F_3 plants for the heat survivability assay described above. Genomic DNA was then extracted from individual mutant plants from the F_2 generation for PCR-based gene mapping. Initially, published simple sequence length polymorphism (SSLP) and cleaved amplified polymorphic sequence (CAPS) markers were used for rough mapping (Konieczny and Ausubel, 1993; Bell and Ecker, 1994). Subsequently, new SSLP and CAPS markers in the vicinity of *hit4-1* were developed on the basis of insertions/deletions and single-nucleotide polymorphisms that were identified by comparing the genomes of Col-0 and Cereon L*er*-0 (Jander *et al.*, 2002).

Genotyping of T-DNA insertion lines

Three primers were used in combination to genotype *hit4-2*. Whereas SALK-ETp-F and SALK-ETp-R amplified a 269-bp wild-type sequence, SALK-ETp-F and LBb1.3 amplified a 518-bp sequence from the junction of the insert and plant DNA. Similarly, for *hit4-3*, primers SALK_081564-F and SALK_081564-R amplified a 1.4-kb wild-type sequence, whereas SALK_081564-F and LBb1 amplified a 900-bp sequence from the insert-plant DNA junction. Furthermore, for SAIL_610_G01, primers mom1-503-F and mom1-1197-R amplified a 694-bp wild-type sequence, and mom1-503-F and LB3 amplified a 499-bp sequence from the insert-plant DNA junction. To analyse the effect of T-DNA insertion on gene expression in *hit4-3*, total RNA was extracted from plants homozygous for the T-DNA insertion using a GeneMark Plant Total RNA Miniprep Purification Kit (Hopegen Biotechnology, Taichung, Taiwan) and reverse transcribed using Moloney murine leukaemia virus HP Reverse Transcriptase (Epicentre Technologies, Madison, WI, USA) to synthesize cDNA for reverse-transcription PCR (RT-PCR).

Cloning of HIT4 *genomic DNA and plant transformation*

For the complementation assay, a 6.2-kb genomic DNA fragment of *HIT4* was amplified by PCR with the primers HIT4gDNA-F and HIT4gDNA-R. The resultant amplicon was first cloned into the T-vector using a TA cloning kit (Yeastern Biotech, Taipei, Taiwan), then digested with *Nhe*I/*Sca*I and subcloned into the Ti binary vector pCAMBIA1301 (CAMBIA, Canberra, Australia). *Agrobacterium tumefaciens* GV3103 was then used to deliver this construct into *hit4-1* plants by the floral dipping method (Clough and Bent, 1998). Transformed seedlings were selected on medium that contained 25 μg/ml hygromycin. The hygromycin-resistant T1 seedlings were transferred to soil and grown to maturity. Homozygous T2 plants were selected by growth of their T3 generations on medium that contained hygromycin. T3 seeds derived from homozygous T2 plants were used for subsequent heat stress tests.

Phylogenetic analysis

Protein sequences of HIT4 homologues were obtained by BLASTp analysis of *Arabidopsis* HIT4 against a non-redundant database (http://blast.ncbi.nlm.nih.gov). The sequences were then aligned using ClustalW (http://www.ebi.ac.uk/clustalw/). The aligned sequences were assembled into a phylogenetic tree using the boot-strapped neighbour-joining algorithm and the Jones, Taylor, and Thornton amino acid substitution model in MEGA 5.05 with 1000 trials (Tamura *et al*., 2011). The percentage of HIT4 protein sequence similarity and identity were calculated by using MatGAT 2.0 (Campanella *et al*., 2003).

Subcellular localization of HIT4

For the localization assay, the GFP cDNA fragment was amplified from the vector p326-GFP (Choi *et al*., 2005) using the primers GFP-*Xba*I-F and GFP-*Bam*HI-R. The amplified fragment was digested with *Xba*I/*Bam*HI and cloned into the pLOLA vector (Ferrando *et al*., 2001) to create pLOLA-GFP. The cDNA for HIT4 was amplified using gene-specific primers (HIT4-*Bam*HI-F and HIT4-*Pst*I-R) and cloned into the *Bam*HI and *Pst*I sites of pLOLA-GFP, which resulted in a GFP–HIT4 fusion flanked by a 35S promoter and the nopaline synthase (nos) 3'-polyadenylation signal. Protoplasts were prepared in accordance with Wu *et al*. (2009). Polyethylene glycol-mediated transformation was performed as described by Yoo *et al*. (2007). Extraction of nuclei and 4',6'-diamidino-2-phenylindole (DAPI) staining were carried out as reported by Tirichine *et al*. (2009).

Gene expression analysis by quantitative real-time PCR

Seven-day-old seedlings that had been grown on MS medium were heat stressed at 37°C for various times. After the stress treatments, approximately 50 seedlings from each treatment were frozen immediately in liquid nitrogen. Total RNA was extracted using the GeneMark Plant Total RNA Miniprep Purification Kit (Hopegen Biotechnology) and processed in accordance with the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was then performed using the KAPA SYBR FAST one-step qRT-PCR Kit (KAPA Biosystems, Woburn, MA, USA) in accordance with the manufacturer's instructions. The *UBC28* gene was selected as an internal control. All qRT-PCR amplifications were performed in four independent biological replicates. Data were collected and analysed with a BioRad iCycler IQ5 Real-Time PCR Detection System (Life Science Research, Hercules, CA, USA).

GUS staining of seedlings and DAPI staining of root cells

The *hit4-1* mutant was crossed with line L5 (Morel *et al*., 2000), and the F_3 progeny that carried the L5 locus within a *hit4-1* background (L5/*hit4-1*) were collected for analysis. Fourteen-day-old L5/*hit4-1* seedlings were heat-stressed at 37°C for various times. GUS activity was detected according to Wang *et al*. (2008). For DAPI staining of root cell nuclei, seedlings were stained with 1 μg ml^{-1} DAPI for 15 min at room temperature before observation under an Olympus IX71 fluorescence microscope (Center Valley, PA, USA).

Fluorescence in situ hybridization

The protocols for extraction of nuclei from *Arabidopsis* seedlings and fluorescence *in situ* hybridization were as reported previously (Tirichine *et al*., 2009). The probe for the centromeric 180-bp repeat was amplified by PCR from genomic DNA using Alexa Fluor 488-labelled primers. The nuclei were counterstained with DAPI (1 μg ml^{-1}) for 5 min before observation and image acquisition.

Primers

The primers used in the study are listed in Supplementary Table S1 (available at *JXB* online).

Results

The hit4-1 *heat-hypersensitive phenotype is caused by a single recessive mutation*

Arabidopsis M_2 seedlings that had been mutagenized with EMS were examined under permissive heat stress conditions to screen for heat-hypersensitive mutants (Wu *et al*., 2010). One selected mutant, *hit4-1*, was later confirmed by retesting the M_3 generation with a heat survivability assay. There was no difference in appearance between the mutant and wild-type seedlings under normal growth conditions. For 7-day-old seedlings, incubation at 37°C for 4 days was lethal to *hit4-1* but not the wild type (Fig. 1A). This treatment was employed subsequently for genetic analysis and the results showed that all of the F_1 plants from a cross between *hit4-1* and the wild type could tolerate the heat treatment. Furthermore, analysis of the F_2 progeny from the self-pollinated F_1 showed a 3:1 segregation ratio for heat-tolerant to heat-sensitive forms (Table 1). Thus, the heat-intolerant phenotype of *hit4-1* is attributable to a single recessive nuclear mutation.

hit4-1 *is defective only in terms of basal and not acquired thermotolerance*

Depending on the intensity, duration, and rate of temperature change, different types of heat stress can impose diverse effects on plants, and plants have evolved a variety of protective

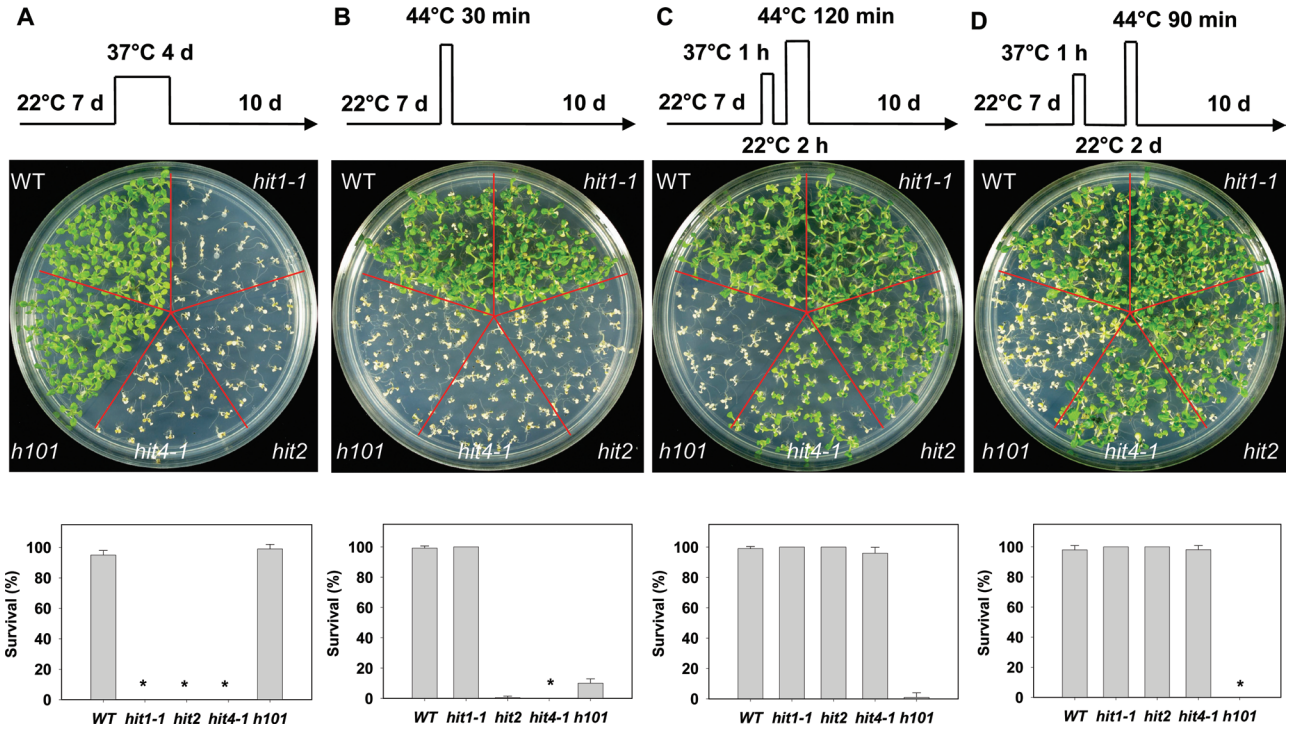

Fig. 1. *Arabidopsis hit4-1* seedlings were defective in terms of basal but not acquired thermotolerance. Seven-day-old seedlings of the wild type (WT), *hit1-1, hit4-1* and *hsp101 (h101)* were subjected to treatment under different heat stress regimes, as shown schematically above each photograph. The survival rates of seedlings after each treatment were calculated at the time that the photographs were taken and are shown in bar charts below each photograph. Values are mean ± SD of three replicates of each treatment. *Zero survival rates.

Table 1. Genetic analysis of the *Arabidopsis hit4-1* mutant. The calculated χ^2 value was based on the expected ratio of 3:1 for tolerant to sensitive individuals, assuming that *hit4-1* was a single recessive mutation ($P > 0.05$).

Strains or crosses (♀ × ♂)	Total	Tolerant	Sensitive	χ^2
Wild-type Col-0	110	110	0	
hit4-1/hit4-1	121	0	121	
Wild-type × *hit4-1/hit4-1*				
F_1	65	65	0	
F_2	616	470	146 (23.7%)	0.554

mechanisms that contribute to survival during these different types of heat stress. To obtain a better understanding of the responses of *hit4-1* to other forms of heat stress, this study compared the thermotolerance of *hit4-1* with those of *hit1-1*, *hit2*, and *hsp101* mutants. These mutants were chosen because they have been shown to be defective specifically in terms of tolerance to sustained heat stress (*hit1-1* and *hit2*), sudden heat shock (*hit2* and *hsp101*), and acquired thermotolerance (*hsp101*) (Wu *et al.*, 2010; Wang *et al.*, 2011; Hu *et al.*, 2012). The survivability assay used in the genetic analysis indicated that *hit4-1* was hypersensitive to sustained heat stress. For the sudden heat shock test, 7-day-old seedlings were exposed directly to 44 °C for 30 min and then returned to room temperature for recovery. After recovery for 10 days, whereas leaves of the wild type and *hit1-1* remained green and showed visible expansion, the leaves of *hit2*, *hsp101*, and *hit4-1* seedlings were bleached (Fig. 1B). For the acquired thermotolerance tests, seedlings were pre-treated at 37°C for 60 min before being subjected to 44°C for at least 90 min, and *hit4-1* was found to be able to acquire tolerance to severe heat shock to a similar extent to the wild type (Fig. 1C and D). Therefore, the protective role of *HIT4* in the heat tolerance of plants was shown to be more specific to basal than to acquired thermotolerance.

The hit4-1 *mutation does not affect HSP gene expression*

Given that *hit4-1* is defective in terms of only basal and not acquired thermotolerance, and that acquired thermotolerance is correlated strongly with the production of HSPs (Sung *et al.*, 2003), this study then monitored the transcripts of major species of *HSPs* in heat-exposed *hit4-1* plants and found that *HSP101*, *HSP70*, and *HSP17.6A* could all be induced to levels equivalent to or even in excess of those in the wild type (Fig. 2). These results are consistent with those for the *hit4-1* phenotype. A noteworthy observation is that the expression of *HIT4* was itself heat inducible (Fig. 2).

Mutation of the At5g10010 gene accounts for the hit4-1 *phenotype*

To identify the genetic determinant that underlies the *hit4-1* heat-intolerant phenotype, the locus of the *hit4-1* mutation

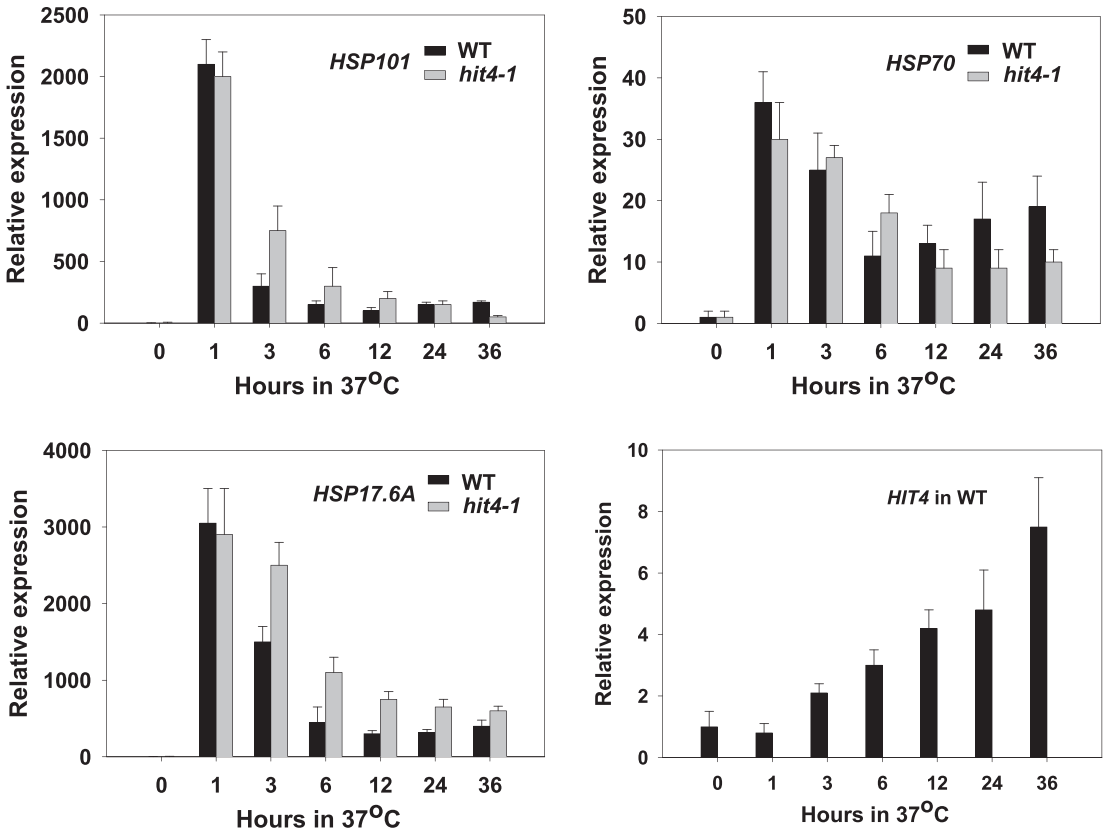

Fig. 2. Induction of *HSP* expression in *hit4-1* after heat exposure was as efficient as that in the wild type, and expression of *HIT4* itself was heat inducible. qRT-PCR analysis was performed and relative expression levels were calculated and normalized with respect to *UBC28* transcripts. Values are mean ± SD of three biological replicates (i.e. three independently isolated RNA samples at each time point, $n = 3$).

was mapped by positional cloning. Initially, coarse mapping of ~100 *hit4-1* mutants derived from an F_2 segregating population of a cross between *hit4-1* (Col-5) and the wild type (L*er*-0) placed the mutation on the upper arm of chromosome V approximately 2.6 cM from the microsatellite marker *nga151* (Fig. 3A). Subsequent fine mapping of ~2000 F_2 individuals characterized the location of the *hit4-1* locus further to a 38.2-kb region defined by the molecular markers Cer441572 and Cer477143, which are located on two overlapping BAC clones, MYH9 and T31P16, respectively (Fig. 3B). This region comprises at least eight candidate genes with AGI annotation numbers from At5g09950 to At5g10020 (Fig. 3C). The entire 38.2-kb region from *hit4-1* was sequenced by PCR and the sequence compared to that from wild-type plants. The results revealed a base transition from C to A in the sixth exon of At5g10010, which led to an amino acid substitution of serine227 to tyrosine (Fig. 3D). This result indicated that *hit4-1* mutation is likely to yield a functionally altered protein product. To confirm the identity of *HIT4*, a 6.2-kb genomic DNA fragment of At5g10010, including the promoter and the 5' and 3' untranslated regions (UTRs), was cloned into the Ti binary vector pCAMBIA1301. This construct was introduced into agrobacteria and then transformed into the *hit4-1* mutant. Five independent hygromycin-resistant lines were obtained and all transformants had regained the ability to tolerate both sustained high temperature and sudden heat shock stress (Fig. 3E), which demonstrated that a defect in the At5G10010 gene causes the heat-intolerant phenotype in *hit4-1*.

HIT4 *is required for embryo development*

To learn more about the possible role of *HIT4,* we searched the T-DNA insertion collections from the ABRC and found two possible *hit4* alleles, *hit4-2* (SALK_047656) and *hit4-3* (SALK_081564C), in which T-DNA was inserted into the 5' and 3' UTRs, respectively (Fig. 3D). For *hit4-2*, the attempt to isolate homozygous mutant plants was unsuccessful. Genotyping of the progeny from a heterozygous parental plant showed a 2:1 ratio of heterozygous to homozygous progeny with no insert (T/–, – /–). Meanwhile, genotyping of F_1 progeny from reciprocal crosses, in which either the wild-type plant was used as a pollen donor to pollinate mutant pistils (T/–) or the heterozygous plant was used as a pollen donor to pollinate wild-type pistils, showed a nearly 1:1 ratio of heterozygous to no-insert progeny (Supplementary Table S2). In contrast, homozygous mutant plants of *hit4-3* could be isolated, but showed a heat-tolerant phenotype. RT-PCR analysis showed that the level of *HIT4* mRNA was the same in *hit4-3* as in wild-type plants (Fig. 3F). These results, together with the finding that *HIT4* is expressed predominately at the early stages during embryogenesis

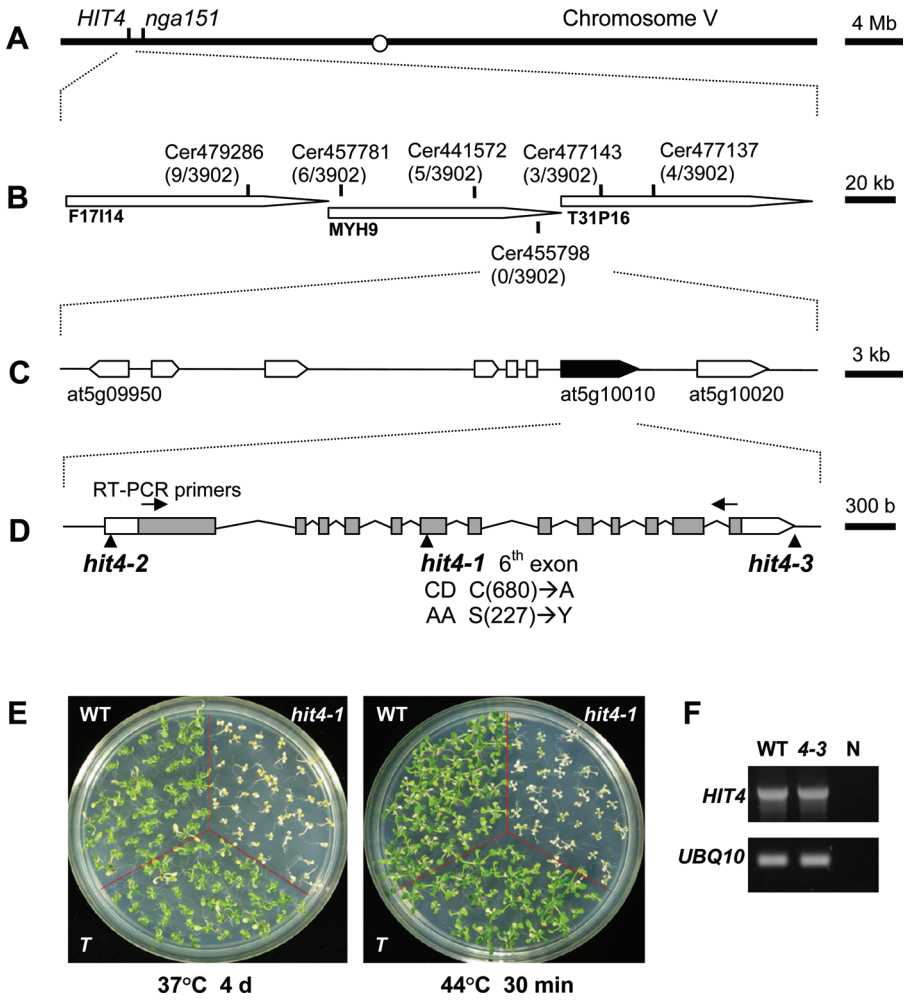

Fig. 3. Map-based cloning of *HIT4*. (A) *Arabidopsis* chromosome 5 with the location of the SSLP marker *nga151*, to which the *HIT4* locus is adjacent. (B) An expansion of the region that encompasses the *HIT4* locus within three overlapping bacterial artificial chromosomes: relative positions of the molecular markers are indicated; values in parentheses are numbers of recombinants / total number of chromosomes tested. (C) The ~40kb interval delimited by the molecular markers Cer441572 and Cer477143 comprises genes with AGI annotation numbers from At5g09950 to At5g10020; the gene shown in black is At5g10010, in which a single base substitution was identified in *hit4-1* mutant plants. (D) Exon/intron structure of *HIT4*: coding regions and 5' and 3' untranslated regions are shown as shaded and open boxes, respectively; the *hit4-1* mutation was a C>A mutation within the sixth exon, which changed the codon for Ser227 to Tyr; two T-DNA insertion mutations within At5g10010, *hit4-2* and *hit4-3*, are also indicated. (E) Restoration of the phenotype of *hit4-1* to the wild type by transformation with a *HIT4* genomic DNA clone; seedlings were transferred either to 37°C for 4 days or to 44°C for 30 min and were allowed to recover at 23°C for 5 or 10 days, respectively, before photography; WT, untransformed wild type; *T*, *hit4-1* transformed with *HIT4* gDNA. (F) RT-PCR analysis showing that the T-DNA insertion within the 3' untranslated region in *hit4-3* had no effect on *HIT4* expression; the locations and orientations of RT-PCR primers are indicated in panel (D); WT, wild type; *4-3, hit4-3*; N, negative control lacking reverse transcriptase.

(Supplementary Fig. S1), indicate that the T-DNA insertion in *hit4-2* causes embryonic lethality, that the *hit4-1* mutation is a partially functional allele, and that the insertion in *hit4-3* does not affect the normal expression and function of *HIT4*.

HIT4 *encodes a highly conserved plant-specific protein*

The predicted HIT4 protein comprises 434 amino acids with a molecular mass of 50.5 kDa. According to the ABRC, HIT4 is an uncharacterized protein for which the cellular function is unknown. BLASTp analysis of HIT4 against a non-redundant protein sequence database showed that the HIT4 protein exhibits a high degree of sequence conservation among land plants (Supplementary Fig. S2), with between 39.7% (*Selaginella moellendorffii*) and 75.7% (*Brassica rapa*) identity and between 52.3% (*Physcomitrella patens*) and 85.7% (*B. rapa*) similarity, but is not found in organisms in other kingdoms (Supplementary Table S3). An unrooted neighbour-joining tree computed from the multiple protein sequence alignment showed that *HIT4* orthologues can be grouped into at least two clusters (Fig. 4). Clade I contains exclusively monocotyledonous plants, whereas clade

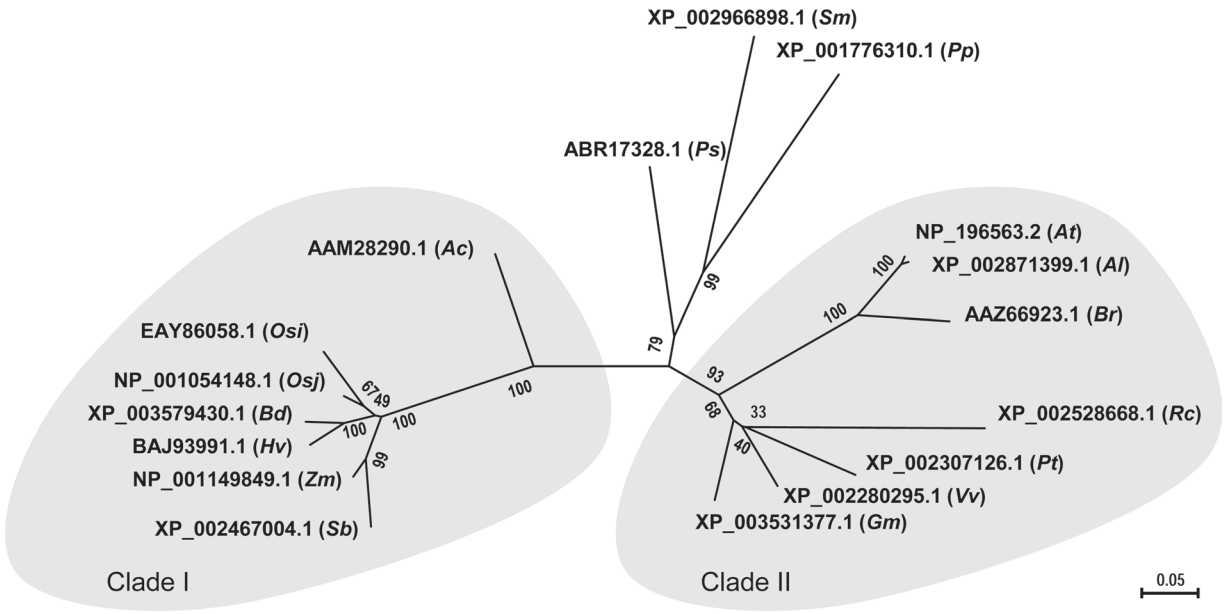

Fig. 4. Evolutionary relationships of HIT4 homologues. The unrooted tree is based on the amino acid sequence alignment of 17 homologues from different plant species or varieties that were identified by a BLAST search using *Arabidopsis* HIT4 as the query sequence. The tree was generated using the boot-strapped neighbour-joining algorithm in MEGA 5.05 with 1000 trials (Tamura *et al*., 2011). Bootstrap values are indicated as percentages of 1000 trials at nodes. The distance scale is shown on the bottom right in the units of the number of amino acid substitutions per site. Two major clades, composed of sequences from monocots (clade I) and dicots (clade II), are encircled. Included in this analysis are sequences from *Arabidopsis thaliana* (*At*), *A. lyrate* (*Al*), *Brassica rapa* (*Br*), *Glycine max* (*Gm*), *Populus trichocarpa* (*Pt*), *Vitis vinifera* (*Vv*), *Ananas comosus* (*Ac*), *Brachypodium distachyon* (*Bd*), *Sorghum bicolor* (*Sb*), *Hordeum vulgare* (*Hv*), *Zea mays* (*Zm*), *Oryza sativa* ssp. *japonica* (*Osj*), *O. sativa* ssp. *indica* (*Osi*), *Ricinus communis* (*Rc*), *Picea sitchensis* (*Ps*), *Physcomitrella patens* (*Pp*), and *Selaginella moellendorffii* (*Sm*).

II contains exclusively dicotyledonous ones. On the other hand, the small number of homologues in certain taxa, only one in each of gymnosperms (*Picea sitchensis*), lycophytes (*S. moellendorffii*), and bryophytes (*Physcomitrella patens*), means that these taxa cannot be grouped phylogenetically, but perhaps represents the early evolution of *HIT4* in Plantae. Together, these results suggest that *HIT4* evolved independently in the plant lineage and might contribute to plant function on land.

HIT4 has a subnuclear localization to chromocentres

To elucidate the function of HIT4, the protein sequence was analysed for known motifs and domains using Search Pfam (http://pfam.sanger.ac.uk/search) and ScanProsite (http://prosite.expasy.org/scanprosite). However, no significant hits could be identified. Nevertheless, previous proteomic analysis detected the presence of HIT4 in the nucleus (Pendle *et al*., 2005). To verify that HIT4 is a nuclear protein, a reporter construct was created by in-frame fusion of the C-terminal end of the green fluorescent protein (GFP) to the N-terminal end of HIT4 for transient expression analysis in protoplasts. The results showed that fluorescently tagged HIT4 was indeed localized to the nucleus. More specifically, the GFP-HIT4 appeared to be located in conspicuous nuclear bodies, of which there were up to 10 in number per nucleus (Fig. 5B). Given that the heterochromatic regions of each chromosome in *Arabidopsis* interphase nuclei are known to cluster into compact chromocentres (Fransz *et al*., 2003) and that diploid *Arabidopsis* cells contain 10 chromosomes, the GFP-HIT4 fluorescent signals are likely to represent the locations of chromocentres. To verify this, the nuclei of protoplasts that expressed GFP-HIT4 were isolated and stained with DNA-binding dye, DAPI. Clear localization of HIT4 at chromocentres was indicated by the perfect overlay of the fluorescent signals of GFP and DAPI (Fig. 5C).

The hit4-1 *mutation restricts heat-induced chromocentre decondensation*

The effect of heat stress on the organization of chromatin in *Arabidopsis* has been studied only recently and it was found that prolonged heat stress can result in substantial decondensation of chromocentres (Pecinka *et al*., 2010). Consequently, the findings that *hit4-1* was a heat-intolerant mutant and that HIT4 localized to chromocentres prompted this study to investigate the potential role of HIT4 in the heat-induced changes in the compaction of heterochromatin. To this end, first, DAPI-stained nuclei of root cells of 10-day-old seedlings were examined in the zones of elongation and maturation. Before heat stress, compact chromocentres were observed in more than 70% of nuclei from both the wild type and *hit4-1* (Fig. 6A and B). After heat exposure for 36 h at 37°C, the compact chromocentres from the wild type were

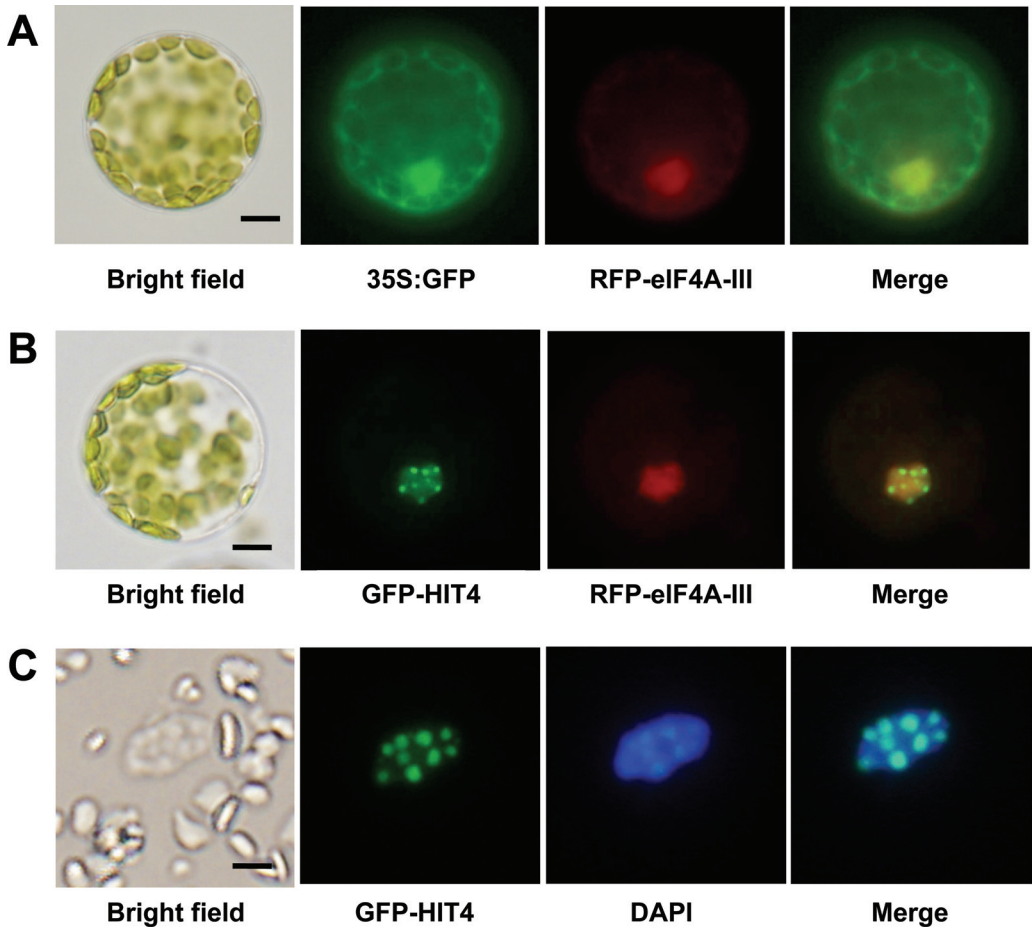

Fig. 5. HIT4 was localized in a subnuclear manner to chromocentres. (A and B) Protoplasts from *Arabidopsis* leaf tissue were co-transformed with expression vectors for 35S:GFP as control (A) or GFP-HIT4 and the nuclear marker RFP-eIF4A-III (B) (Koroleva *et al*., 2009). At 16 h after transformation by polyethylene glycol-mediated transfection, each fluorescent signal was observed with a fluorescent microscope. Bar, 10 μm. (C) Nuclei of GFP-HIT4-expressing protoplasts were isolated and counterstained with DAPI to indicate the locations of chromocentres. Bar, 5 μm.

dispersed significantly, as judged by diffuse nucleoplasmic labelling of DAPI. In contrast, approximately 70% of nuclei from heat-treated *hit4-1* cells maintained conspicuous DAPI-positive nuclear domains (Fig. 6A and B). Since the expression of *HIT4* is heat inducible (Fig. 2), the difference in the compaction degree of chromocentres between WT and *hit4-1* in response to heat stress occurring at 24 h reflects a potential dosage-dependent regulatory effect of HIT4. Subsequently, similar results were observed when nuclei were extracted from entire plants and examined by FISH using oligonucleotide probes that targeted the centromeric 180-bp satellite repeat, the major constituent of each chromocentre (Fig. 6C). These results suggested that the attenuation of heat-induced chromocentre decondensation in *hit4-1* occurred systemically throughout the plant. Given that *hit4-1* was also intolerant to sudden heat shock, the phenotypes of nuclei in root cells after heat exposure for 30 min at 44°C were also examined. Again, whereas ~70% of nuclei from the wild type showed a diffuse DAPI signal, ~70% of nuclei from heat-shocked *hit4-1* maintained compact chromocentres (Fig. 6A). Interestingly, chromocentre decondensation could be induced in *hit4-1* to a level similar to that in the wild type if the plants were acclimated at 37°C for 60 min before the heat shock treatment (Fig. 6A). These observations, together with the finding that *hit4-1* retained the ability to acquire thermotolerance, suggested that the reorganization of chromocentres is a critical response for heat tolerance in plants.

The hit4-1 *mutation attenuates the release of transcriptional gene silencing by heat exposure*

To elucidate the association between HIT4-mediated decondensation of heterochromatin and heat-mediated reactivation of TGS, the transcript levels of TGS markers for which silencing is known to be alleviated by heat were compared between the wild type and *hit4-1* by qRT-PCR. Initially, clear inhibition of the reactivation of the centromeric 180-bp repeat was observed in *hit4-1* after exposure for 36 h to 37°C (Fig. 7A). The strongest disassociation of the chromocentre in wild-type nuclei was observed at this time point (Fig. 6B). To determine whether the *hit4-1* mutation also affects the transcriptional release of silenced, multicopy sequences outside

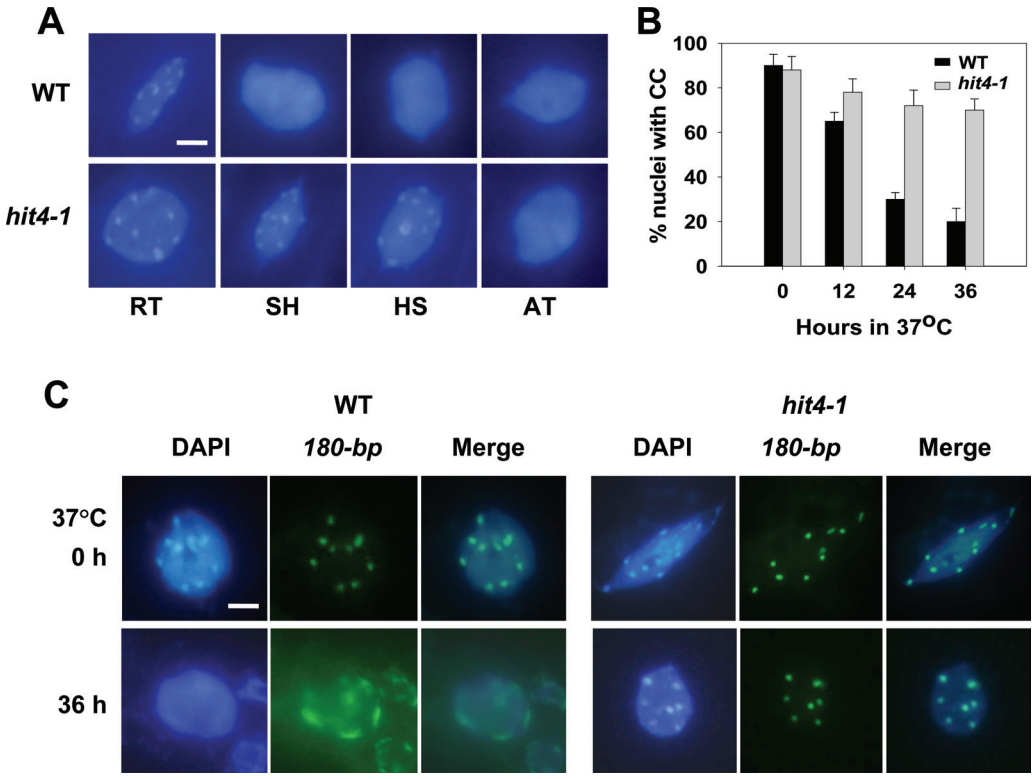

Fig. 6. Chromocentre decondensation induced by sustained heat stress or sudden heat shock was attenuated in *hit4-1*. (A) Phenotype of representative DAPI-stained root interphase nuclei in WT and *hit4-1* before and after various heat treatments; chromocentre decondensation was assessed by diffuse labelling of the nucleoplasm with DAPI; AT, acquisition of thermotolerance at 37°C for 60 min, recovery at 23°C for 2 h, and then heat shock at 44°C for 120 min; HS, sudden heat shock at 44°C for 30 min; RT, room temperature control; SH, sustained heat stress at 37°C for 36 h. Bar, 5 μm. (B) Quantification of nuclei with condensed chromocentres in the WT and *hit4-1* before and after heat stress at 37°C for the time indicated; the total number of nuclei and the number of nuclei with condensed chromocentres were counted from the zone of elongation toward the zone of maturation, and are expressed as the percentage of nuclei with condensed chromocentres (CC); 100 nuclei were counted from each seedling root; values are mean ± SD of five seedlings from each treatment. (C) Chromocentre decondensation was analysed by FISH with a centromeric 180-bp probe in nuclei extracted from WT and *hit4-1* plants before and after heat stress at 37°C for 36 h.

the centromeric core, this study next determined the levels of RNA from *ONSEN*, a *copia*-like retrotransposon family (Ito *et al.*, 2011; Matsunaga *et al.*, 2012) and *TSI* (transcriptionally silent information), an endogenous repeat that is present in pericentromeric regions (Steimer *et al.*, 2000). The results again showed clear repression of reactivation in *hit4-1* for both sets of repeats after exposure for 36 h to 37°C. The same also applied to a single-copy TGS locus, *MULE-F19G14* (Fig. 7A), which resides outside the constitutive heterochromatic region (Habu *et al.*, 2006). Again, the differences of gene expression levels between WT and *hit4-1* in response to heat stress at 37°C occurring after 24 h reflects the potential dosage-dependent regulatory function of HIT4.

The effect of the *hit4-1* mutation on a transcriptionally silenced transgene was then tested. This was achieved by crossing *hit4-1* with transgenic plants that harboured locus L5, which consists of a multicopy silenced marker gene that encodes β-glucuronidase (GUS) that can be reactivated by heat stress (Morel *et al.*, 2000; Elmayan *et al.*, 2005; Tittel-Elmer *et al.*, 2010); offspring that contained the L5 locus in the *hit4-1* background were isolated for analysis. The results indicated that reactivation of the silenced GUS transgene was also suppressed in *hit4-1*, as revealed by qRT-PCR for GUS transcripts and histochemical staining for GUS activity (Fig. 7B and C). Collectively, these results suggest that HIT4-mediated dissociation of heterochromatin is necessary, although it might not be sufficient, for the heat stress-induced reactivation of TGS loci, regardless of whether these loci are endogenous or exogenous or exist as a single copy or repeats or where they are located in the genome.

Discussion

In interphase *Arabidopsis* nuclei, heterochromatin is usually confined to chromocentres, which harbour tandemly repeated DNA and dispersed transposons. However, chromocentre decondensation occurs at certain developmental stages, for example, during flower transition, leaf aging, and protoplast dedifferentiation (Tessadori *et al.*, 2004, 2007a, b). This suggests that, despite the fact that chromocentres are gene-poor regions of the genome, their organization influences or reflects the capacity for reprogramming of genetic information. More recently, studies have shown that prolonged exposure

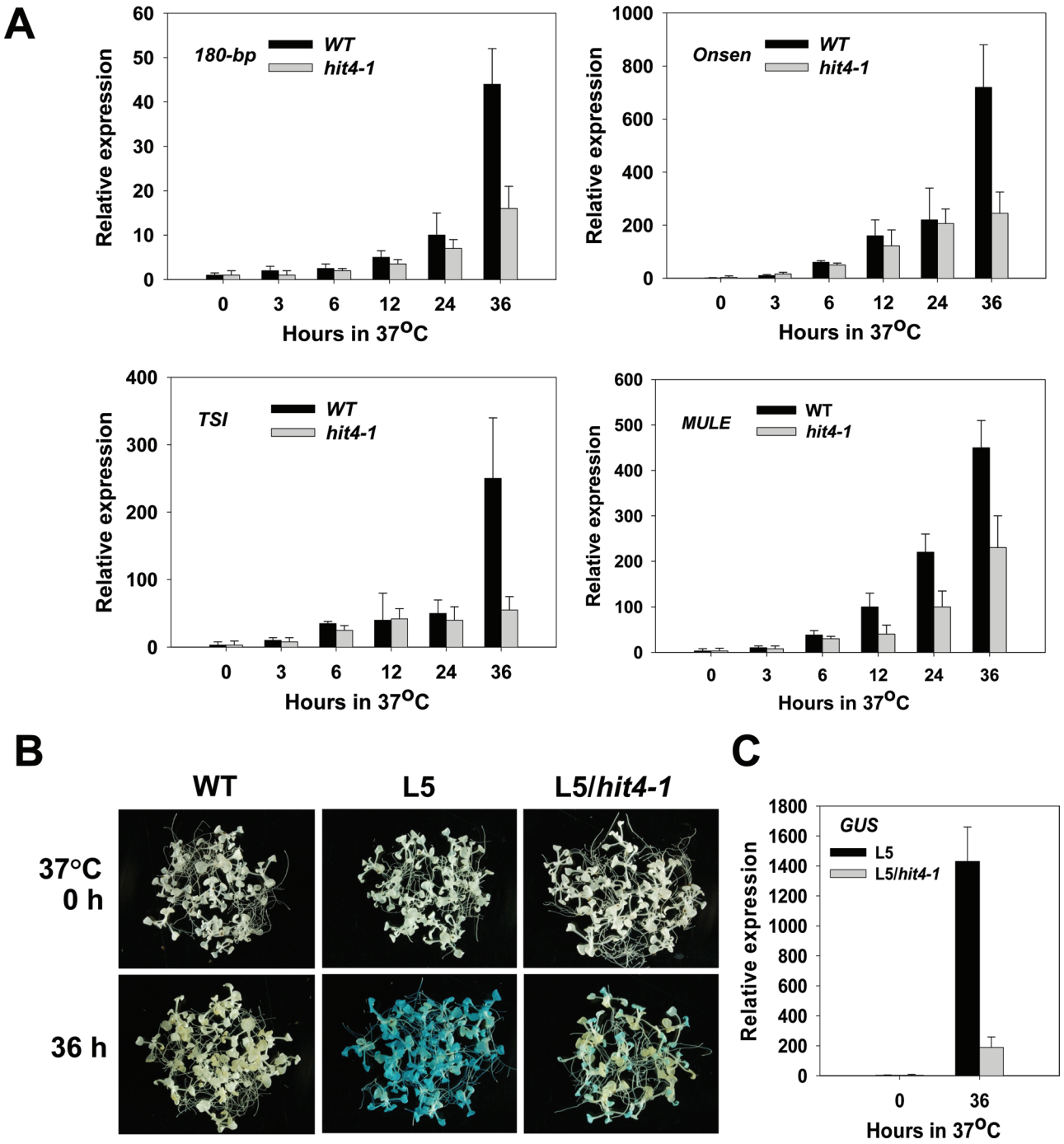

Fig. 7. Alleviation of TGS by prolonged heat stress was attenuated in *hit4-1*. (A) Kinetics of expression of the 180-bp, *ONSEN*, *TSI*, and *MULE* sequences measured by qRT-PCR after exposure at 37°C. (B) Representative images of histochemical staining for GUS activity performed on seedlings of wild type (WT), L5, and L5 in a *hit4-1* background (L5/*hit4-1*) before and after heat exposure at 37°C for 36 h. (C) qRT-PCR detection of GUS transcripts within total RNA from the seedlings as indicated in (B). Values are mean ± SD of four independent biological replicates.

to heat induces substantial decondensation of chromocentres and reactivates genes within them that are normally silent (Pecinka *et al.*, 2010; Tittel-Elmer *et al.*, 2010). These findings indicate that chromatin reorganization is not restricted to developmental needs, but is also a response to environmental stress. Nevertheless, the genetic determinants that govern heat-triggered chromatin reorganization and the extent to which the process of chromatin reorganization affects TGS destabilization and plant survival under heat stress conditions were completely unknown. Consequently, the isolation and characterization of the *hit4-1* mutant, in which the mutation restricted heat-induced decompaction of chromocentres, attenuated the release of TGS, and caused a heat-intolerant phenotype, sheds new and important light on this issue.

One of the characteristics associated frequently with chromatin regions that are subject to TGS regulation is the methylation of cytosine residues. Consequently, chromocentres, which have numerous TGS loci, are enriched with hypermethylated DNA. *Arabidopsis* that carry mutations that interfere with the maintenance of TGS or methylation of DNA have been used to study the relationship between these two processes. For example, mutations in the SWI/SNF2 ATP-dependent chromatin remodeller gene *DDM1*, which were isolated originally as DNA hypomethylation mutants (Vongs *et al*., 1993), result in the loss of DNA methylation, the release of TGS, and the dispersion of chromocentres (Jeddeloh *et al*., 1999; Soppe *et al*., 2002; Probst *et al*., 2003; Lippman *et al*., 2004). Similar results were also observed in the *met1* mutant, which is defective in a DNA methyltransferase (Soppe *et al*., 2002; Kankel *et al*., 2003). In contrast, the *mom1* mutant was isolated on the basis of the reactivation of silent loci (Amedeo *et al*., 2000); however, the reactivation occurred without changes in chromocentre organization and the levels and distributions of DNA methylation (Amedeo *et al*., 2000; Probst *et al*., 2003). These results led to the idea that at least two regulatory levels of TGS, methylation-dependent and -independent, exist in *Arabidopsis*. This was confirmed later by the additive effect seen in *ddm1*/*mom1* double mutants and by other cytogenetic analyses (Mittelsten Scheid *et al*., 2002; Probst *et al*., 2003). As regards the heat-triggered destabilization of TGS, it has been shown to be methylation independent (Pecinka *et al*., 2010; Tittel-Elmer *et al*., 2010). This suggests that HIT4 might be a part of the MOM1 regulatory pathway. Nevertheless, *mom1* and *hit4-1* involve phenotypes with opposite effects: whereas *mom1* promotes the release of TGS, *hit4-1* inhibits this release. Moreover, chromocentres in *mom1* nuclei did decondense in response to heat exposure (Supplementary Fig. S3), which implies that HIT4 defines a new class of TGS regulator that functions at a level associated with chromocentre organization.

In addition to HIT4, many identified regulators that are involved in the remodeling and epigenetic modification of chromatin are present solely within the plant lineage. These include the aforementioned MOM1, the RNA polymerases PolIV and PolV, which mediate RNA-directed DNA methylation, and the HD2 family of histone deacetylases (HDTs). MOM1 emerged at the origin of vascular plants (Čaikovski *et al*., 2008). PolIV exists in algae and land plants, but PolV is specific to angiosperms (Luo and Hall, 2007). As for HDTs, sequences from monocots and dicots can be separated in two distinct clades in phylogenetic analysis (Pandey *et al*., 2002). This resembles the situation for HIT4 homologues and implies that both HDT and HIT4 originated before the divergence of the monocot and dicot lineages. Considering that terrestrial environments show much greater fluctuations in abiotic factors than aquatic environments and that plants are sessile organisms that cannot escape from their habitats, it is plausible to assume that these regulators have evolved to increase the flexibility of execution of the information stored in the genome in response to environmental cues and to optimize the survival of land-based plants under stress conditions.

The HIT4 homologues are highly conserved; however, not all species that contain *HIT4* display chromocentres. For example, there are no reports of chromocentres in cereals (Fransz, 2009). Thus, it has been suggested that the appearance of chromocentres reflects the organization of heterochromatic and euchromatic domains along the linear chromosome (de Jong *et al*., 1999; Fransz and de Jong, 2011). From this perspective and considering the fact that HIT4 affects the silenced GUS transgene at the L5 locus (Fig. 7B and C), the function of HIT4 is not to promote chromocentre decondensation *per se*, but rather the resolution of heterochromatin or the reactivation of silenced genes that reside within heterochromatin. However, the mechanism by which HIT4-mediated destabilization of heterochromatic TGS loci enhances heat tolerance in plants remains obscure. One possible explanation lies in observations of the *ONSEN* family of long terminal repeat retrotransposons. Unlike other heat-induced TGS repeats, which can be activated by mutation of *MOM1* or *DDM1*, the transcription of *ONSEN* is triggered exclusively by heat stress (Pecinka *et al*., 2010; Ito *et al*., 2011). Both full-length transcripts and extrachromosomal *ONSEN* DNA were detected in wild-type plants after heat exposure. Meanwhile, a high frequency of retrotransposition could be detected in the progeny of heat-stressed mutants that were deficient in siRNA biogenesis. More importantly, genes in the vicinity of *ONSEN* neo-insertions were found to become more heat-responsive (Ito *et al*., 2011). These observations correlate well with the processes of plant genome evolution in that bursts of retrotransposition help to reshape gene regulatory networks and potentially create novel traits for adaptation to stress (Mirouze and Paszkowski, 2011). Nevertheless, heat-triggered retrotransposition of *ONSEN* did not occur in the vegetative tissues of wild-type plants subjected to heat stress (Ito *et al*., 2011), and the *hit4-1* mutant itself could not endure heat stress, especially heat shock treatment, with its reproductive capacity intact, which suggests that a protective mechanism that responds more rapidly than retrotransposition is involved in the HIT4-mediated response. Further study is clearly necessary to elucidate the novel regulation of chromatin organization with regard to stress tolerance of plants, and the *hit4-1* mutant can serve as an invaluable tool to this end.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Table S1. Primer list.

Supplementary Table S2. Inheritance of the *hit4-2* T-DNA insertion.

Supplementary Table S3. HIT4 protein sequence similarity and identity matrix.

Supplementary Fig. S1. *HIT4* is expressed predominately at the early stages during embryogenesis.

Supplementary Fig. S2. Amino acid sequence alignments of HIT4 orthologues from land plants.

Supplementary Fig. S3. Phenotype of DAPI-stained root interphase nuclei in *mom1*.

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Supplementary data for

Arabidopsis HIT4 encodes a novel chromocenter-localized protein involved in the heat reactivation of transcriptionally silent loci and is essential for heat tolerance in plants

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Table S1. Inheritance of the *hit4-2* T-DNA insertion

	No. of progeny tested[a]	Genotype of progeny[b]			χ^2	*P*
		– / –	– / T	T/T		
Natural self cross						
		25.0%	50.0%	25.0%	*Expected*	
– / T	405	34.8%	65.2%	0%	34.283	≤ 0.001
Outcross						
		50.0%	50.0%	--	*Expected*	
– / – (♀) × – / T (♂)	248	56.0%	44.0%	--	3.629	> 0.05
– / T (♀) × – / – (♂)	289	50.9%	49.1%	--	0.087	> 0.05

[a]Samples are from three or more individual crosses
[b]–, wild-type allele; T, T-DNA insertion allele

Table S2. HIT4 protein sequence similarity and identity matrix. The percentage of similarity (in light blue boxes) and identity (in gray boxes) were calculated by using MatGat v2.0 software (Campanella et al., 2003).

Species	Accession	HIT4	*Al*	*Br*	*Gm*	*Pt*	*Vv*	*Ac*	*Bd*	*Sb*	*Hv*	*Zm*	*Osj*	*Osi*	*Rc*	*Ps*	*Pp*	*Sm*
Arabidopsis thaliana (HIT4)	NP_196563.2		94.7	75.7	56.3	54.3	52.6	48.8	46.2	43.5	45.2	43.5	45.9	43.7	42.3	49.3	40.3	39.7
Arabidopsis lyrata (*Al*)	XP_002871399.1	97.5		75.1	56.2	54.8	53.2	49.7	46.2	44.1	45.3	43.6	46.7	43.8	42.6	49.9	40.6	39.8
Brassica rapa (*Br*)	AAZ66923.1	85.7	85.5		58.4	56.9	54.1	51.4	44.7	42.7	43.9	43.6	45.8	42.0	45.4	50.0	41.3	40.9
Glycine max (*Gm*)	XP_003531377.1	66.8	66.1	68.9		78.5	80.9	60.9	54.5	55.3	57.7	56.6	58.6	56.9	60.4	66.9	52.6	52.5
Populus trichocarpa (*Pt*)	XP_002307126.1	67.5	67.7	68.4	88.2		76.8	59.6	52.0	54.3	53.9	54.5	55.6	54.0	59.1	61.4	50.1	51.4
Vitis vinifera (*Vv*)	XP_002280295.1	65.7	65.8	67.5	90.6	86.5		63.9	53.1	54.5	57.9	56.6	57.5	57.9	59.0	66.6	54.9	54.8
Ananas comosus (*Ac*)	AAM28290.1	65.0	64.9	66.7	75.3	75.8	76.1		63.7	62.7	66.2	64.3	68.2	64.9	49.2	62.7	44.9	46.6
Brachypodium distachyon (*Bd*)	XP_003579430.1	62.4	62.1	64.2	72.5	72.5	71.6	78.3		75.0	85.4	77.9	84.3	77.2	43.6	55.9	44.2	44.2
Sorghum bicolor (*Sb*)	XP_002467004.1	60.1	60.3	62.5	72.0	73.2	72.6	79.1	85.4		76.8	87.9	81.4	77.7	43.7	55.5	42.2	43.3
Hordeum vulgare (*Hv*)	BAJ93991.1	61.3	61.0	63.0	76.5	73.5	77.0	79.9	89.6	87.6		81.0	86.7	81.5	45.2	57.4	47.5	47.5
Zea mays (*Zm*)	NP_001149849.1	59.9	60.0	61.6	73.0	72.4	73.9	79.7	87.9	93.5	90.2		85.7	82.8	44.5	55.6	42.9	44.9
Oryza sativa ssp. *japonica* (*Osj*)	NP_001054148.1	62.2	62.1	64.4	74.9	74.4	74.3	80.2	90.2	90.4	93.1	92.8		87.3	45.7	57.6	44.3	46.6
Oryza sativa ssp. *indica* (*Osi*)	EAY86058.1	59.0	58.9	59.7	73.2	71.8	76.0	78.8	86.0	85.9	92.2	88.5	92.2		46.0	54.8	45.1	46.3
Ricinus communis (*Rc*)	XP_002528668.1	55.1	55.9	57.5	69.4	71.2	72.1	62.9	61.5	61.9	64.2	61.8	63.3	63.1		48.8	42.0	43.4
Picea sitchensis (*Ps*)	ABR17328.1	62.4	62.1	63.2	79.0	75.5	80.8	76.9	72.8	74.0	75.5	75.9	76.0	73.2	61.5		53.9	54.9
Physcomitrella patens (*Pp*)	XP_001776310.1	52.3	52.2	53.3	65.6	65.7	68.8	60.4	61.0	59.6	65.7	61.5	62.4	63.4	62.0	66.8		65.9
Selaginella moellendorffii (*Sm*)	XP_002966898.1	53.2	53.3	54.2	66.8	66.6	70.9	63.2	60.7	59.6	65.1	61.5	62.1	62.8	63.7	69.1	82.5	

Table S3. Primer list

Primer name	**Primer sequences (5' to 3')**	**Note**
Nga151-F Nga151-R	GTTTTGGGAAGTTTTGCTGG CAGTCTAAAAGCGAGAGTATGATG	SSLP Col/L*er* 150/122 bp
Cer479286-F Cer479286-R	ATGTGATCATTTGTATTTAG CACACAAAACCGCAACAG	SSLP Col/L*er* 97/89 bp
Cer457781-F Cer457781-R	CGTGTGATTAAGATTGGACT GGTCAATCGTATTCACAACG	SSLP Col/L*er* 111/103 bp
Cer441572-F Cer441572-R	CCTTGCAATATGTGAAGCGATTC CAAAGCAACGATTACTTAAAAGCT	CAPS, *Hind*III, Col/L*er* 94/118 bp
Cer455798-F Cer455798-R	GCGCCAAATGAGCTGTTATTAG GCTAAGTGAGAGACTGAGGTG	SSLP Col/L*er* 127/110 bp
Cer477143-F Cer477143-R	TCGCCAAATGAGCTGTTATTAG AATGTCGCAAAGACTTCC	SSLP Col/L*er* 179/151 bp
Cer477137-F Cer477137-R	AGGCACCAAAGAAACAAG CGTTGTGTTCATTTATGAC	SSLP Col/L*er* 184/214 bp
SALK-ETp-F SALK-ETp-R LBa1.3	GATGAAAATATTAAACATGATG CCGGAGGAGACACAAATTTGG CCGGAGGAGACACAAATTTGG	
SALK_081564 –F SALK_081564 –R LBb1	GGAAGCATTACCTGCTGAACAAG TGTCCGTACACTGTACTACTCC GCGTGGACCGCTTGCTGCAACT	
MOM1-503bp-F MOM1-1197bp-R LB3	GCATACCTGCAGGCAATGATGA ACCCAGATGGAGATGGAATGAC TAGCATCTGAATTTCATAACCAATCTCGATACAC	
UBQ10-F UBQ10-R	AACTTTCTCTCAATTCTCTCTACC CTTCTTAAGCATAACAGAGACGAG	
*HIT4-Bam*HI-F *HIT4-Pst*I-R *HIT4*gDNA-F *HIT4*gDNA-R	CG*GGATCC*ATGAAGAAAGGAGCGAAGAG AA*CTGCAG*AAGGACTTCATGAGCCTTTC AA*GCTAGC*GGTACCGTTACACATGCCTGCCTTGAA GCGAGCTCGGTACCATGGTATGACATTTTTTTCTA	Restriction sites are underlined
GFP-*Xba*I-F GFP-*Bam*HI-R	GC*TCTAGA*ATGAGTAAAGGAGAAGAACT GC*GGATCC*TTTGTATAGTTCATCCATGCCA	Restriction sites are underlined
180bp (all)-F 180bp (all)-R	ACCATCAAAGCCTTGAGAAGCA CCGTATGAGTCTTTGTCTTTGTATCTTCT	
Onsen-qF Onsen-qR	CGGTGCTCACAAAGAGCAACTATG ATCCTTGATAGATTAGACAGAGAGCT	

TSI-qF TSI-qR	CTCTACCCTTTGCATTCATGAATCCTT GATGGGCAAAAGCCCTCGGTTTTAAAATG	
MULE2-F MULE2-R	TACAAGCTTCCAGAAGAGGAAATCTAT TGCAGGCTCCTCGTCTATGATATCTTC	
qPCR-GUS-F qPCR-GUS-RqP	TTAACTATGCCGGAATCCATCGC CACCACCTGCCAGTCAACAGACGC	
HSP101-F HSP101-R	TGAGCTAGCTGTGAATGCAGGACATGCTC ATCACTCTTTCAGCAGATTGAGCTGCGTT	
HSP70-qF HSP70-qR	TCAAGCGGATAAGAGTCACT CCAGCGTCCTTAGTCGCTTG	
HSP17.6-qF HSP17.6-qR	CGAAGACATGCTTGAAGCCCCTG CGTACGCATCCGGGTGCTCG	
UBC28q-F UBC28q-R	TCCAGAAGGATCCTCCAACTTCCTGCAGT ATGGTTACGAGAAAGACACCGCCTGAATA	
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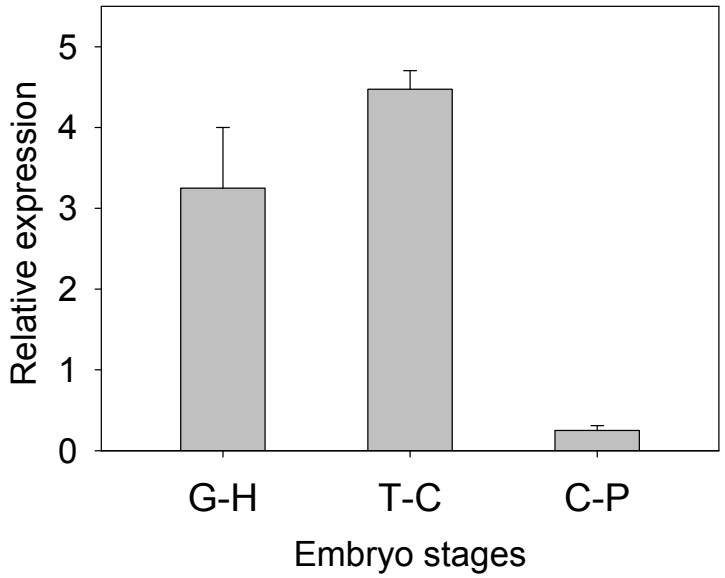
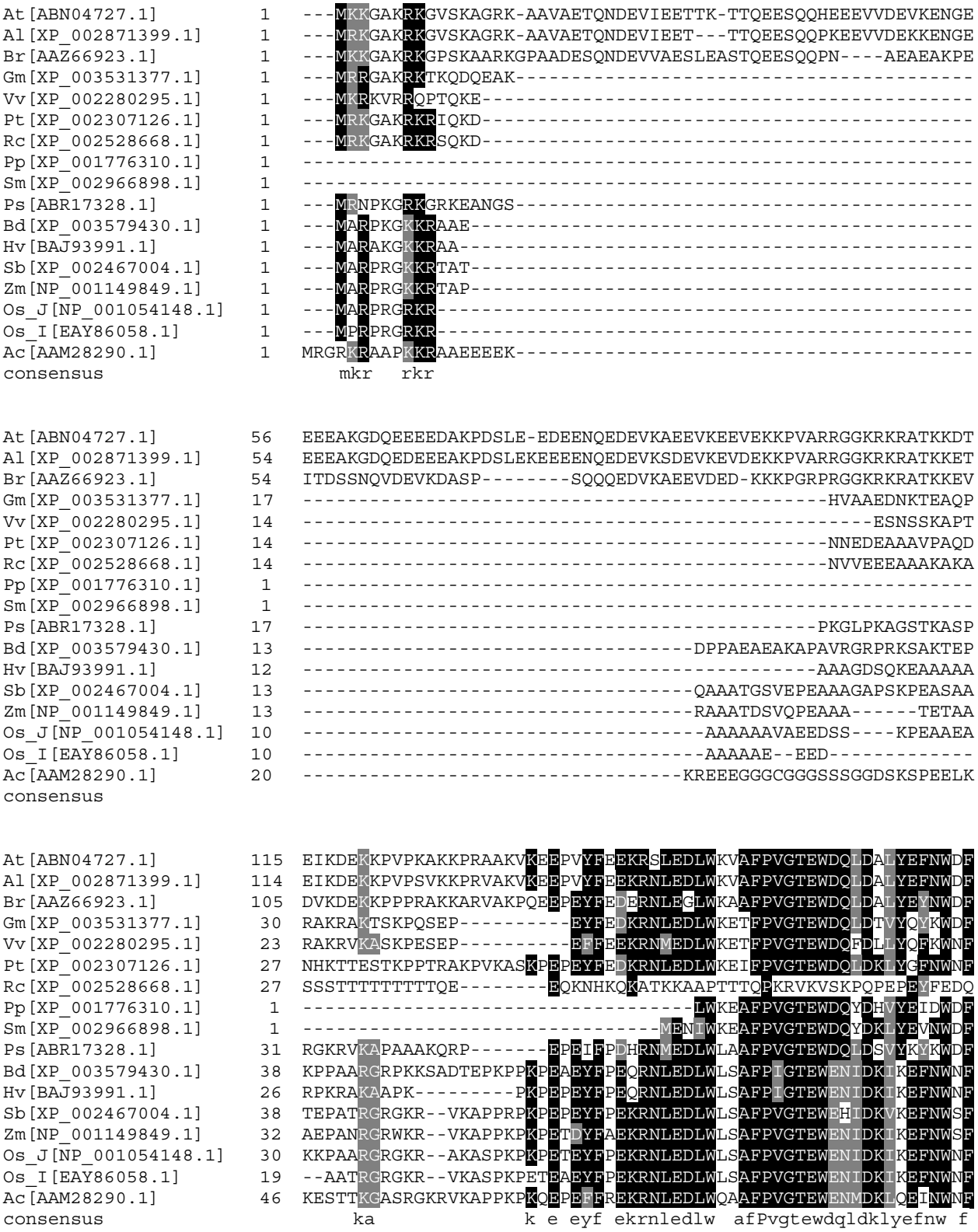


Fig. S1. *HIT4* is expressed predominately at the early stages during embryogenesis. Seeds containing embryos at different stages of development were collected from siliques at various days after pollination (Brandon *et al.,* 2012. PNAS USA 107:8063-8070). qRT-PCR analyses were then performed, and relative expression levels of *HIT4* were calculated and normalized with the respective actin levels. Three biological replicates (i.e., 3 independently isolated RNA samples at each embryo stage) were used. Bars are mean±SD (n=3). G-H, globular to heart transition stages; T-C, torpedo to curled stages; C-P, cotyledons to postmature green stages.

Fig. S2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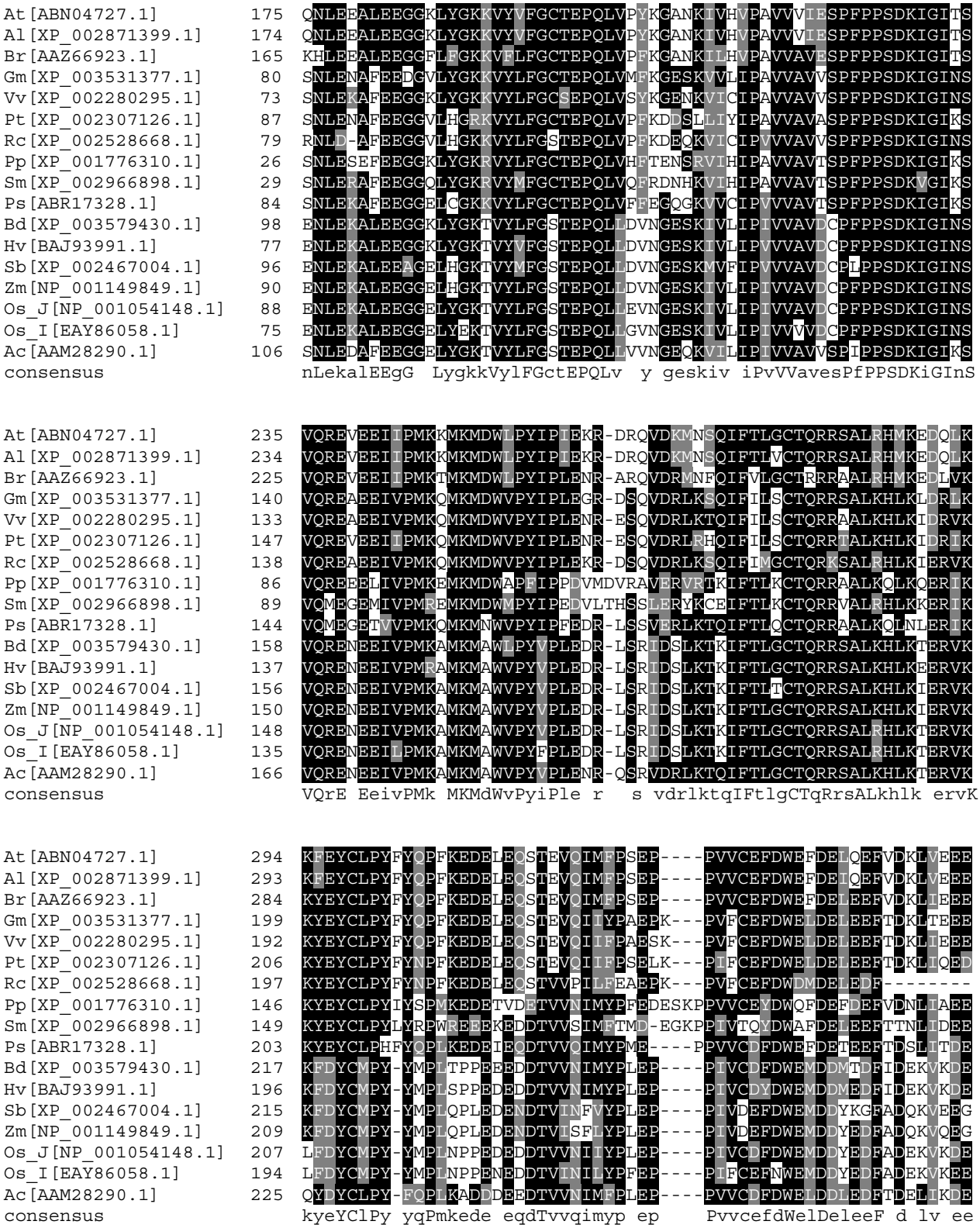
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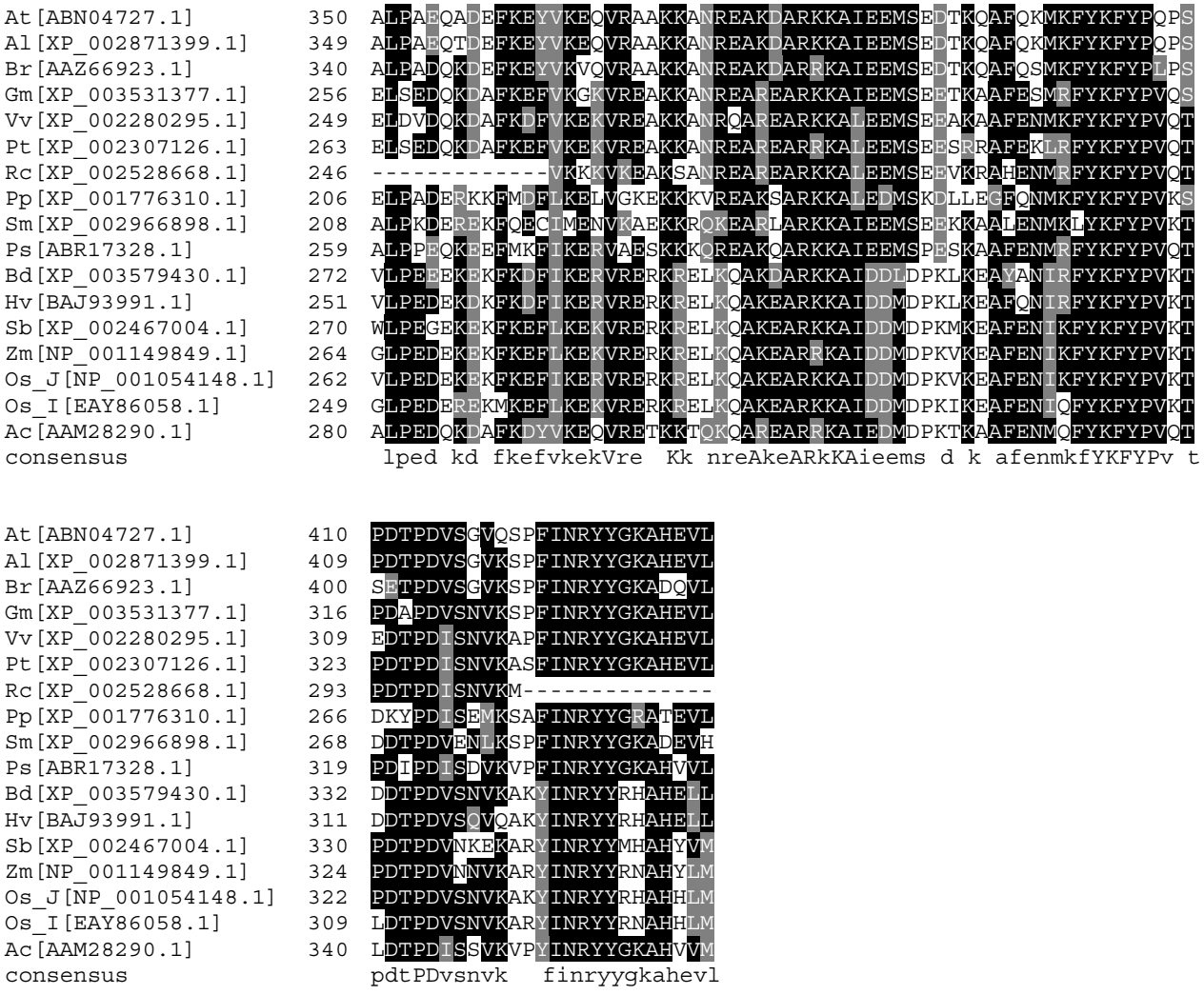

Fig. S2. Amino acid sequence alignments of HIT4 orthologs from land plants. Identical amino acids are shaded in black, and similar amino acids are shaded in gray. Alignment was performed using ClustalW (http://www.ebi.ac.uk/clustalw/). Boxshade was produced by BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html). At, *Arabidopsis thaliana*; Al, *Arabidopsis lyrata*; Br, *Brassica rapa*; Gm, *Glycine max*; Pt, *Populus trichocarpa*; Vv, *Vitis vinifera*; Ac, *Ananas comosus*; Bd, *Brachypodium distachyon*; Sb, *Sorghum bicolor*; Hv, *Hordeum vulgare*; Zm, *Zea mays*; Os_J, *Oryza sativa* ssp. *japonica*; Os_I, *Oryza sativa* ssp. *indica*; Rc, *Ricinus communis*; Ps, *Picea sitchensis*; Pp, *Physcomitrella patens*; Sm, *Selaginella moellendorffii*.

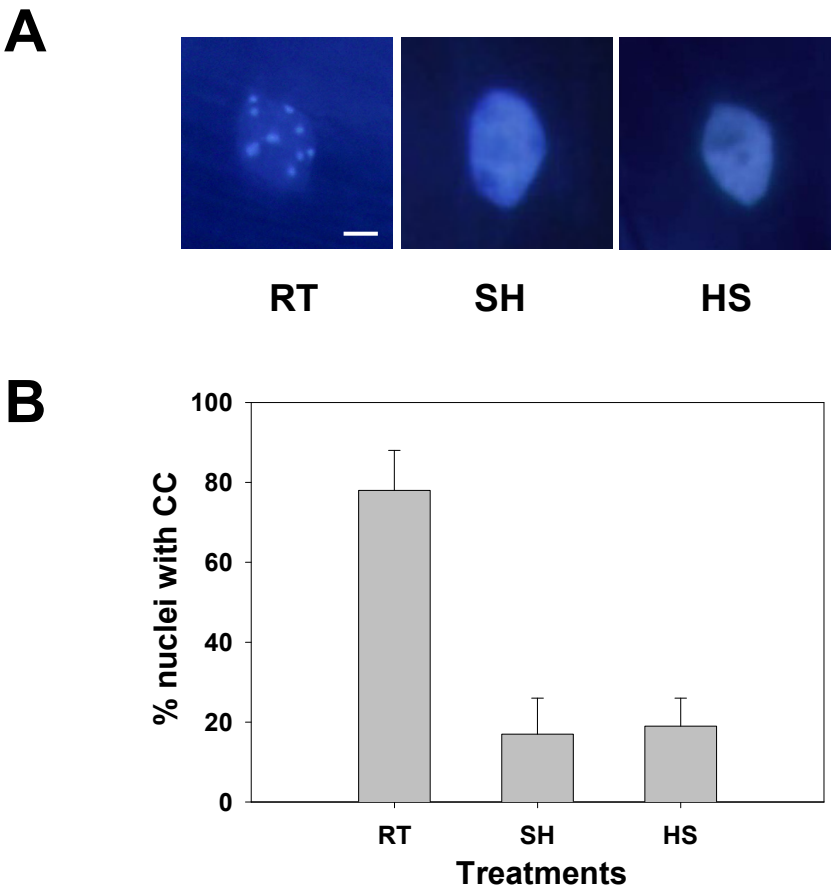

Fig. S3. Phenotype of DAPI-stained root interphase nuclei in *mom1*. (A) Representative DAPI-stained root interphase nuclei in *mom1* before and after various temperature treatments. Chromocenter decondensation was assessed by diffuse nucleoplasmic labeling with DAPI. Treatments were as follows: RT, room temperature control; SH, sustained heat stress at 37°C for 24 h; HS, sudden heat shock at 44°C for 30 min. Bar = 5 μM. (B) Quantification of the observations shown in (A). The total number of nuclei and the number of nuclei with condensed chromocenters were counted from the zone of elongation toward the zone of maturation, and are expressed as the percentage of nuclei with condensed chromocenters (CC). One hundred nuclei were counted from each seedling root, and the error bars represent the SD of five seedlings from each treatment.