

LHT1/MAC7 contributes to proper alternative splicing under long-term heat stress and mediates variation in the heat tolerance of *Arabidopsis*

Kazuho Isono^a, Kotaro Nakamura^a, Kousuke Hanada^b, Kazumasa Shirai^b, Mao Ueki^a, Keisuke Tanaka^c, Takashi Tsuchimatsu^d, Satoshi Iuchi^e, Masatomo Kobayashi^e, Izumi Yotsui^a, Yoichi Sakata^a and Teruaki Taji^{a,*}

^aDepartment of Bioscience, Tokyo University of Agriculture, Tokyo 156-8502, Japan

^bDepartment of Bioscience and Bioinformatics, Kyushu Institute of Technology, Iizuka, Fukuoka 820-8502, Japan

^cNODAI Genome Center, Tokyo University of Agriculture, Tokyo 156-8502, Japan

^dDepartment of Biological Sciences, University of Tokyo, Tokyo 113-0033, Japan

^eRIKEN BioResource Research Center, Tsukuba, Ibaraki 305-0074, Japan

*To whom correspondence should be addressed: Email: t3teruak@nodai.ac.jp

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Abstract

Natural genetic variation has facilitated the identification of genes underlying complex traits such as stress tolerances. We here evaluated the long-term (L-) heat tolerance (37°C for 5 days) of 174 *Arabidopsis thaliana* accessions and short-term (S-) heat tolerance (42°C, 50 min) of 88 accessions and found extensive variation, respectively. Interestingly, L-heat-tolerant accessions are not necessarily S-heat tolerant, suggesting that the tolerance mechanisms are different. To elucidate the mechanisms underlying the variation, we performed a chromosomal mapping using the F₂ progeny of a cross between Ms-0 (a hypersensitive accession) and Col-0 (a tolerant accession) and found a single locus responsible for the difference in L-heat tolerance between them, which we named *Long-term Heat Tolerance 1* (*LHT1*). *LHT1* is identical to *MAC7*, which encodes a putative RNA helicase involved in mRNA splicing as a component of the MOS4 complex. We found one amino acid deletion in *LHT1* of Ms-0 that causes a loss of function. *Arabidopsis* mutants of other core components of the MOS4 complex—*mos4-2*, *cdc5-1*, *mac3a mac3b*, and *prl1 prl2*—also showed hypersensitivity to L-heat stress, suggesting that the MOS4 complex plays an important role in L-heat stress responses. L-heat stress induced mRNA processing-related genes and compromised alternative splicing. Loss of *LHT1* function caused genome-wide detrimental splicing events, which are thought to produce nonfunctional mRNAs that include retained introns under L-heat stress. These findings suggest that maintaining proper alternative splicing under L-heat stress is important in the heat tolerance of *A. thaliana*.

Keywords: selective splicing, natural variation, *Arabidopsis thaliana* accessions, abiotic stress tolerance

Significance Statement

Plants are often exposed not only to short-term (S-) heat stress but also to diurnal long-term (L-) heat stress over several consecutive days. However, it is unclear whether plant responses to both S- and L-heat stresses are common or not. We evaluated L-heat tolerances of 170 *Arabidopsis thaliana* accessions and S-heat tolerances of 88 accessions and found wide variation, respectively. L-heat-tolerant accessions are not necessarily S-heat tolerant, suggesting that the tolerance mechanisms are different. We identified *LHT1* as responsible for the variation in L-heat tolerance between Ms-0 and Col-0. *LHT1*, identical to *MAC7*, encodes a putative RNA helicase involved in mRNA splicing. Loss of function of *LHT1* causes detrimental splicing events, suggesting that *LHT1* contributes to maintaining proper alternative splicing under L-heat stress.

Introduction

Since plants cannot move about, they must adapt quickly to environmental stresses. Plants can be subjected to both short-term (S-) severe heat stress, such as at daytime maximum temperatures, and long-term (L-) heat stress over several consecutive days. Nevertheless, most studies of heat tolerance using *Arabidopsis thaliana* have focused on evaluating S-heat stress, such as 42°C

for 30–60 min. Among plant responses to S-heat stress, the accumulation of heat shock proteins (HSPs) and the detoxification of excessive reactive oxygen species (ROS) are thought to be of particular importance and are highly conserved from bacteria to eukaryotes (1–5). Acquired thermotolerance or heat acclimatization is known to increase plant tolerance to subsequent lethal S-heat stress following a nonlethal high-temperature stress event.

Competing Interest: The authors declare no competing interest.

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Epigenetic regulation to maintain higher HSP expression is considered to be important for this acquired thermotolerance (6–8). There are few reports on the isolation of L-heat stress mutants and the identification of their causal genes (9–13), so the mechanism for L-heat stress tolerance is not fully understood. However, these reports show differences in the mechanisms between S-heat and L-heat tolerances. Defects in *Arabidopsis* Mitochondrial GrpE 2, encoding a nucleotide exchange factor of the HSP70/DnaK complex, result in high sensitivity to L-heat stress, whereas the S-heat tolerance is comparable to that of the wild type (WT) (12). HSP101 plays an important role in S-heat tolerance, but mutation in this gene does not affect L-heat tolerance (12, 14). In contrast, the *Arabidopsis* heat intolerant 2 (*hit2*) mutant has defects in both S- and L-heat tolerances, suggesting the existence of common factors in these tolerance mechanisms (11). Indeed, the *Arabidopsis* *hsf1a/b/d/e* quadruple mutant had significantly impaired S- and L-heat tolerances, suggesting that the HSF1s-mediated heat stress response is also important in the L-heat stress response (15). However, several *Arabidopsis* mutants such as sensitive to long-term heat (*sloh*) and *hit4* have impaired tolerance without reduced expression of HSFs or HSPs, indicating the existence of an HSF-independent pathway (16–18).

Arabidopsis thaliana is distributed widely across the world, and more than 2000 accessions have been collected (19). This broad geographic distribution encompasses substantial variation in nucleotide sequences, and even subtle differences induce large variation in phenotypic traits such as stress tolerance (20–23). Studies of *A. thaliana* accessions have provided new insights into genome evolution, differentiation among geographic populations, and selective mechanisms that shape complex trait variation in nature (21, 24, 25). Quantitative trait loci for high-temperature germination and fertility under heat stress have been reported (26, 27), yet the variation in heat tolerance during postgermination growth and the underlying mechanisms remain underexplored.

Here, we evaluated L- and S-heat tolerances among *A. thaliana* accessions. We found wide variation in both, with different mechanisms. We focused on the difference between the L-heat-tolerant Col-0 and that of the L-heat-sensitive Ms-0 to identify the locus contributing to L-heat tolerance and identified *LHT1* as responsible for the variation in L-heat tolerance between Ms-0 and Col-0.

Results

Natural variation in L-heat tolerance among *A. thaliana* accessions

To investigate the natural variation in L-heat tolerance among *A. thaliana* accessions, we evaluated 170 accessions using the L-heat stress assay (37°C for 5 days) and found nearly a 10-fold variation, represented by the relative chlorophyll content (Fig. 1A, Fig. S1). The reference accession Col-0 had moderate L-heat tolerance. Cvi-0, Ms-0, and Be-1 (as examples) were L-heat sensitive (Fig. 1B). Bs-2, Na-1, Hi-0, and Old-1 were markedly L-heat tolerant (Fig. S2A and B). The L-heat-tolerant accessions were able to survive at 37°C for 7 days, but Col-0 showed complete chlorosis (Fig. S2A). A genome-wide association study (GWAS) using the data of the 170 accessions to identify the genetic loci responsible for the variation found no single-nucleotide polymorphisms (SNPs) that satisfied the genome-wide significance among the 250 k SNP data set (Fig. S3). This result may suggest that L-heat tolerance is a polygenic trait, regulated by multiple loci with relatively small effects, or that the loci contributing to the tolerance differ among accessions. To compare the S- and L-heat tolerances, we evaluated the tolerance of 88 accessions to S-heat stress (42°C for 50 min) and again found ~10-fold variation in heat tolerance (Fig. 1A, Fig. S4). Ty-0 and Vi-0, among others, proved S-heat tolerant, and Col-0 and Na-1, among many others, proved sensitive (Fig. S4). Interestingly, the L-heat-tolerant accessions were not necessarily S-heat tolerant and vice versa (Fig. 1A, Fig. S5). This result suggests that two traits are governed by different mechanisms.

Identification of Long-term Heat Tolerance 1 locus

To identify the locus responsible for the variation in L-heat tolerance, we generated F₁ progeny of Col-0 and Ms-0, a L-heat-sensitive accession with relatively early flowering. Using F₂ progeny, we mapped a single locus on chromosome 2, which we named *Long-term Heat Tolerance 1* (*LHT1*). To narrow down the *LHT1* region, we generated near-isogenic lines (NILs) by five rounds of backcrossing of plants showing the L-heat-sensitive phenotype to Col-0 plants. We screened the BC₅F₂ plants for recombination events within the mapped region containing *LHT1* and found NILs of Col-0 and Ms-0 that respectively showed L-heat-tolerant or L-heat-sensitive phenotypes, though they carried a small

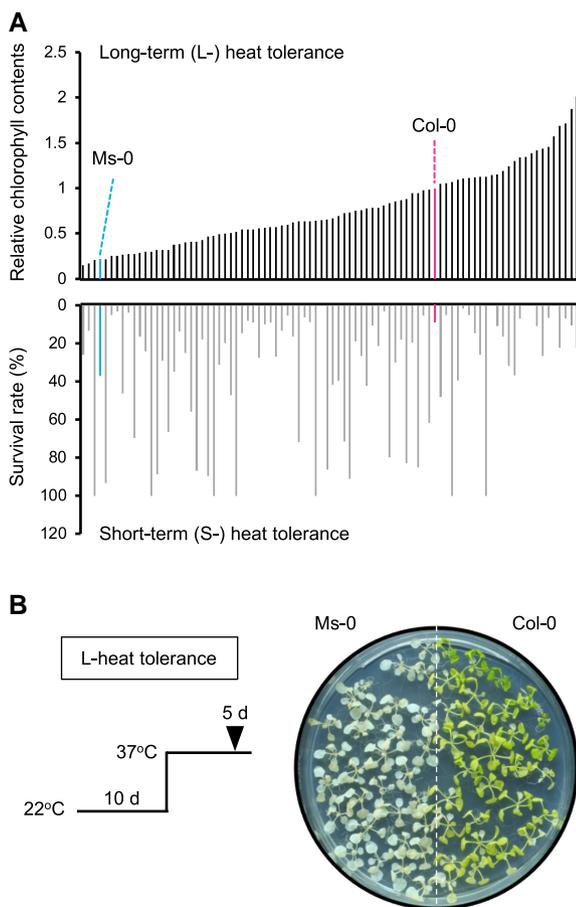


Fig. 1. Relationship of L-heat tolerance to S-heat tolerance. **A)** Comparison of L-heat (37°C for 5 days) and S-heat (42°C for 50 min) tolerances among 88 *A. thaliana* accessions. Upper panel: L-heat tolerance ranked in order. L-heat tolerance was calculated as chlorophyll content divided by that of Col-0 on the same plate. Lower panel: S-heat tolerance (survival rate after 5 days of S-heat stress) of same accessions as in the upper panel. Accession names are shown in Fig. S5. **B)** L-heat tolerance of Ms-0 and Col-0.

chromosomal segment from Ms-0 containing the *LHT1* region in the genetic background of Col-0 (Fig. 2A and B). To narrow down the *LHT1* locus, we determined genotypes within the *LHT1* region in NILs #1, #2 (referred to as NIL_Ms-0), #3, and #4 using simple sequence length polymorphism (SSLP) markers. Correlation of these genotypes with L-heat tolerance successfully narrowed the *LHT1* locus to 33 kbp (Fig. 2A and B). We next sequenced Ms-0 to detect all polymorphisms at the *LHT1* locus between Col-0 and Ms-0. Comparison within the *LHT1* region of Col-0 and Ms-0 revealed that of the 11 genes in the region, 3 were polymorphic with nonsynonymous amino acid substitutions and 1 had 2 nonsynonymous substitutions and a 3 bp (1 amino acid) deletion (Fig. 2B). Since F₁ progeny between Col-0 and Ms-0 showed L-heat tolerance, we considered that a recessive mutation occurred in Ms-0. Therefore, we cloned the region from promoter to 3' untranslated region (UTR) of each of four candidate genes from Col-0 and introduced them into L-heat-sensitive NIL_Ms-0. Only the Col-0 gene with a 3 bp deletion in Ms-0 was able to complement the L-heat sensitivity of NIL_Ms-0, suggesting that this is the *LHT1* gene responsible for L-heat tolerance (Fig. 2C).

Variation in *LHT1* gene among *A. thaliana* accessions

We identified *LHT1* by genetic analysis using Col-0 and Ms-0. There were two nonsynonymous substitutions (Pro74Thr and Phe1395Leu) and one amino acid deletion (Leu1385del) in the sequence of *LHT1* between Col-0 and Ms-0 (Fig. 3). We examined the phylogenetic tree of *A. thaliana* accessions based on 143 SNPs and found that Kas-1, Truk-7, and Rome-1 are closely related to Ms-0. To find accessions with an Ms-0-type *LHT1* gene considered to have a loss of function, we examined L-heat tolerance and genotypes in Kas-1, Truk-7, and Rome-1. Sequencing revealed that Kas-1 has the same polymorphisms in *LHT1* as Ms-0, whereas Truk-7 and Rome-1 had one or two Ms-0-type nonsynonymous substitutions in *LHT1* (Fig. 3). Evaluation of L-heat tolerance revealed that only Kas-1, like Ms-0, is sensitive to stress, but Truk-7 and Rome-1 show L-heat tolerance (Fig. 3). These results strongly suggest that one amino acid deletion found in Ms-0 and Kas-1 is responsible for the function of *LHT1*.

Functional analyses of *LHT1* in L-heat response of *A. thaliana*

LHT1 is identical to MOS4-ASSOCIATED COMPLEX 7 (*MAC7*), which encodes an RNA-binding protein with an RNA helicase domain and an intron-binding domain. One amino acid deletion found in *LHT1* of Ms-0 was not located in either of these domains but was situated near the RNA helicase domain. T-DNA insertion mutants of *LHT1* have an embryonic lethal phenotype, suggesting an important role of *LHT1* in plant developmental stages (28). *LHT1* has been reported as a component of the MOS4 complex, which is widely conserved in eukaryotes and has been suggested to interact with spliceosomes (29–32). While *LHT1* has been suggested to regulate the expression levels of plant disease response genes and to be involved in miRNA biosynthesis and mRNA splicing (33), its involvement in heat or other abiotic stress responses is unknown.

To understand the L-heat stress response of *LHT1*, we examined the expression profiles under L-heat stress. Induction of *LHT1* by L-heat stress suggests that it contributes to the stress response (Fig. 4A). The similar expression pattern in NIL_Ms-0 suggests that the defect in L-heat tolerance in NIL_Ms-0 was not due to reduced expression of *LHT1* itself. The fact that the S-heat tolerance of NIL_Ms-0 was comparable to that of Col-0

(Fig. S6) suggests that Ms-0-type *LHT1* is specifically defective in mediating L-heat stress response. On the other hand, the better heat tolerance of Ms-0 than of Col-0 suggests that it has loci that contribute to S-heat tolerance independent of *LHT1* (Fig. S6).

We further investigated the contribution of other MOS4 complex components to L-heat tolerance. These factors are known to be involved in plant immune responses, but little is known about abiotic stress responses (33–36). We evaluated the L-heat tolerance of MOS4 core component mutants including *MOS4*, *CDC5*, *PRL1/2*, and *MAC3A/B*. We generated double mutants for *MAC3A* and *MAC3B* and for *PRL1* and *PRL2* because they have redundant roles in splicing (29, 37). Among them, *mos4-2*, *prl1 + prl2*, and *mac3a + mac3b* had higher sensitivity to L-heat stress than WT (Fig. 4B and C). These results suggest that the MOS4 complex plays an important role in L-heat stress responses.

Effects of Ms-0-type *LHT1* on transcriptional regulation

We sampled 10-day-old seedlings of Col-0 and NIL_Ms-0 grown under normal conditions (22°C) and under L-heat stress (37°C for 1, 24, and 72 h) and performed RNA sequencing (RNA-seq) to determine the effect of *LHT1* on gene expression. Paired-end sequencing obtained about 60 million reads for each sample. The data were appropriate for analysis because they were clustered within treatment plots and biological repeats by clustering analysis (data not shown).

First, we detected heat-induced genes (\log_2 -fold change >2, false discovery rate [FDR] <0.05) of Col-0 at 1, 24, and 72 h (Fig. 5A and Table S1). The group of genes highly expressed at 1 and 24 h included genes that are well known to be induced by heat, such as HSPs and HSFs. On the other hand, the group of genes highly expressed at 72 h included many genes related to RNA processing (Fig. 5B). These results suggest that RNA processing is important for the L-heat stress response. Among genes differentially expressed between Col-0 and NIL_Ms-0 at each time point, the number of genes was significantly higher at 72 h (Fig. 6A and Table S1). Gene Ontology (GO) using Araport software (<https://www.araport.org>) analysis showed that the up-regulated genes in NIL_Ms-0 at 72 h after heat stress included genes related to defense responses (Fig. 6B and C).

Our findings that *LHT1* encodes an RNA-binding protein and that L-heat stress induces RNA processing-related genes implied that the loss of *LHT1* function affects alternative splicing under L-heat stress. To test this possibility, we used the RNA-seq data to detect alternative splicing. Alternative splicing can be categorized into four types: exon skipping (SE), alternative 5' splice site (A5SS), alternative 3' splice site (A3SS), and retained intron (RI) (38). Alternative splicing events in Col-0 and NIL_Ms-0 were compared at 22 and 37°C. At both temperatures at 1 h, there was little difference in the number of splicing events between Col-0 and NIL_Ms-0. During L-heat stress (37°C for 24 and 72 h), however, the number of splicing events increased in NIL_Ms-0, with SE and RI significantly increased (Fig. 7 and Tables S2, S3, and S4). RI is an alternative splicing in which intronic regions are retained, suggesting that increased RI increases nonfunctional mRNAs in NIL_Ms-0 under L-heat stress. These data indicate that all four types of alternative splicing were induced in Col-0 after prolonged heat stress (24 and 72 h; Fig. 7). qRT-PCR to detect the RI of *HsfB1* and *HSA32* genes in Col-0 and NIL_Ms-0 in order to confirm the validity of the RNA-seq analyses showed that premature RNA levels of both *HsfB1* and *HSA32* in NIL_Ms-0 under L-heat stress were higher than those in WT (Fig. S7). To characterize the genes

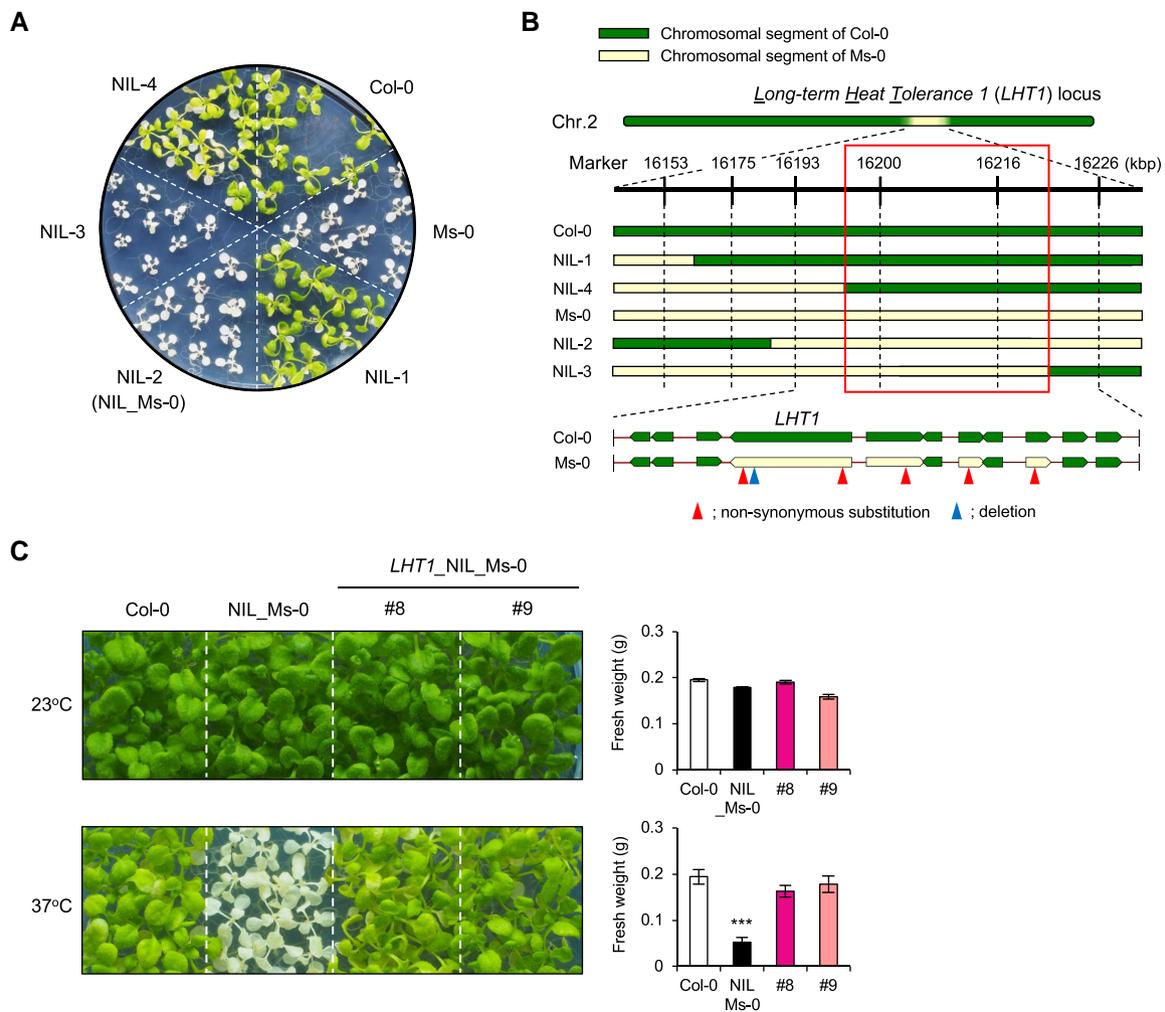


Fig. 2. Identification of *LHT1*. A) High-resolution mapping of the *LHT1* locus using NILs. L-heat tolerance of Col-0, Ms-0, and NIL-1, 2 (NIL_Ms-0), 3, and 4. B) Graphical genotypes of NILs. The number of the marker represents the position (kbp) on chromosome 2. C) Complementation test for L-heat tolerance using Col-0 type *LHT1/MAC7*. Ten-day-old seedlings grown at 22°C were placed at 22°C (upper panel) or 37°C (lower panel) for 5 days and then grown at 22°C for 5 days. Right panel: fresh weight of the aerial parts of plants as depicted in the left panel. Differences between Col-0 and NIL_Ms-0 or *LHT1_NIL_Ms-0*#8 and #9 were analyzed by Student's t test. *** $P < 0.001$.

showing RI in NIL_Ms-0 at 72 h after heat stress, a GO enrichment analysis was performed. As a result, GO terms including mRNA metabolic process, RNA processing, defense response, and immune response were enriched (Fig. 7B). These GO terms were found as those of genes induced by L-heat stress in Col-0 WT (Fig. 5B) or NIL_Ms-0 (Fig. 6B). It suggests that the genes showing RI in NIL_Ms-0 are consistent with those that were highly expressed under L-heat stress in NIL_Ms-0. It is possible that the effect on splicing caused by one amino acid deletion of *LHT1/MAC7* in Ms-0 does not occur in a specific gene but rather in general.

Discussion

Here, we revealed wide variation in both L- and S-heat tolerances of *A. thaliana* accessions. Interestingly, tolerances to the two stresses were not necessarily related, suggesting that the QTLs responsible for each are different and that the accessions have acquired each stress tolerance independently in their evolution.

Genetic analyses using L-heat-tolerant Col-0 and L-heat-sensitive Ms-0 led us to identify a role of *LHT1*, a core component of the MOS4 complex, in establishing the L-heat tolerance of *A. thaliana*. As far as we could determine, the only accession with the Ms-0

genotype with *LHT1* was Kas-1, and no GWAS peak was identified at the *LHT1* locus, suggesting that this *LHT1* genotype is very rare among *A. thaliana* accessions. The fact that a complete deficiency of *LHT1* is embryonic lethal (28) also suggests that there are several accessions in which *LHT1* is functionally defective. The Ms-0 genotype with *LHT1* is disadvantageous because it impairs L-heat tolerance, but it is unclear what the advantage of having it is. Ms-0 was collected in Moscow, Russia, and Kas-1 was collected in Kashmir, northern India. Since both regions are cold, the disadvantage of the L-heat-sensitive Ms-0 genotype may not have been apparent there.

The absence of SNPs significantly associated with L-heat tolerance in this study suggests that L-heat tolerance is regulated by multiple loci or that the contributing loci differ among accessions. Previously, we isolated several *sloh* mutants defective in L-heat tolerance, in which the causal genes are different from *LHT1* (17, 18). A considerable number of genes may contribute to the variation in L-heat tolerance among *A. thaliana* accessions. Our large-scale evaluation of L-heat tolerance using *A. thaliana* accessions revealed that there are several accessions showing more tolerance to L-heat stress than Col-0. The mechanism by which accessions exhibit marked L-heat tolerance is intriguing. Future studies

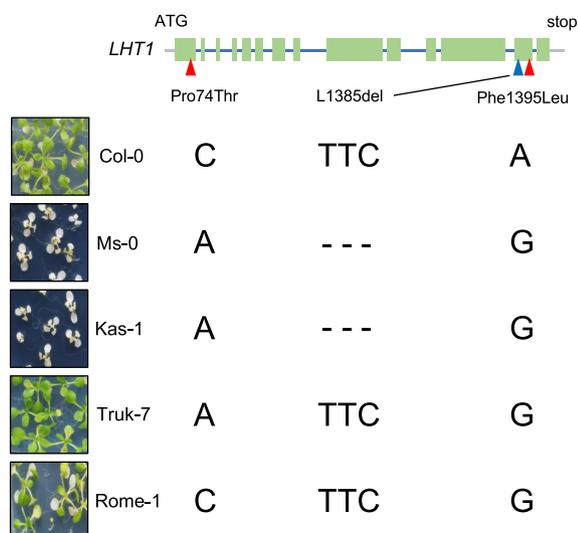


Fig. 3. Nucleotide diversity of *LHT1* gene. Upper panel: Schematic representation of *LHT1* gene. Arrowheads indicate polymorphisms with nonsynonymous substitutions (Pro74Thr and Phe1395Leu) and a deletion (L1385del). Left photos: L-heat tolerance of Col-0, Ms-0, Kas-1, Truk-7, and Rome-1. Right nucleotides: genotypes at the Pro74Thr, L1385del, and Phe1395Leu sites.

to identify the causal genes in these L-heat-tolerant accessions will lead to further elucidation of the mechanism of L-heat tolerance in *A. thaliana*. On the other hand, we recently isolated *sloh3* and *sloh63* mutants, which are hypersensitive to L-heat but not to S-heat stress. *SLOH3* and *SLOH63* were identical to the splicing-related factor genes *MAC9* and *MAC17*, respectively (Endo et al., in submission as back-to-back article). Interestingly, *LHT1/MAC7* interacts with *SLOH3/MAC9* and *SLOH63/MAC17* (28). Both quantitative trait locus (QTL) analysis and forward genetic screening revealed the importance of maintaining precise mRNA splicing under L-heat stress in Arabidopsis.

QTL analyses have detected loci that contribute to heat tolerance in crops, although the causal gene has not yet been identified. L-heat stress (>35°C for 5 days) at the ear emergence and flowering in rice affects pollen tube elongation and normal pollen dispersal, resulting in the formation of empty or unfertilized grains (39). Thus, most of the recently published heat-tolerant QTLs in rice are related to the ear emergence and flowering stages, and like *LHT1*, only a few are related to seedling stage QTL (40). A QTL analysis was conducted to identify the loci contributing to heat tolerance in rice at vegetative stage using the recombinant inbred lines obtained by crossing heat-tolerant N22 and heat-sensitive IR64 varieties. A total of 15 loci were detected, of which *rlht5.1* was a major QTL contributing 20% toward variation for

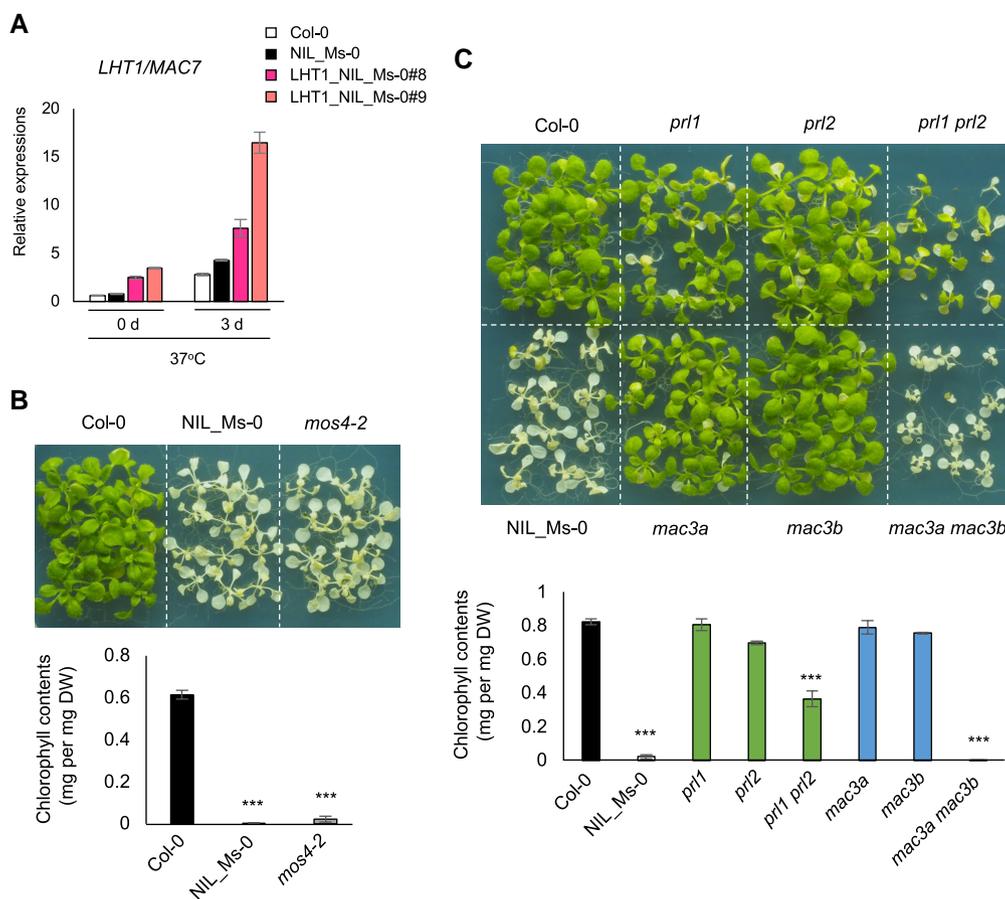


Fig. 4. L-heat tolerance of *mos4* and mutants of *MAC* genes. A) Expression of *LHT1* in Col-0, NIL_Ms-0, and *LHT1_NIL_Ms-0*#8 and #9 under L-heat conditions, determined by qRT-PCR (mean \pm SE, $n = 3$). B, C) L-heat tolerance of mutants and wild type. Photos show L-heat tolerance of (B) Col-0, NIL_Ms-0, and *mos4-2* and (C) *prl1*, *prl2*, *prl1 prl2*, *mac3a*, *mac3b*, and *mac3a mac3b* mutants. Ten-day-old seedlings grown at 22°C were placed at 37°C for 5 days and then grown at 22°C for 5 days. Graphs show chlorophyll contents of plants above. DW, dried weight. Differences between Col-0 wild type and each mutant were analyzed by Student's *t* test. *** $P < 0.001$.

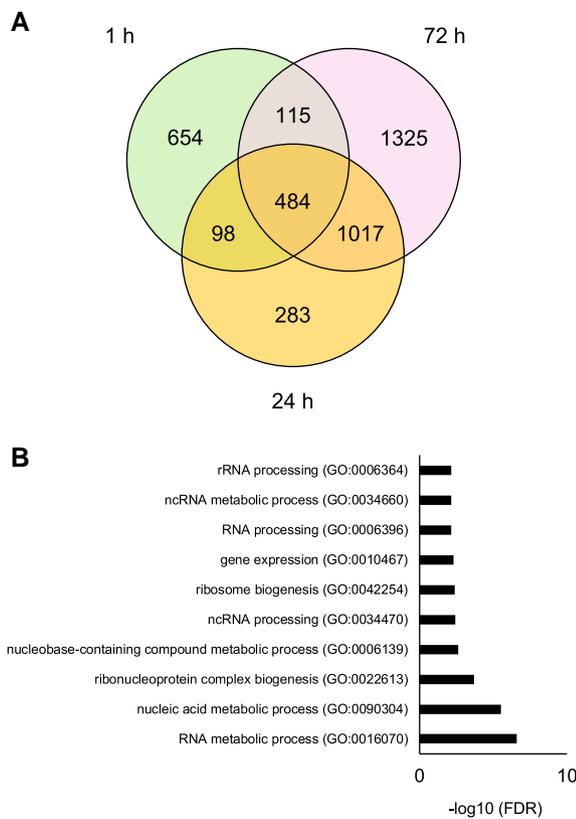


Fig. 5. Genes induced by L-heat stress in Col-0 WT. A) Venn diagram showing the overlaps between heat-induced genes at 1, 24, and 72 h after heat stress treatment. B) GO analysis of the heat-induced genes specifically at 72 h after heat stress in Col-0. The 1,325 genes were classified according to the GO terms in Araport software into categories based on biological processes.

root length under heat stress (41). In wheat, QTL analysis was also conducted to detect loci affecting wheat yield under drought and heat stress. Of the identified QTL, 17 were related to both stresses, while 16 were related only to heat stress (42). In addition to the fact that no SNPs significantly associated with L-heat tolerance was detected in the GWAS of this study, many QTLs were detected in both wheat and rice, suggesting the complexity of the heat tolerance mechanism in plants. On the other hand, several genes that play a positive role in high-temperature tolerance in rice have been isolated by forward genetics. A heat-sensitive dwarf mutant, *thermotolerant growth required1-1*, *togr1-1*, was isolated from an indica variety, Zhongxian 3037. Under L-heat stress (daily maximum temperatures up to 36°C, 15 days exceeding 34°C and 72 days exceeding 30°C), *togr1-1* was extremely dwarf with narrow leaf blades and did not set any seed (43). *TOGR1* encodes a DEAD-box RNA helicase and functions as a key chaperone for rRNA homeostasis required for rice heat-tolerant growth. It is interesting to note that a different type of RNA helicase than *LHT1* contributes to L-heat tolerance in rice.

RNA-seq analyses in this study revealed that L-heat stress induces the expression of genes related to RNA processing. In other studies, transcriptome analysis of *Vitis vinifera* (European grape) under heat stress conditions suggested that heat stress induces RNA processing-related gene expression, supporting the importance of these genes in the response of plants to heat stress. It has also been reported that deletion of the Arabidopsis splicing factor gene *STA1* removes heat acclimatization ability and regulates the splicing of *HSF* and *HSP* under heat stress conditions

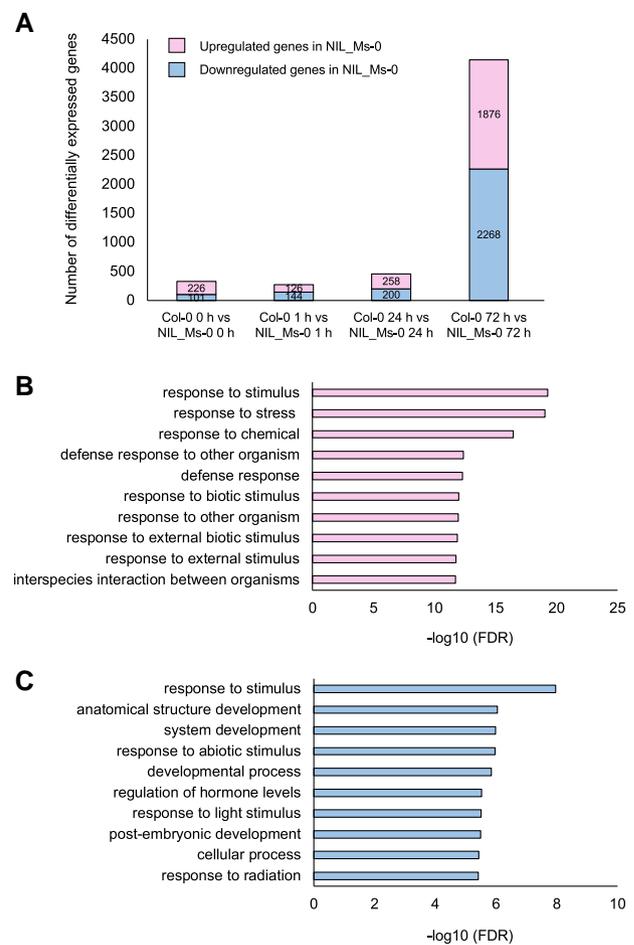


Fig. 6. DEGs between Col-0 and NIL_Ms-0 under heat stress. A) Number of DEGs (\log_2 -fold change >2, FDR <0.05) between Col-0 and NIL_Ms-0 at indicated times after heat stress treatment. B, C) GO analysis of the (B) up-regulated and (C) down-regulated genes in NIL_Ms-0 at 72 h after heat stress. The 1,876 genes were classified according to the GO terms in Araport software into categories based on biological processes.

(44). However, *HSFs* and *HSPs* were not in the group of genes showing alternative splicing under L-heat stress in this study. These results suggest the existence of a pathway that is important for L-heat response and that is independent of *HSP/HSF* splicing.

Since *LHT1* has been identified as an mRNA-binding protein (28), it may be directly involved in mRNA recognition and regulate L-heat tolerance. Although our results suggest that the precise mRNA splicing regulated by *MOS4* complex under L-heat stress is important for stress tolerance, the direct target genes of *LHT1* and how *LHT1* contributes to the tolerance remain unclear. To elucidate this information, it will be necessary to identify the mRNA to which *LHT1* binds and to analyze the function of its splicing variants. It is known that just one splicing variant can critically affect the phenotype (45, 46). Here, *NIL_Ms-0*, with impaired *LHT1* function, affected the splicing of over 3,000 mRNAs. This enormous effect of *LHT1* on splicing may affect L-heat tolerance, or there may be a splicing variant that is critical for L-heat tolerance. L-heat stress induced disease-responsive genes in *NIL_Ms-0*. Mis-activated immunity often results in stunted growth and necrotic lesioning (47). Defects in *LHT1* may cause detrimental autoimmunity, thereby reducing L-heat tolerance.

LHT1 is widely conserved in eukaryotes, including in *Caenorhabditis elegans* as *emb-4* (48, 49). *EMB-4* binds to introns of

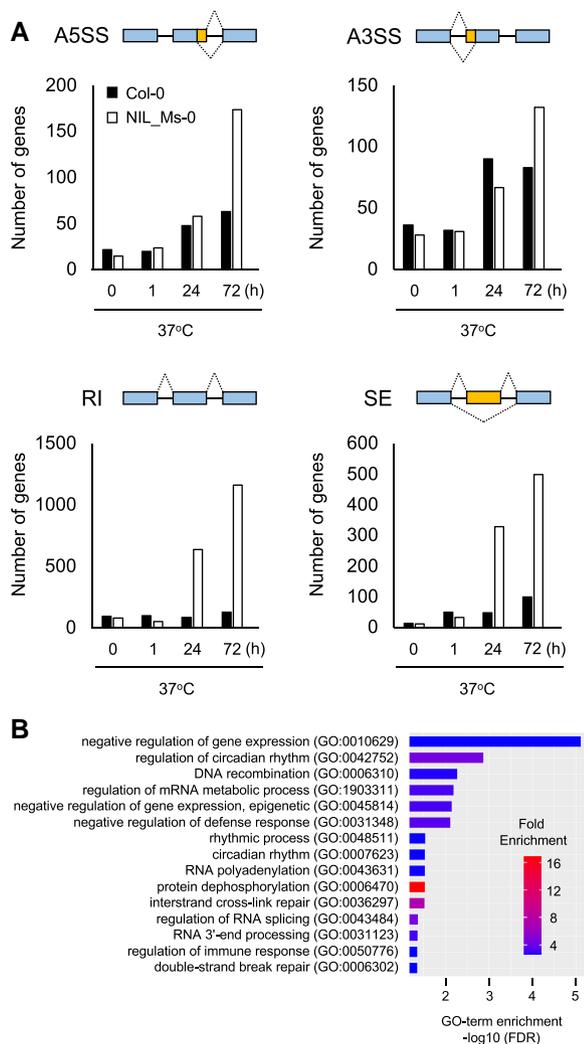


Fig. 7. Effects on selective splicing between Col-0 and the NIL_Ms-0 under L-heat stress. **A)** Number of genes showing a specific splicing pattern—selective 5' splice site (A5SS), selective 3' splice site (A3SS), RI, and SE under heat stress—at 0, 1, 24, and 48 h. Selective splicing was detected in rMAT software. Genes with a FDR of <0.05 in each comparison were identified as showing selective splicing. **B)** Applying GO enrichment analysis to the genes showing RI in NIL_Ms-0 at 72 h after heat stress for top 15 biological processes based on positive fold enrichment. The 1,161 genes were classified according to the GO terms in GENEONTOLOGY software (<https://geneontology.org/>) into categories based on biological processes. The color represents the fold enrichment of the DEGs.

target RNAs and is involved in RNA processing and germline gene expression and therefore may also be involved in heat tolerance in *C. elegans* (48, 49). Indeed, a simultaneous report from our laboratory reveals that the *emb-4* mutant and knockdown lines exhibit hypersensitivity to heat stress (Sato et al., in submission as back-to-back article). Taken together, these observations suggest that the heat response mediated by *LHT1/emb-4* is evolutionarily conserved between plants and animals.

Materials and methods

Plant materials and growth conditions

We used *A. thaliana* (L.) Heynh. accessions obtained from the RIKEN BioResource Center, identified by their JA stock number (Table S5). Seeds of the following *A. thaliana* accessions and mutants were obtained from the Arabidopsis Biological Resource

Center (Ohio State University): Kas-1(CS28376), Truk-7(CS22805), Rome-1(CS76590), *mos4-2* (SALK_090851), *cdc5* (SAIL_207_F03), *prl1* (SALK_008466), *prl2* (SALK_075970), *mac3a* (SALK_089300), and *mac3b* (SALK_050811). Seeds were sown on agar (0.8%, w/v) plates (90 mm × 20 mm; Bio-Bik) with 20 mL full-strength Murashige and Skoog (MS) medium containing vitamins (10 mg L⁻¹ myoinositol, 200 μg L⁻¹ glycine, 50 μg L⁻¹ nicotinic acid, 50 μg L⁻¹ pyridoxine hydrochloride, and 10 μg L⁻¹ thiamine hydrochloride, pH 5.7) and 1% sucrose. Plates were sealed with surgical tape. The seeds were stratified at 4°C for 4–7 days and then transferred to a growth chamber (80 μmol photons m² s⁻¹; 16/8 h light/dark cycle; 22°C) for germination and growth.

Genome-wide association study

GWAS was performed as described (25) to find loci associated with L-heat tolerance in 170 accessions (Table S5).

Plasmid construction and transformation

Plasmids pGreen0029 (50) and pRI909 (Takara, Japan) were used as vectors for plant transformation. DNA fragments from the promoter region to the coding sequence region were amplified using KOD-Plus-Neo (Toyobo, Japan) and introduced into the vector by an In-Fusion HD Cloning Kit (Takara, Japan). pGreen0029 and pRI909 constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 in the presence of pSoup, a helper plasmid for pGreen replication. Primers for cloning are listed in Table S6. *Agrobacterium* were then used for plant transformation by the floral dip method. Transgenic plants were selected on MS agar plates containing 200 μg mL⁻¹ claforan and 25 μg mL⁻¹ kanamycin or 20 μg mL⁻¹ hygromycin. Ten-day-old seedlings (T₁ plants) were transferred to soil pots.

Heat stress assay

For the L-heat tolerance assay, plates with 10-day-old seedlings were held at 37°C for 5 or 7 days. Aerial parts were harvested and homogenized in cold acetone. To quantify the stress tolerance, chlorophyll content (51) or fresh weight was measured. For the S-heat tolerance assay, plates with 10-day-old seedlings were put into a water bath at 42°C for 40 or 50 min and moved back to 22°C (normal growth conditions) for 5 days. To quantify the stress tolerance, survival rates were measured.

Genetic mapping

The L-heat stress-sensitive Ms-0 was crossed with the tolerant Col-0, and the F₁ progeny were self-fertilized to generate F₂ populations. Genomic DNA was prepared from individual F₂ plants with the recessive L-heat-sensitive phenotype for use as PCR templates. To generate NILs, BC₅F₂ plants were generated by backcrossing L-heat-sensitive F₂ plants (derived from Ms-0 × Col-0) to Col-0 plants five times. We screened the BC₅F₂ plants for recombination events at the *LHT1* locus. Plants were genotyped using SSLP markers listed in Table S6. PCR conditions were consisted of an initial 94°C for 2 min; 30–35 cycles of 94°C for 20 s, 54–57°C for 20 s, and 72°C for 10 s; and a final 72°C for 2 min. The microsatellites were fractionated in 6% agarose gels, and the recombinant value was calculated from the band pattern.

RNA extraction and qRT-PCR

Total RNA was isolated in RNAiso Plus (TaKaRa, Japan) and used as a template to synthesize first-strand cDNA using ReverTra Ace (Toyobo). qRT-PCR was performed on a LightCycler 96 (Roche Diagnostics, Switzerland) with Thunderbird SYBR qPCR

Mix (Toyobo) as described (17). ACTIN2 was used an internal standard. Primers are listed in Table S6.

RNA-seq

Total RNA of the extracted samples was quality-checked on an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Libraries were prepared with 500 ng of total RNA obtained from samples with RNA Integrity Number (RIN) >7.0 according to the TruSeq RNA Sample Preparation v. 2 Guide (Illumina, San Diego, CA, USA). Adapters were removed, and quality was filtered in Trimmomatic v. 0.38 software (52), and quality was checked in FastQC v. 0.11.7 software (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Cleaned reads were mapped to the TAIR10 Arabidopsis genome in HISAT2 (53). The output SAM files were converted to BAM files and sorted in SAMtools v. 1.1 (54). Reads were counted in featureCounts (55) using *A. thaliana* gene annotation information (<http://plants.ensembl.org>). Differentially expressed genes (DEGs) were detected in edgeR software (50) from the count data. The BAM files output from the detection of DEGs were used in rMATS software (56) to detect genes with altered selective splicing patterns. The read data were submitted to the DNA Data Bank of Japan (DDBJ) Read Archive (accession number DRA 016172).

GO enrichment analysis

The genes showing RI in NIL_Ms-0 at 72 h after heat stress were applied to GENEONTOLOGY software (<https://geneontology.org/>) into categories based on biological processes to obtain the GO terms. The obtained GO terms were ordered in descending order for the top 15 biological processes based on positive fold enrichment. These top 15 GO terms are then arranged in descending order of the FDR.

Supplementary Material

Supplementary material is available at PNAS Nexus online.

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Author Contributions

K.I. and Te.T. initiated, conceived, and coordinated the project; K.N. and K.I. evaluated the L-heat tolerance of Arabidopsis; Ta.T. performed GWAS; K.I. generated NILs and identified the *LHT1/MAC7* gene responsible for L-heat-sensitive phenotype of Ms-0; K.I. performed physiological analyses; S.I. and M.K. provided seeds of *A. thaliana* accessions and found a closely related accession of Ms-0; K.I. and K.T. performed RNA-seq and analysis; K.I. and K.H. detected splicing variants; Te.T. wrote the manuscript with assistance from I.Y. and Y.S.

Data Availability

All data that support the findings of this study are available within its [supplementary materials](#).

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