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Functional analysis of the Hikeshi-like protein and its interaction with HSP70 in Arabidopsis

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ABSTRACT

Heat shock proteins (HSPs) refold damaged proteins and are an essential component of the heat shock response. Previously, the 70 kDa heat shock protein (HSP70) has been reported to translocate into the nucleus in a heat-dependent manner in many organisms. In humans, the heat-induced translocation of HSP70 requires the nuclear carrier protein Hikeshi. In the Arabidopsis genome, only one gene encodes a protein with high homology to Hikeshi, and we named this homolog *Hikeshi-like* (HKL) *protein*. In this study, we show that two Arabidopsis HSP70 isoforms accumulate in the nucleus in response to heat shock and that HKL interacts with these HSP70s. Our histochemical analysis revealed that *HKL* is predominantly expressed in meristematic tissues, suggesting the potential importance of HKL during cell division in Arabidopsis. In addition, we show that HKL regulates HSP70 localization, and *HKL* overexpression conferred thermotolerance to transgenic Arabidopsis plants. Our results suggest that HKL plays a positive role in the thermotolerance of Arabidopsis plants and cooperatively interacts with HSP70.

1. Introduction

As sessile organisms, plants are exposed to various environmental stresses, among which heat is one of the most critical stressors for the growth and productivity of plants. Heat stress damages plant cells by provoking protein aggregation, reactive oxygen species (ROS) production, and the hyperfluidization of membranes [1]. To adapt to elevated temperatures, plants have developed a wide range of strategies at the cellular and molecular levels. When plants are exposed to heat stress, many heat-inducible genes are upregulated. The transcriptional response to heat stress has been extensively characterized in many model organisms [2]. The heat shock transcription factors (HSFs) are the most well documented protein family involved in the heat stress response [3]. HSFs bind to heat shock elements (HSEs) found in the promoter regions of heat-inducible genes to activate their expression [3].

The expression of heat shock proteins (HSPs) is the most highly conserved mechanism for protecting cells from heat shock damage. HSPs interact with damaged proteins and assist in properly refolding them [2]. HSPs can be classified into several groups based on their protein weight (small, 60, 70, 90, and 100 kDa HSPs), and they are involved in many cellular processes [2]. The HSP70 protein family is highly conserved in eukaryotes, and its biological functions are diverse. Because protein misfolding and aggregation disrupt cellular homeostasis, the HSP70 chaperone system is crucial during both the stress response and development, and it has therefore been a therapeutic target in humans [4–6]. Five cytosolic/nuclear HSP70s have been identified in Arabidopsis, and their amino acid sequences are highly conserved [7,8]. Because cytosolic/nuclear HSP70 isoforms most likely function in a redundant manner, loss-of-function analyses of cytosolic/nuclear HSP70 may be difficult [9]. Consequently, little is known about HSP70-mediated cellular stress responses in plants despite their essential role in thermotolerance [9].

Among all organelles, the nucleus is the most essential compartment. Protecting nuclear proteins from heat shock damage is likely an important mechanism for survival under heat stress conditions [6,10]. Recently, an HSP70 nuclear import carrier protein named Hikeshi was identified in human cells [11]. Hikeshi plays an important role in cellular protection against heat-induced damage via the nuclear translocation of HSP70 in human cells. Because this Hikeshi-mediated HSP70 nuclear import pathway appeared to be a fundamental mechanism for heat stress tolerance, we searched for a Hikeshi ortholog in the Arabidopsis genome.

In this study, we identify an Arabidopsis ortholog to Hikeshi, *Hikeshi-like protein* (*HKL*), and show that it interacts with Arabidopsis HSP70 isoforms and is involved in the regulation of the subcellular localization of HSP70. Our histochemical expression analysis

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reveals that *HKL* is predominantly expressed in meristematic tissues in a heat-dependent manner. Moreover, transgenic plants overexpressing HKL showed higher thermotolerance. Our results suggest that HKL plays a positive role in the protection of cells under heat stress in cooperation with HSP70 proteins.

2. Materials and methods

2.1. Plant materials and growth conditions

The plants (*Arabidopsis thaliana* Columbia ecotype) were grown on GM agar plates under a 16 h light/8 h dark cycle at 22 °C for 2 weeks. Transgenic plants and control plants were grown on GM agar plates containing kanamycin (20 mg/l) for 2 weeks.

2.2. Plant transformation

To generate the *35S:sGFP-HKL* construct, full-length coding sequences were amplified using the primers listed in the Supplemental Table 1, and the fragments were cloned into the pGreen0029 El2-35S-Ω Ns-GFP vector [12]. After confirming the sequence, the constructed plasmids were introduced into *Agrobacterium tumefaciens* GV3101 cells. Plants were transformed as previously described with minor modifications [13].

2.3. RNA analysis

Total RNA was isolated from 14-day-old Arabidopsis seedlings using the RNAiso reagent (TaKaRa, Kyoto, Japan) according to the manufacturer's instructions. For semi-quantitative or quantitative PCR analysis, cDNA was synthesized from 1 μg total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, http://www.appliedbiosystems.com/) with random primers according to the manufacturer's instructions. Semi-quantitative RT-PCR was performed using KOD FX DNA polymerase (Toyobo, Osaka, Japan) with gene-specific primers. Quantitative RT-PCR was performed as previously described [14]. The specific primer pairs used for real-time PCR are listed in the Supplemental Table.

2.4. Yeast two-hybrid analysis

Yeast two-hybrid analysis was performed using MatchMaker GAL4 Two-Hybrid System 3 (Clontech, http://www.clontech.com/) according to the manufacturer's instructions. The AH109 yeast strain was transformed using pairs of pGBKT7 vectors (Clontech, http://www.clontech.com/) that encoded HSP70 and HSC70-1 and pGADT7 vectors (Clontech) that encoded HKL. The transformants were tested on SD (synthetic dextrose) screening medium.

2.5. Transient expression assay

Transient expression assays using protoplasts derived from Arabidopsis mesophyll were performed as previously described [15] with minor modifications. Transient expression analysis was performed as previously described [16] with minor alterations. Fluorescence images of protoplasts were obtained using a confocal laser scanning microscope (LSM 5 PASCAL; Carl Zeiss) as previously described [14].

2.6. Histochemical analysis

Histochemical staining for GUS activity was performed as previously described [12]. We isolated the 1193 bp region upstream of *HKL* from genomic DNA using PCR and introduced the fragment into the pGreenII0029GUS vector. The constructed plasmid was transformed into wild-type plants. T2 transgenic plants grown on GM medium for 10 days were submitted to analysis. GUS staining was observed under an MZ APO stereomicroscope (Leica; http://www.leica-microsystems.co.jp/). The images were captured using AxioVision 4.4 digital imaging processing software (Zeiss; http://www.zeiss.com/).

2.7. Thermotolerance test

For thermotolerance testing, 5-day-old seedlings plated on 1/2MS medium containing 1% sucrose were exposed to 42 °C for 95 min and then allowed to recover at 22 °C for an additional 20 days. Seedlings that showed no severe growth defects were scored as survivors.

3. Results and discussion

3.1. Analysis of HKL expression and tissue specificity

To identify a Hikeshi gene in Arabidopsis, we searched the Arabidopsis genome for genes that encoded proteins similar to Hikeshi. Our database search indicated that only one gene encodes a protein with high homology to Hikeshi, and the amino acid sequence of this protein was partially conserved with that of Hikeshi (Fig. S1A). We named the Hikeshi homolog Hikeshi-like protein (HKL). To determine whether this homologous protein is conserved in both animals and plants, we searched for orthologous peptide sequences using the Phytozome (http://www.phytozome.net/index.php) and National Center for Biotechnology Information BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) databases. Many proteins orthologous to HKL were found, and a neighbor-joining tree analysis was performed based on the conserved regions. Phylogenetic analysis revealed that the *Hikeshi*-like genes were conserved across a range of animals and plants (Fig. S1B).

To analyze the responsiveness of *HKL* to heat stress, we performed a quantitative RT-PCR analysis of its expression under heat stress conditions using wild-type plants (Fig. 1A). The expression of *HKL* was rapidly and transiently induced by heat stress treatment (37 °C) and peaked after approximately 1 h, suggesting an important role for HKL under heat stress conditions similar to that of Hikeshi. Then, to examine the tissue-specific expression pattern of the *HKL* gene, we isolated the *HKL* promoter region (including the heat shock element motif) and generated transgenic plants that included *HKLpro:GUS*. Under non-stress conditions, GUS expression was not detected in the transgenic plants (Fig. 1B). However, after exposure to heat stress treatment (37 °C, 3 h), strong GUS expression was observed in the shoot and root meristem and lateral root primordia; weak GUS staining was observed in the leaf tips (Fig. 1C). These results indicate that *HKL* is a heat-inducible gene but that its responsiveness is primarily limited to meristematic tissues. The meristematic region is essential for normal plant growth. Therefore, the specific expression pattern of *HKL* suggests that HKL functions to protect cells during cell division under heat stress.

3.2. Subcellular localization and interaction analysis of HKL and two HSP70 isoforms

Heat-induced HSP70 accumulation in the nucleus is commonly observed in animals, and Hikeshi-mediated HSP70 accumulation is reported to be critical for the protection of cells from heat shock damage in humans [8]. In plants, however, the dynamic nuclear translocation of HSP70 proteins has not yet been reported. To test whether plant HSP70 proteins translocate into the nucleus in response to heat stress, we selected two major HSP70 isoforms, HSP70 and HSC70-1, and generated transgenic plants that overexpressed each HSP70

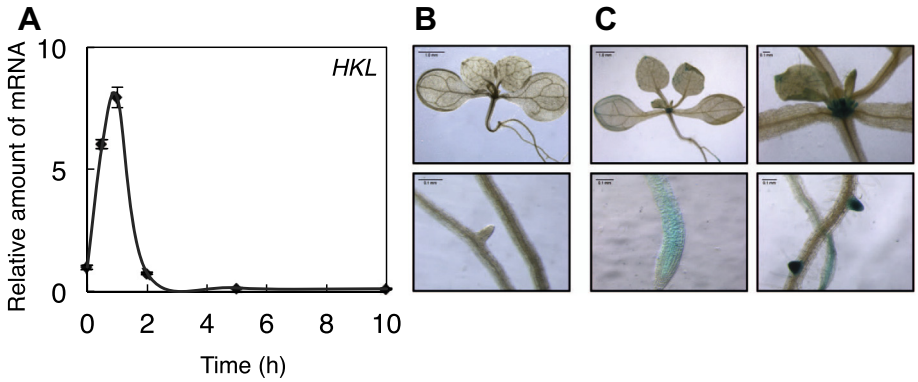

Fig. 1. Heat-inducible and tissue-specific *HKL* gene expression. (A) Analysis of *HKL* gene expression under heat conditions using real-time quantitative PCR. The data represent the means ± SD of triplicate results. The expression level under non-stress conditions was set to 1. (B and C) Histochemical localization of the GUS activity of 10-day-old T2 transgenic plants containing an *HKL* promoter:*GUS* fusion. Transgenic plants were incubated at 37 °C for 3 h. Plants in (B) and (C) were stained before and after incubation, respectively.

protein fused to the C-terminus of sGFP (sGFP-HSP70 and sGFP-HSC70-1) driven by the cauliflower mosaic virus (CaMV) *35S* promoter. Under normal conditions, the fluorescence signal of these sGFP fusion proteins was detected in the nucleus and cytoplasm, which is consistent with a previous study in *Nicotiana tabacum* [6]. However, following heat stress treatment (37 °C, 2 h), the fluorescence signals of sGFP-HSP70 and sGFP-HSC70-1 accumulated in the nucleus (Fig. 2A). The heat-induced accumulation of the HSP70 isoforms was also observed in Arabidopsis mesophyll protoplasts (Fig. S2A). This HSP70 translocation into the nucleus also appeared to be important for plant cell survival under heat stress conditions.

Next, to investigate the subcellular localization of HKL, sGFP fusion proteins driven by the CaMV *35S* promoter were expressed in Arabidopsis mesophyll protoplasts. Confocal microscopic analysis revealed that HKL localized to the nucleus and cytosol in plant cells (Fig. S2B). To confirm the subcellular localization of HKL *in planta*, we generated transgenic Arabidopsis plants harboring the *sGFP–HKL* fusion gene driven by the CaMV 35S promoter (*35S:sGFP–HKL*). The *HKL* expression level was assessed using RT-PCR (Fig. S2C), and two transgenic lines were selected for further analysis. We examined the subcellular localization of HKL in 10-day-old transgenic plants. HKL proteins were localized in the nucleus and cytosol, which is consistent with protoplasts, and the localization did not change after heat stress treatment (37 °C, 1 h) (Fig. 2B). Nuclear carrier proteins bind their cargo in the cytosol and import the cargo into the nucleus through nuclear pore

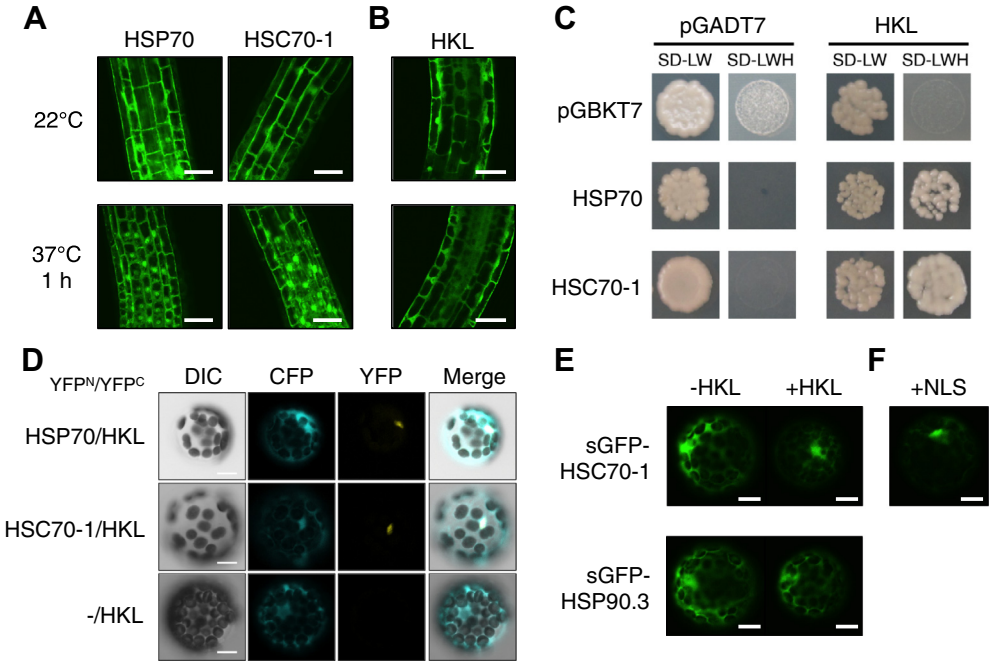

Fig. 2. Subcellular localization and interaction analysis of *HKL* and two HSP70 isoforms. (A) Subcellular localization of the HSP70 and HSC70-1 proteins. Ten-day-old T2 transgenic plants harboring *35S:sGFP-HSP70* and *35S:sGFP-HSC70-1* fusions were examined with or without heat shock treatment (37 °C, 1 h). Bars = 50 μm. (B) Subcellular localization of HKL proteins. Ten-day-old T2 transgenic plants harboring a *35S:sGFP-HKL* fusion were assessed with or without heat shock treatment (37 °C, 1 h). Bars = 50 μm. (C) Yeast two-hybrid assay of the interaction between HKL and two HSP70 isoforms. Transformed yeast cells were grown on SD/-Leu/-Trp medium (left panel) and SD/-His/-Leu/-Trp medium (right panel). (D) BiFC analysis of the interaction between HKL and two HSP70 isoforms. YFP and cyan fluorescent protein (CFP) fluorescence and merged images from the same field of transfected cells are shown for each transfection combination. A control plasmid (*35S:CFP*) was co-transfected to identify transformed cells prior to analyzing YFP fluorescence. Bars = 10 μm. (E) Subcellular localization of HSC70-1 with or without HKL co-expression in Arabidopsis mesophyll protoplasts. The *35S:sGFP-HSC70-1* construct was transfected with the *35S:HKL* construct or an empty vector. Bar = 10 μm. (F) Subcellular localization of HSC70-1 with a nuclear localization signal in Arabidopsis mesophyll protoplasts. Bar = 10 μm.

complexes [17]. Therefore, the observed HKL localization appears to be reasonable if HKL functions as a carrier protein in Arabidopsis.

To investigate whether HKL functions as a regulator of HSP70 localization, we examined the interaction of the two HSP70 isoforms and HKL in a yeast two-hybrid assay. Sequences encoding the full-length HSP70 isoforms and HKL were fused to DNA binding and transcriptional activation domains. The assay demonstrated that both HSP70 proteins interacted with HKL in yeast cells (Fig. 2C). To further confirm the HSP70–HKL interaction, we performed bimolecular fluorescence complementation (BiFC) assays using Arabidopsis mesophyll protoplasts. YFP fluorescence signals were detected in the nucleus for both HSP70 isoforms, which contained fusions to the N-terminal and C-terminal halves of YFP (Fig. 2D). These yeast two-hybrid and BiFC experiments demonstrated that HKL physically interacts with the HSP70 proteins.

To reveal the function of HKL as a HSP70 carrier protein, we introduced the *35S:sGFP-HSC70-1* and *35S:HKL* constructs into Arabidopsis mesophyll protoplasts. The *35S:sGFP-HSP90.3* construct [14] was tested as a negative control. When HSC70-1 was expressed alone, it localized to the cytosol and nucleus. In contrast, co-expression of HSC70-1 and HKL resulted in the accumulation of HSC70-1 in the nucleus even under non-stress conditions (Fig. 2E). We then constructed *GFP-HSC70-1*, which contains the SV40 nuclear localization signal driven by the CaMV *35S* promoter (*35S:GFP-NLS-HSC70-1*) and introduced this construct into Arabidopsis mesophyll protoplasts. GFP signals were detected mainly in the nucleus as well as HSC70-1 and HKL were co-expressed (Fig. 2F). These results suggest that HKL regulates the subcellular localization of HSP70 proteins.

3.3. Overexpression of HKL in transgenic Arabidopsis plants

To analyze the function of HKL in plants, we tested the *35S:sGFP–HKL* transgenic Arabidopsis plants which were used in the subcellular localization analysis. Two lines of *35S:sGFP–HKL* transgenic plants were grown on agar at 22 °C for 3 weeks, but no significant change was observed between the *35S:sGFP–HKL* lines and the vector control line (Fig. S2D). We then evaluated the thermotolerance of *HKL* overexpressors. The vector control line and *35S:sGFP–HKL* lines were grown on 1/2MS plates for 5 days, and the seedlings were heated to 42 °C for 95 min, followed by recovery at 22 °C for 20 days. The *35S:sGFP–HKL* lines exhibited higher survival rates than did the control plants (Fig. 3). These results indicate that HKL plays an important role in the acquisition of thermotolerance in Arabidopsis plants. Moreover, because ectopic HKL expression did not affect normal plant growth (Fig. S2D), HKL could be used as a useful genetic tool for improving thermotolerance in plants.

In this study, we identified the Hikeshi homologous protein HKL in Arabidopsis and showed that HKL interacts with Arabidopsis HSP70 proteins (Fig. 2C and D). We also observed that HSP70 proteins accumulate in the nuclei of Arabidopsis cells in response to heat stress (Fig. 2A). Moreover, transient expression of HKL in protoplasts caused an accumulation of HSP70 protein in the nucleus (Fig. 2E), indicating that HKL plays an important role in the translocation of HSP70 into the nucleus in response to heat stress in Arabidopsis plants. Considering that Hikeshi was shown to function as a nuclear import carrier for HSP70s in human cells, HKL may be a functional homolog of Hikeshi and mediate the nuclear import of HSP70s in Arabidopsis cells. There appears to be a nuclear import pathway mediated by Hikeshi homologous proteins under heat stress conditions in plants similar to that in animals. Moreover, our subsequent analysis revealed that the Hikeshi homologs in monocots lack the amino acid sequence that corresponds to the middle region of Hikeshi and HKL (Fig. S1A). If the monocot Hikeshi homologs also function as carrier proteins, the conserved region that does not include the Hikeshi and HKL middle region may be essential for HSP70 nuclear import.

We observed that the overexpression of *HKL* increases thermotolerance in transgenic Arabidopsis plants (Fig. 3). Heat stress causes protein misfolding and denaturation in various cellular compartments, which triggers the induction of genes for various molecular chaperones to restore protein homeostasis. HSP70 proteins are central components of the cellular network of molecular chaperones [18], and HKL appears to function as a nuclear import carrier for HSP70s. Therefore, HKL overexpression most likely increases the accumulation of HSP70s in the nucleus, and the accumulated HSP70s minimize heat-induced damage by acting inside the nucleus. Similarly to various nuclear events, such as DNA replication and RNA transcription, that have been shown to be stress sensitive [19], the accumulation of HSPs in the nucleus may be important for improving the thermotolerance of transgenic plants. However, the precise molecular mechanism of HSP70 transport by HKL from the cytoplasm to nucleus in response to heat stress remains unknown in plant cells. HSP70 co-chaperones, such as the HSP40 and HSP110 families, have been reported to be required

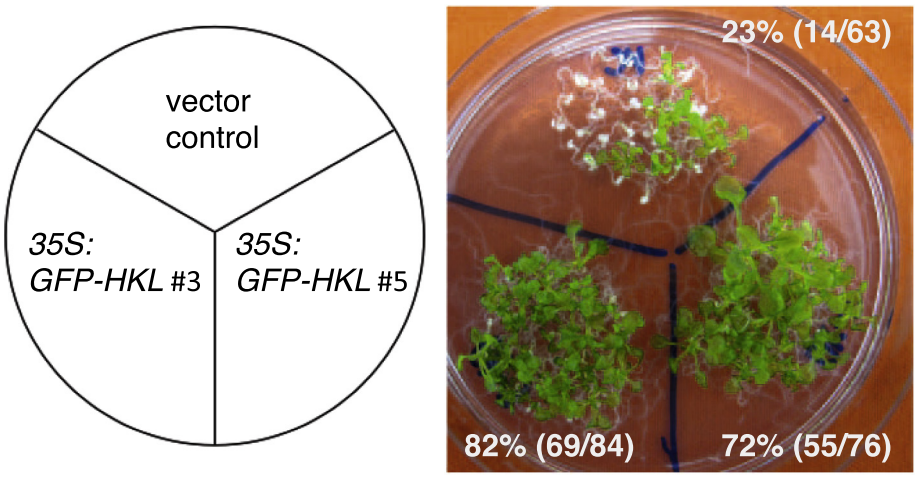

Fig. 3. Effects of overexpressing *HKL* in transgenic Arabidopsis. Five-day-old vector control plants and HKL-overexpressing transgenic plants were subjected to 42 °C for 95 min. The photograph was taken after a 20-day recovery period at 22 °C. The percentage and number of surviving plants per the total number of tested plants are shown within the images. Over 30 plants were used per test, and each test was repeated twice.

for the regulation of HSP70 transport in human cells [11,17]. Further analysis is needed to understand the molecular mechanism underlying HSP70-mediated cellular stress responses in plants.

Our histochemical study revealed that *HKL* expression is limited to the meristematic tissues that are responsible for the division of new cells and essential for plant growth and differentiation. The protection of meristematic cells in response to heat stress is likely a key mechanism for normal plant growth and development during and/or after heat stress conditions. To date, only HeLa cells and fission yeast have been employed to analyze the molecular function of Hikeshi [11,20]; consequently, the tissue-specific expression of this protein has not been examined. Because the molecular function of HKL may be similar to that of human Hikeshi, we are interested in the tissue-specific expression patterns of *Hikeshi* in animals. We also consider that Arabidopsis cultured cells, such as T87 cells, can be used to analyze the physiological functions of HKL during the division of cells under heat stress conditions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.05.128.

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