博士論文（要約）

Roles of 70-kDa heat shock proteins in the regulation of heat-stress-responsive gene expression in *Arabidopsis*

(シロイヌナズナの熱ストレス応答性遺伝子発現制御における
70-kDa 熱ショックタンパク質の役割)

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５年以内に出版予定。

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(シロイヌナズナの熱ストレス応答性遺伝子発現制御における 70-kDa 熱ショックタンパク質の役割)

Plants are typically sessile organisms, which are spending their whole lives at almost the same place. They face to changing surroundings and unsuitable growth situations such as high light, low or high temperature and osmotic stress. Temperature is known as one of the most serious physical parameters that affect plant growth and productivity. The last decade has produced record-breaking heat waves in many parts of the world, which caused severe damages to plant growth and reproductive. Plants can rapidly respond to environmental conditions and unsuitable environmental factors, such as high temperatures. Heat stress transcription factors (Hsfs) are the initial components of a signal transduction chain mediating the transcriptional activation of heat stress-responsive genes. In previous studies, HsfA1 family proteins were reported to be the master regulators of heat stress response in *Arabidopsis*. 70kDa Heat Shock Proteins (HSP70s) were identified as interacting proteins of HsfA1d, a member of the HsfA1 family, both under normal and high temperature conditions by utilizing the co-immunoprecipitation coupled with LC-MS/MS analysis. The HSP70 protein family is one of the main chaperone families among both prokaryotic and eukaryotic organisms that contribute to protein folding, stress response, protein homeostasis, etc. In my master thesis, I identified cytosolic DnaK HSP70s as the main interactors of HsfA1 family proteins in *Arabidopsis*. In this study, the molecular roles of the cytosolic DnaK HSP70s were examined in heat stress-responsive genes expression via modulating the transcriptional activity of HsfA1s, and their physical roles were also analyzed in heat stress responses in plants.

1. Phylogenetic analyses of cytosolic DnaK HSP70s in plants.

There are 18 HSP70s in *Arabidopsis* genome. The full-length amino acid sequences of HSP70 proteins were used in phylogenetic analyses. Cytosolic DnaK HSP70s are highly conserved and separated from other DnaK HSP70s in *Arabidopsis*. While comparing with HSP70s in other plants, cytosolic DnaK HSP70s could be divided into three different groups, HSP70B group; HSP70 group; HSC70 group (HSC70-1, 2 and 3, and HSP70T-1). In each group, these cytosolic DnaK HSP70s have their own homologous proteins in other plants. Through the domain analyses of the cytosolic DnaK HSP70s, *Arabidopsis* HSC70-1, 2 and 3 were classified into the same group when the C-terminal conserved subdomains were compared. I conjectured that the cytosolic DnaK HSP70s in *Arabidopsis* may have functional variations and three HSC70s (designated as HSC70-1, 70-2, and 70-3) may have some overlapping functions in heat stress responses, since they are categorized into the same subgroup in a phylogenetic analysis.

2. Cytosolic DnaK HSC70s are main interactors and repressors of HsfA1d in *Arabidopsis*.

Cytosolic DnaK HSP70s (HSC70-1, HSC70-2, HSC70-3, HSP70, HSP70B, and HSP70T-1) were reported to localize at cytoplasm and nucleus in *Arabidopsis* cells under normal growth conditions. In this study, they are also confirmed to localize at cytoplasm and nucleus by using the transgenic *Arabidopsis* plants harboring the GFP-fused CDS constructs. In histochemical assay, promoter activities of *HSC70-1, HSC70-2* and *HSC70-3* could be observed in whole seedling plants both under normal and heat stress conditions. *HSP70*, *HSP70B*, and *HSP70T-1* showed less or no promoter activities under normal growth condition. High heat-inductivities were observed in quantitative RT-PCR analyses of *HSP70* and *HSP70B*, while *HSC70-1, 2, 3*, and *HSP70T-1* showed lower heat-inductivities. According to the analyses of expression patterns of cytosolic DnaK *HSP70s* genes, HSC70-1, 2, and 3 may be possible interactors of HsfA1d in *Arabidopsis* under normal growth conditions. In my master thesis, yeast two-hybrid analyses and reporter assays were performed. HSP70, HSC70-1, HSC70-2, and HSC70-3 were found to physically interact with HsfA1d in yeast two-hybrid systems, while no obvious physical interactions of HSP70B and HSP70T-1 with HsfA1d were detected. All cytosolic HSP70s repressed the heat stress-responsive, HsfA1d-mediated up-regulation of *HSP18.2* promoter activity. Further analyses were performed in this study. The interaction between HsfA1a and cytosolic HSP70s was also confirmed. Furthermore, the repression activities of these cytosolic HSP70s toward HsfA1d were also observed using *HSP17.6* promoter as another heat stress-responsive reporter gene.

3. Generation and heat tolerance analyses of *hsc70* knockout mutants

hsc70-1, 2, 3 single, *hsc70-1/2, 1/3, 2/3* double and *hsc70-1/2/3* triple mutants were generated and used in this study. Then, thermotolerance tests were performed. The multiple *hsc70s* knockout mutant plants showed significant elevation in thermotolerance at 42°C when compared to wild-type plants. To explore the causal events for their enhanced thermotolerant phenotypes, physiological parameters, such as ion leakage and Fv/Fm were analyzed in *hsc70-1/2/3*. Considering that the cytosolic DnaK HSC70s are the repressors of HsfA1d in *Arabidopsis*, the expression of the HsfA1-downstream genes was analyzed in the *hsc70-1/2/3* triple mutant plants by quantitative RT-PCR. We found that some of the HsfA1-downstream, heat stress-responsive genes are up-regulated in the *hsc70-1/2/3* triple mutant under normal growth conditions. By contrast, no significant changes of the expression levels of these HsfA1d-downstream genes were observed in *hsc70-1/2/3* under heat stress conditions and recovery phases. These results indicate the possibility that HsfA1-regulated transcriptional cascade may change in the *hsc70-1/2/3* mutant plants under normal growth conditions.

4. Transcriptome analyses of *hsc70-1/2/3* mutant plants.

Based on the expression analyses of the HsfA1d-downstream genes in *hsc70-1/2/3*, which are explained in the chapter 3, other HsfA1d-downstream genes may also be up-regulated in the *hsc70-1/2/3* mutant plants under normal conditions. To test the above hypothesis, transcriptome analysis was performed. The analysis indicated that many of heat stress-responsive genes are up-regulated in the *hsc70-1/2/3* mutant plants, and the enrichment analysis showed that genes responsive to abiotic stimulus, such as heat and high light are most significantly up-regulated in the *hsc70-1/2/3* mutant even under a normal growth condition (at 22°C). Then, down-regulated genes in the *hsc70-1/2/3* mutant plants were analyzed. The result of GO enrichment assay showed that biotic and elicitor-related genes are down-regulated in the *hsc70-1/2/3* mutant. Considering that the thermotolerance of the *hsc70-1/2/3* is related to the genes up-regulated in the *hsc70* triple mutant plants, down-regulation of biotic related genes may lead a weak phenotype of *hsc70-1/2/3* under biotic stress conditions.

5. Phenotypic analyses of *hsc70-1/2/3* mutant plants under pathogen and salt treatments

According to the result of the transcriptome analysis, biotic related genes are down-regulated in the mutant plants of *hsc70-1/2/3*. Although the biotic related genes are down-regulated, the *hsc70-1/2/3* mutant plants showed no significant phenotype after pathogen treatment. Since HsfA1 was also reported to function in salt tolerance

responses, I wondered if cytosolic HSP70s also participate in regulating salt tolerance in plants. In germination stages, no significant changes were observed between wild-type and *hsc70-1/2/3* mutant plants under high salinity stress conditions. After a long-term treatment of salt stresses, the *hsc70-1/2/3* mutant plants displayed severer growth retardation when compared to wild-type plants.

Conclusion

In this study, cytosolic DnaK HSC70s were identified to be the interactors and repressors of HsfA1d in *Arabidopsis* under normal growth conditions. *hsc70-1/2/3* triple knockout mutant plants showed higher thermotolerance. The transcriptome analysis showed that heat stress-responsive, HsfA1d-downstream genes and biotic related genes are up- and down-regulated, in the *hsc70-1/2/3* triple mutant, respectively. After a pathogen stress treatment, no significant difference between wild-type and *hsc70-1/2/3* mutant plants were observed. An enhanced growth retardation was observed in *hsc70-1/2/3* mutant plants under salt stress conditions. Further analyses are required to reveal the causal events for the impaired phenotypes of *hsc70-1/2/3* and physiological functions of HSC70s under salt stress conditions in plants. Considering all the consequences, the molecular roles of the cytosolic DnaK HSC70s are to act as repressors of HsfA1s in the heat-responsive regulation via repressing the transactivation activities of HsfA1s. Knock-out of these three HSC70s caused an up-regulation of HsfA1-downstream genes, which directly affects the thermotolerance of mutant *Arabidopsis* plants. The phenotype analyses under salt condition also showed that HSC70s may have other physiological or molecular roles during different developmental stages and stress conditions.