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Functional analysis of an *Arabidopsis* heat-shock transcription factor *HsfA3* in the transcriptional cascade downstream of the DREB2A stress-regulatory system

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Abstract

A transcription factor DREB2A functions as a key regulator not only in drought stress responses but also in heat stress (HS) responses, and activates expression of many abiotic stress-responsive-genes involved in drought and HS tolerance. *HsfA3* is one of the most up-regulated heat-inducible genes in transgenic plants overexpressing DREB2A. In this study, the analyses of *HsfA3* expression profile and the transactivation analysis of *HsfA3* showed that the expression of *HsfA3* was directly regulated by DREB2A under HS. Microarray analysis using transgenic plants overexpressing *HsfA3* also showed that overexpression of *HsfA3* induces many heat-inducible genes. Furthermore, we showed that thermotolerance of the *HsfA3* overexpressors was increased, and that of the *hsfA3* T-DNA tagged mutants was decreased. These results indicate that *HsfA3* regulates expression of many heat-inducible genes in the transcriptional cascade downstream of the DREB2A stress-regulatory system and functions in acquisition of thermotolerance under the control of the DREB2A cascade.
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High temperature is a key stress factor with a negative impact on the growth of plants and the productivity of crops. Many genes involved in the stress tolerance and response are induced under the high temperature stress condition. These genes include heat-shock transcription factors (HSFs) and heat-shock proteins (HSPs). HSFs regulate the expression of heat-shock-regulated genes including HSPs as transcription factors [1]. In *Arabidopsis*, 21 members of HSFs were identified and divided into three classes, A, B, and C, based on structural features of their oligomerization domains [2]. Among them function of the several HSFs has been reported. Using knockout mutants, HsfA1a and HsfA1b were shown to be important for the immediate stress-induced activation of heat-shock-responsive gene expression [3] and heat-inducible HsfA2 was characterized to be essential for extending the duration of acquired thermotolerance [4,5]. HsfA4a and HsfA8 are hypothesized to function as sensors of reactive oxygen species (ROS) [6] and

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HsfA5 was reported to function as a specific repressor of *HsfA4* [7]. Furthermore, *HsfA9* was shown to be regulated by ABI3 and function as a master regulator for expression of *Hsp* genes during seed development [8]. Thus, HSFs form a complex regulatory network in *Arabidopsis* and their complex regulation of gene expression is considered to be important for causing flexible responses of plant to environmental conditions.

The dehydration-responsive element (DRE) with the core sequence A/GCCGAC was identified as a *cis*-acting promoter element that regulates gene expression in response to drought, high-salinity, and cold stresses in *Arabidopsis* [9]. A similar motif was identified as the C-repeat and low-temperature-responsive element in cold-inducible genes [10]. *Arabidopsis* cDNAs encoding DRE-binding proteins, *DREB1A/CBF3*, and *DREB2A* were isolated by using the yeast one-hybrid screening method [9,10]. These proteins are AP2/ERF type transcription factors binding the DRE sequence and activate the expression of genes driven by the DRE sequence. In *Arabidopsis*, there are three DREB1/CBF proteins, DREB1A/CBF3, DREB1B/CBF1, and DREB1C/CBF2 [9,10] and two DREB2 proteins, DREB2A and DREB2B [11]. While expression of the *DREB1/CBF* genes is induced by cold stress, expression of the *DREB2* genes is induced by drought and high-salinity [9,12]. These *DREB2* genes are also induced by heat stress (HS) [13]. However, overexpression of the intact *DREB2A* gene did not result in any remarkable alterations in plant phenotype and expression of the downstream genes [9]. Recently, a negative regulatory domain was found in the central region of DREB2A and deletion of this region transformed DREB2A to a constitutive active form (DREB2A-CA) [12]. Overexpression of DREB2A-CA resulted in improved tolerance to both drought stress and HS in the plants [12,13]. Microarray analysis indicated that the expression of many drought- and/or heat-shock-inducible genes are up-regulated in the DREB2A-CA overexpressor [12,13]. The highest up-regulated gene was *HsfA3* which is a member of the *HSF* gene family and belongs to class A. *HsfA3* is a unique *HSF* gene up-regulated by DREB2A-CA and phylogenetically distinct from other *HsfAs* (Supplementary Fig. 1). In the transgenic plants many HSPs were also up-regulated, which allows us to expect that *HsfA3* contributes to expression of these HSPs in the transcriptional cascade downstream of the DREB2A regulatory system and improves thermotolerance.

In this study, we carried out expression and transactivation analyses of *HsfA3* and demonstrated that expression of *HsfA3* is directly regulated by *DREB2A* under the HS condition. We also characterized the function of *HsfA3* using *HsfA3* overexpressors and knockout mutants and found that *HsfA3* regulates many heat-shock-related genes in response to HS. Furthermore, we report thermotolerance of the overexpressors and the mutants, and discuss the role of *HsfA3* in the transcriptional cascade in response to HS.

Materials and methods

Plant materials. Plants (*Arabidopsis thaliana* ecotype Columbia) were grown on germination medium agar plates for 10–48 days, as described previously [12]. The dreb2a-1 (379F02), dreb2a-2 (179C04) and hsfa3-2 (208B08) mutants were obtained from the GABI-KAT [14]. The hsfa3-1 (SALK_011107) was obtained from ABRC. For HS treatment, Arabidopsis seedlings were grown on agar plates at 22 °C for 2–3 weeks and then transferred to 37 °C. Arabidopsis T87 suspension-cultured cells were maintained as described previously [12].

Quantitative RT-PCR analysis. Total RNA was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Quantitative RT-PCR was carried out as described on a 7300 Real Time PCR system (Applied Biosystems) using POWER SYBR GREEN PCR MASTER MIX (Applied Biosystems) [13].

Plant transformation. The 35S:*HsfA3* and 35S:sGFP-*HsfA3* plasmids were constructed by cloning of cDNAs encoding the *HsfA3* protein into a pGreenII0029 El2-35S-Ω vector and a pGreenII0029 El2-35S-Ω NsGFP vector [13], respectively. The constructed plasmids were introduced into *Agrobacterium tumefaciens* GV3101 cells. Plants were transformed as described previously [12].

Histochemical analysis. For the *HsfA3* promoter:GUS plasmid, a 1000-bp fragment of the *HsfA3* promoter was inserted into the pGreenII0029GUS vector, which has a GUS sequence in the ApaI site. Histochemical localization signals of GUS activity were detected as described [15].

Observation of subcellular localization of green fluorescent signals in transgenic plants. The 1000-bp *HsfA3* promoter and the sGFP fused with the *HsfA3* coding sequence and the Nos terminator of the 35S:sGFP-*HsfA3* plasmid were cloned into the pGreenII0029 vector. GFP fluorescence was analyzed with a confocal laser-scanning microscope LSM5 PASCAL (Zeiss, Oberkochen, Germany).

Transient expression experiments. For effector plasmids, we used 35S:DREB2A-CA [12] and 35S:DREB1A [11]. For reporter plasmids two copies of the 50-bp fragments containing DRE or mutated DRE core motifs of the *HsfA3* promoter were inserted into the RD29AmTATA-GUS plasmid [12]. The *HsfA3* promoter:GUS plasmid was also used for a reporter plasmid. Transient expression assay was performed as described previously [12].

Microarray analysis. Total RNA was isolated from two lines of transgenic plants overexpressing *HsfA3*, *35S:HsfA3-a*, and *35S:HsfA3-b* and used for the preparation of Cy5- and Cy3-labeled cRNA probes. All microarray experiments, including the data analysis, were performed

Fig. 1. Stress-inducible and tissue-specific expression of the *HsfA3* gene and subcellular localization of the *HsfA3* protein. (A) Expression of *HsfA3* and *DREB2A* in response to HS (37 °C) treatment. Total RNA was prepared from about 3-week-old *Arabidopsis* plants that had been heated at 37 °C or incubated at 22 °C for several hours. Accumulation of the *HsfA3* and *DREB2A* mRNAs was analyzed by quantitative RT-PCR. Bars indicate SD ($n = 3$). The expression level under control condition was defined as 1.0. (B) Histochemical localization of GUS activity in the 10-day-old T2 transgenic plants containing the *HsfA3 promoter:GUS* fusion gene. Transgenic plants were incubated at 37 °C for 10 h and stained for 2 h. Plants (a–d) and (e–g) were stained after incubation and before incubation, respectively. Scale bar = 5 mm (a) and 0.4 mm (b–g). (C) Confocal microscope images of sGFP fluorescence (a,c, and e) and Nomarski microscope images (b,d, and f) of the *HsfA3 promoter:sGFP-HsfA3* plants and the 35 S:*sGFP* plants. The root tissues were observed under microscope before (a,b,e, and f) or immediately after (c and d) incubation at 37 °C for 5 h. Scale bar = 100 μm.

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