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Short Communication

Ca^{2+}-dependent protein kinases and their substrate HsfB2a are differently involved in the heat response signaling pathway in Arabidopsis

Chidananda Nagamangala Kanchiswamy[1,2,3], Atsushi Muroi[1,2], Massimo E. Maffei[3], Hirofumi Yoshioka[4], Tatsuya Sawasaki[5], Gen-ichiro Arimura[1,2,*]

[1] Global COE Program: Evolution and Biodiversity, Graduate School of Science, Kyoto University, Kyoto, Kyoto 606-8502, Japan; [2] Center for Ecological Research, Kyoto University, Otsu, Shiga 520-2113, Japan; [3] Plant Physiology Unit, Department of Plant Biology and Innovation Centre, University of Turin, Turin 10135, Italy; [4] Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Aichi 464-8601, Japan; [5] Cell-free Science and Technology Research Center, Ehime University, Matsuyama, Ehime 790-8577, Japan

* E-mail: garimura@ecology.kyoto-u.ac.jp Tel & Fax: +81-77-549-8258

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Abstract Little is known about the mechanisms by which Ca^{2+}-binding sensory proteins direct the plant heat shock (HS) response. Since two Ca^{2+}-dependent protein kinases (CPK3 and CPK13) were recently shown to phosphorylate the heat shock transcription factor HsfB2a, we assessed in the current study whether these kinases are also involved in HS signal transduction, by monitoring the transcriptional profile of HS protein (Hsp) family genes in Arabidopsis Col-0 plants (WT) and the corresponding mutants. Both with and without HS, the gene transcript levels of *Hsp70*, *Hsp101*, *Hsp17.4-CIII* and *Hsp15.7-CI* were found to be lower in *cpk3* and *cpk13* mutants compared to WT, resulting in the impairment of basal thermotolerance in the mutants. To determine the *in vivo* function of CPKs, CPK3/13 and their substrate HsfB2a (heat shock transcription factor) were co-expressed as cofactors for the transient expression of a reporter (GUS) gene under the control of heat shock element (HSE) in *Nicotiana benthamiana* leaves. However, CPK3/13-phosphorylated HsfB2a did not function in the suppression/activation of HSE-promoted expression in the transient expression system. Implications for possible signal trafficking via CPKs and Hsfs are discussed.

Key words: Arabidopsis, Ca^{2+}-dependent protein kinase (CPK), heat shock response, HsfB2a.

Abiotic stresses, such as drought, salinity or extreme temperatures, are serious threats to agriculture and result in deterioration of the environment. Elucidating the various mechanisms of plant responses to stress and their roles in acquired stress tolerance is thus of great practical and basic importance (Wang et al. 2004). Various stress effects result in the appearance of partly denatured proteins in cells that activate a stress response system. Many molecular chaperones are stress proteins and many of them were originally identified as heat shock (HS) proteins (Hsp) (Wang et al. 2004). Most Hsps begin to be expressed as a result of the HS-induced trimerization of an HS transcription factor (Hsf), which enables Hsf to bind to the HS element (HSE) in the promoter region of Hsp genes (Baniwal et al. 2004; von Koskull-Döring et al. 2007), eventually resulting in the potential acquisition of thermotolerance by plants (Montero-Barrientos et al. 2010).

Plants possess several classes of Ca^{2+}-binding sensory proteins, including calmodulins, calmodulin-like proteins, calcineurin B-like proteins, and Ca^{2+}-dependent protein kinases (CPKs) (Sanders et al. 2002). Several Ca^{2+}-binding sensory proteins [e.g., Ca^{2+}/calmodulin-binding protein kinase (AtCBK3), a member of the PPP family (AtPP7)], are known to play roles in heat-shock signal transduction in which Hsf is phosphorylated in both constitutive and HS-induced manners (Li et al. 2004; Liu et al. 2005; Liu et al. 2007; Liu et al. 2008). Moreover, the CPKs are of special interest, since they represent a novel class of Ca^{2+} sensors, having both a protein kinase domain and a calmodulin-like domain (including an EF-hand calcium-binding site) in a single polypeptide (Klimecka and Muszyńska 2007). CPKs constitute a large family of serine/threonine protein kinases that are broadly distributed in the plant kingdom. In a previous study we demonstrated with *in vitro* kinase assays that

Abbreviations: CPK, Ca^{2+}-dependent protein kinase; HS, heat shock; HSE, heat shock element; Hsf, heat shock factor; Hsp, heat shock protein
This article can be found at http://www.jspcmb.jp/

CPK3 and CPK13 proteins phosphorylate the heat shock transcription factor HsfB2a and with *in vivo* agroinfiltration assays that the CPK-mediated phosphorylation of HsfB2a promotes the transcriptional activation of the plant defensin gene *PDF1.2* in a defense response (Nagamangala Kanchiswamy et al. 2010). Moreover, CPK3 kinase activity appeared to be induced by heat stress treatment (Mehlmer et al. 2010). These results prompted us to assess whether the CPK3 and CPK13 cascades are also involved in HS signal transduction. The expression of various Hsp family genes (*Hsp70*, *Hsp101*, *Hsp17.4-CIII* and *Hsp15.7-CI*) was assayed by quantitative reverse transcription (RT)-PCR in Arabidopsis Col-0 plants (WT) and two T-DNA insertion lines: *cpk3* (SALK_022862) and *cpk13* (SALK_057893) that were subjected to heat stress treatment at 40°C for 3 h. Disruption of CPK mRNA expression in leaves of the respective *cpk* mutants was confirmed previously (Nagamangala Kanchiswamy et al. 2010). Compared to the levels in WT seedlings, unheated *cpk3* seedlings contained significantly lower transcript levels for the *Hsp101* and *Hsp15.7-CI* genes, whereas *cpk13* seedlings contained low transcript levels for all of the Hsp genes analyzed (Figure 1A). In both T-DNA insertion lines, however, no visible phenotypical changes were observed in the unheated condition (data not shown).

WT plants reacted to HS by increasing the transcript levels of all Hsps, compared to the respective levels in unheated plants. In contrast, the transcript levels in *cpk3* and *cpk13* plants were significantly lower compared to those in WT plants, with the sole exception of *Hsp15.7-CI* expression in *cpk3* seedlings (Figure 1B). Both *cpk3* and *cpk13* seedlings were impaired, compared to WT, in thermal tolerance at 40°C for 3 h (Figure 1C). Genetic analysis has shown that Hsp101 is absolutely essential for thermal tolerance in Arabidopsis (Tonsor et al. 2008), and this Hsp has been reported to play a role in preventing oxidative stress (Zhang et al. 2009). Hsp70 is one of the major classes of chaperone molecules and is involved in a variety of tasks in eukaryotic cells, and increased Hsp70 synthesis results in a marked increase in stress tolerance (Hu et al. 2010; Montero-Barrientos et al. 2010). Although plants generate an array of high molecular weight Hsps, most of the translation capacity is devoted to the synthesis of the small Hsps (e.g., *Hsp15.7-CI* and *Hsp17.4-CIII*) which also play an important role in the acquisition of thermal tolerance (Yildiz and Terzi 2008). Accordingly, the drastically decreased levels of expression of Hsps in *cpk3* and *cpk13* seedlings, especially, with HS treatment, very likely caused impairment of the plants' basal thermotolerance.

As described above, it was found that CPK3 and CPK13 phosphorylate HsfB2a (heat shock transcription factor) (Nagamangala Kanchiswamy et al. 2010). This

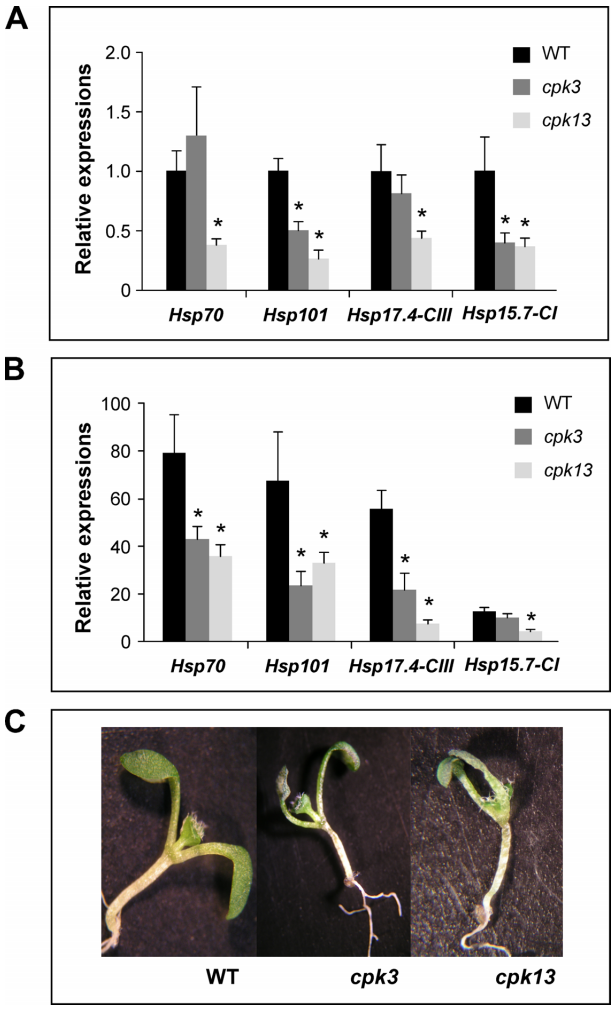

Figure 1. Effects of loss of CPK function on heat response signaling pathway in Arabidopsis seedlings. Arabidopsis seedlings (Col-0) were grown on MS medium containing 2% sucrose and 0.8% agarose in a growth chamber at 22°C. Transcript levels of Hsp genes in seven-day-old seedlings of WT, *cpk3* and *cpk13* were assessed before (A) and after (B) heat stress treatment at 40°C for 3 h. Quantitative reverse transcription (RT)-PCR was done on an ABI Prism® 7000 Sequence Detection System (Applied Biosystems). Primers used for this study are shown in Table 1. Transcript levels of genes were normalized by those of *ACT1* (At2G37620) measured in the samples and expressed relative to the normalized transcript levels in the leaves of unheated WT plants. Data represent the mean+SE ($n>5$). An asterisk (*) indicates that the mutant was significantly different from WT for the indicated gene and treatment ($P<0.05$, ANOVA). (C) Impaired thermal tolerance at 40°C for 3 h both *cpk3* and *cpk13* seedlings with respect to compared to WT phenotype is shown.

Hsf appears to be induced by HS treatment (Li et al. 2009), and belongs to the Hsf class B transcription factors (B-Hsfs). The function of class B-Hsfs differs from that of class A-Hsfs due to a structural variation within the oligomerization domain and the lack of an AHA-motif, which is required for the transcriptional activation function of class A-Hsfs (von Koskull-Döring et al. 2007). Since class B-Hsfs have the capacity to bind to similar or the same sites in the heat shock gene promoters as class A-Hsfs, most of them may act as

Table 1. Primers used for this study

Gene	Purpose	Sequence (5' to 3')
ACT1	Real-time PCR (F)	TGCACTTCCACATGCTATCC
(At2G37620)	Real-time PCR (R)	GAGCTGGTTTTGGCTGTCTC
Hsp15.7-CI	Real-time PCR (F)	TCAACGGCTCTGATTGATTG
(At5g37670)	Real-time PCR (R)	ACTTCCACCACCGGAAAAAG
Hsp17.4-CIII	Real-time PCR (F)	CCCGGAATTTCAAAATCAGATA
(At1g54050)	Real-time PCR (R)	GCCGTTACAGAAGCCATATCA
Hsp101	Real-time PCR (F)	AATTGAACTTCACGCCTTGG
(At1g74310)	Real-time PCR (R)	CTGCCTCCTGCAAAGAAAAC
Hsp70	Real-time PCR (F)	TAAGGTTCTTTCCGGTCCAG
(AT3G12580)	Real-time PCR (R)	CTTGACGCTGAGAGTCGTTG
CPK3	pRE8(XVE) (F)	GAGACTCGAGATGGGCCACAGACACAGCAAG
(At4g23650)	pRE8(XVE) (R)	GAGAACTAGTCTAAATCCACGGATGATTTAGCAC
CPK13	pRE8(XVE) (F)	GAGACTCGAGATGGGAAACTGTTGCAGATCTCC
(At3g51850)	pRE8(XVE) (R)	GAGAACTAGTCTAAGCACTTGCTTTGCAGTCAGC
HsfB2a	pGreen0229 (F)	GAGATTCGAAATGAATTCGCCGCCGGTT
(At5g62020)	pGreen0229 (R)	GAGAGGATCCATTACAAACTCTCTGATT

(F) and (R) indicate the forward and reverse primers, respectively.

repressors of target gene expression (Czarnecka-Verner et al. 2000; Czarnecka-Verner et al. 2004). This assumption was, in fact, supported by our findings that *hsfB2a* mutants showed significantly higher transcript levels of *Hsp101* and *Hsp15.7-CI* but not other Hsp genes (*Hsp70* and *Hsp17.4-CIII*) in heated seedlings, compared to heat-stressed WT seedlings, (Figure 2B). In contrast, in unheated plants, *hsfB2a* mutants showed slightly lower gene expression for *Hsp101* and *Hsp15.7-CI*, compared to WT (Figure 2A). No phenotypic changes were, however, found for the mutant seedlings, irrespective of heated or not-heated conditions, when compared to WT (data not shown).

To confirm the *in vivo* function of the CPK-HsfB2a cascade, constitutively active forms of CPK and HsfB2a were co-expressed as cofactors for the transient expression of a reporter (GUS) gene under the control of HSE (six inverted repeats of nGAAn units) in *Nicotiana benthamiana* leaves, in *Agrobacterium tumefaciens*-mediated transient expression (agroinfiltration) assays. As shown in Figure 3, the activity of the reporter gene was increased when it was co-expressed with HsfB2a as effector (20-fold). Moreover, the activation by HsfB2a was not additionally enhanced when a constitutively active form of CPK3 or CPK13 protein, which lacks the junction and calmodulin-like domains and thus no longer shows Ca^{2+} dependency, was co-expressed. According to these results, we concluded that the CPK3/13-phosphorylated HsfB2a does not function in the suppression/activation of Hsp expression and enhancement of thermotolerance. As described above, since class B-Hsfs generally interact antagonistically with A-Hsfs by binding (or competing for binding) to the HSE consensus sequence, A-Hsfs should be investigated as possible additional cofactors in further studies. It should also be examined whether a suite of A-Hsfs, of which we have not been tested for CPK substrate targeting (i.e., HsfA1d, HsfA8 (Hsf5), HsfA7a, HsfA7b, HsfA5, HsfA1a (Hsf1) and HsfA3), are directly phosphorylated by CPK3 or CPK13, and then potentially activate the Hsp transcription in an HsfB2a- independent manner.

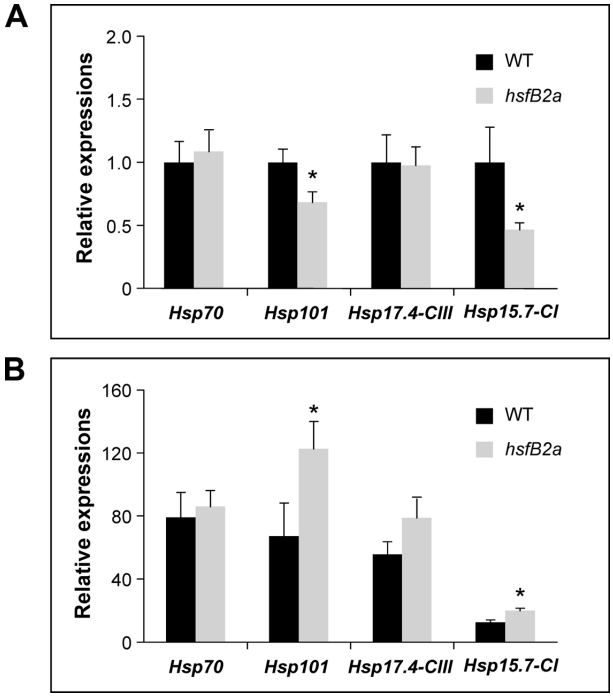

Figure 2. Effects of loss of HsfB2a function on heat response signaling pathway in Arabidopsis seedlings. Transcript levels of Hsp genes in WT and HsfB2a T-DNA insertion mutant (Salk_027578) seedlings before (A) and after (B) heat stress treatment at 40°C for 3 h. Transcript levels of genes were normalized by those of *ACT1* measured in the samples and expressed relative to the normalized transcript levels in the leaves of unheated WT plants. Data represent the mean+SE ($n>5$). An asterisk (*) indicates that the mutant was significantly different from WT for the indicated gene and treatment ($P<0.05$, ANOVA).

Our results show that CPK3 and CPK13 function as positive regulators of Hsp gene up-regulation involved in

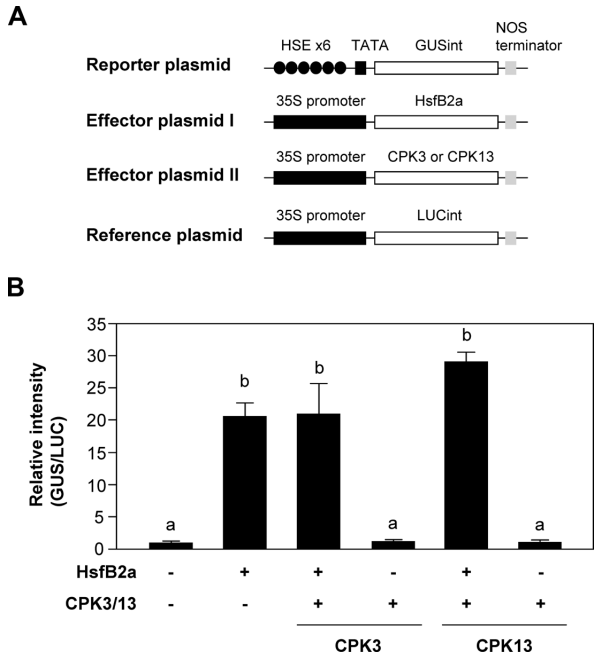

Figure 3. Agroinfiltration assay of a GUS reporter gene under the control of HSE in *Nicotiana benthamiana*. (A) Schematic diagram of the reporter and effector plasmids used in transient assay. An HSE (six inverted repeats of nGAAn units, see underlined sequences) fragment fused to a minimal TATA box: aGAAg cTTCc aGAAc gTTCg aGAAc gTTCg ccc ttc ctc tat ata agg aag ttc att tca ttt gga gag gac tcc ggt) fragment was fused to a minimal TATA box and a GUS reporter gene including the intron (GUSint). Transient activation of the reporter gene according to co-expressed effector(s), HsfB2a, or truncated variant of CPK3 or CPK13, in *N. benthamiana* leaves was assessed according to a modification of the protocol from (Nagamangala Kanchiswamy et al. 2010). *Agrobacterium*, carrying 35S promoter::LUC including intron (LUCint), was used to normalize for the efficiency of agroinfiltration. (B) Transactivation of a GUS reporter gene under the control of HSE. Data represent the mean+SE ($n=5$). Means followed by different small letters are significantly different ($P<0.05$, ANOVA followed by Fisher's PLSD test).

HS tolerance in Arabidopsis. In fact, CPK3 (together with CPK6) is also known to play a role in guard cell ion channel regulation that transduces stomatal abscisic acid signaling (Mori et al. 2006). Moreover, CPK3 has been reported to be associated with the plasma membrane and vacuoles, both depending on its N-terminal myristoylation in the salt stress responses, and thus is able to phosphorylate predominantly membrane-associated proteins (Mehlmer et al. 2010). Since the heat-induced withering of *cpk3* seedlings seem to be due to water-loss (see Figure 1C), a significant portion of CPK3 that function in the heat response signaling pathway may be associated with drought/salt stress-related membrane proteins rather than Hsfs (Arimrua and Sawasaki, 2010). Therefore, it is very likely that CPKs contribute to a wide range of central signal transduction responses in plants, whereas their substrate targets are differently involved or function differently.

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