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SHORT COMMUNICATION

# In vitro reconstitution of temperature-dependent phosphorylation of DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN2A by *Arabidopsis* CASEIN KINASE 1 suggests a potential biochemical basis of thermal sensing

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**Abstract**
Excessive and misregulated stress responses can negatively impact plant growth, highlighting the importance of mechanisms that enable the precise sensing of environmental cues such as temperature. Accurate temperature perception is essential for plant survival under fluctuating environments. DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN (DREB)2A, one of the key transcription factors in plant heat stress response, accumulates and becomes active under heat stress, whereas it is degraded under non-stress conditions. The degradation is triggered by phosphorylation, which is inhibited at 37 °C. However, no molecular thermosensor regulating DREB2A degradation has been identified. In this study, the temperature-dependent phosphorylation of DREB2A was reconstituted in vitro using recombinant proteins. *Arabidopsis* Casein Kinase 1 (CK1) phosphorylates DREB2A at 23 °C but not at 37 °C, reflecting the temperature dependency observed in vivo. The successful reconstitution indicates that no additional thermosensor is required for the temperature-dependent phosphorylation in vitro. Chimera analysis with human CK1 suggests that this temperature dependency is mediated by the middle domain of *Arabidopsis* CK1. Thus, *Arabidopsis* CK1 has the potential to function as a molecular thermosensor in DREB2A-mediated heat stress responses, although in vivo validation remains to be explored.

**Keywords** Thermosensor · Casein kinase 1 · DREB2A · Arabidopsis

**Abbreviations**

| | |
|---|---|
| AP2/ERF | APETALA2/ethylene-responsive element binding factor |
| CK1 | Casein Kinase 1 |
| DREs | Dehydration-responsive elements |
| DREB | DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN |
| ELF3 | EARLY FLOWERING 3 |
| NRD | Negative regulatory domain |
| PIF7 | PHYTOCHROME-INTERACTING FACTOR 7 |

As global temperatures continue to rise, understanding how plants respond to heat stress has become increasingly critical. The initiation of heat response pathways requires thermosensors. Although temperature impacts all molecules, thermosensors are defined as molecules whose temperature sensitivity provides information about the thermal environment that is used to trigger an appropriate physiological or behavioral response (Sengupta and Garrity 2013). In plants, known thermosensors include the photoreceptor phytochrome B, the scaffold protein EARLY FLOWERING 3 (ELF3), the transcriptional co-regulator TWA1, and heat shock proteins that bind to unfolded proteins (Toribio et al. 2024; Jung et al. 2020; Legris et al. 2016; Lin et al. 2022; Bohn et al. 2024). The AFC2 kinase, the RNA of PHYTOCHROME-INTERACTING FACTOR 7 (PIF7) and HSFA2 as well as other molecules such as membrane fluidity and molecular condensation, have also been proposed as thermosensors (Chung et al. 2020, Kebler and Wigge 2023, Geng et al. 2025, Wu et al. 2025).

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Another key component of heat response is the activation of stress-related gene expression, mediated by specific transcription factors such as DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN (DREB)2A. DREB2A, a member of the plant-specific APETALA2/ethylene-responsive element binding factor (AP2/ERF) family, binds to dehydration-responsive elements (DREs) to activate genes involved in heat- and drought-stress responses (Sakuma et al. 2006a; Liu et al. 1998). The importance of DREB2A in heat stress adaptation is highlighted by findings that the *dreb2a* mutants exhibit hypersensitivity to heat, whereas transgenic *Arabidopsis* plants expressing a constitutively active form of DREB2A show enhanced heat tolerance

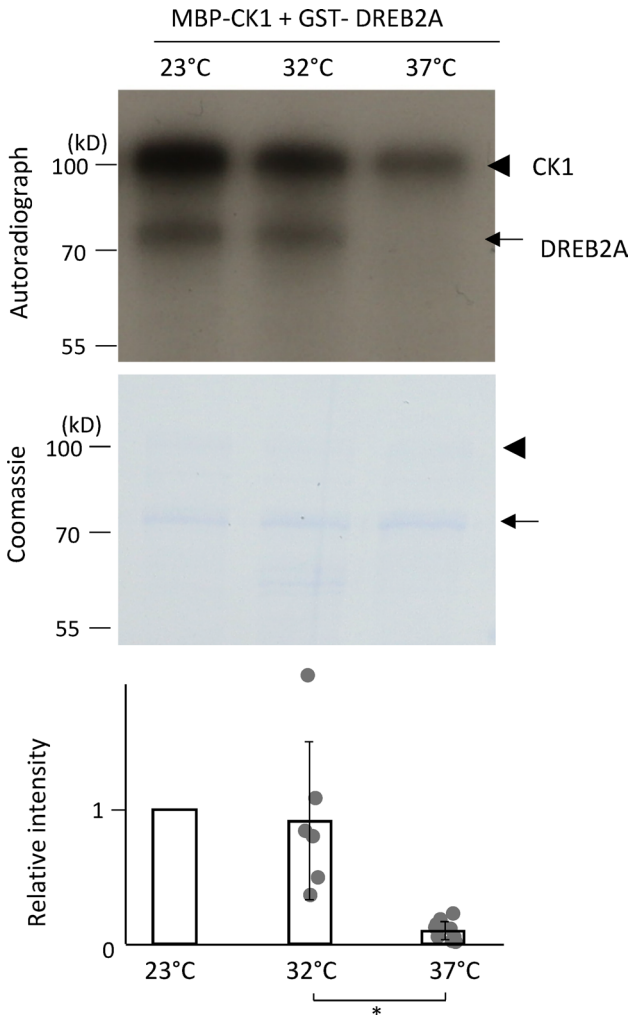


**Fig. 1** In vitro reconstitution of 37 °C -sensitive phosphorylation of DREB2A. MBP-CK1 and GST-DREB2A were incubated in the presence of [$\gamma^{32}$P] ATP at indicated temperature. Autophosphorylation of CK1 (arrowhead) and phosphorylation of DREB2A (arrow) are shown in the autoradiograph (top panel), while protein levels are indicated by Coomassie staining (middle panel). The band intensity of DREB2A was quantified and normalized to that at 23 °C (set to 1, bottom panel). The error bars: SD. The value is 92 ± 58% at 32 °C, n = 6, while it is 9 ± 7% at 37 °C, n = 10. *: $P < 0.05$

(Sakuma et al. 2006a). Similar effects have been observed in other species: Kumar et al. (2022), Singh et al. (2021) and other DREB2 family members contribute to thermotolerance across various species (Lim et al. 2007; Li et al. 2014; Almoguera et al. 2009).

In addition to transcriptional regulation (Sakuma et al. 2006a; Yoshida et al. 2011), post-translational control is crucial for DREB2A function. Under non-stress conditions, full-length DREB2A is rapidly degraded, whereas the protein accumulates during heat or dehydration stress (Liu et al. 1998; Morimoto et al. 2013). Notably, deleting the negative regulatory domain (NRD, residues 136–165) results in DREB2A accumulation even under non-stress conditions, leading to growth inhibition (Sakuma et al. 2006b). This suggests that NRD-mediated degradation prevents unnecessary stress responses in the absence of stress. Specific E3-ligases mediates DREB2A degradation via the proteasome pathway (Qin et al. 2008; Morimoto et al. 2017), while SUMOylation enhances DREB2A stability under high temperatures (Wang et al. 2020). Notably, degradation is initiated by phosphorylation at five Ser/Thr residues (143–147) in the NRD, mediated by nuclear kinases at normal temperatures (23 °C) (Mizoi et al. 2019). However, phosphorylation is reduced at 37 °C, suggesting that inhibiting DREB2A phosphorylation is a key step in activating heat responses. Casein Kinase 1 (CK1) has been proposed as the responsible kinase as phosphorylation is inhibited by CK1-specific inhibitors (Mizoi et al. 2019). CK1, a highly conserved Ser/Thr kinase in eukaryotes (Fulcher and Sapkota 2020), has 13 members in *Arabidopsis*, where it regulates processes such as the circadian clock (Uehara et al. 2019). However, its specific role and regulatory mechanisms in heat stress pathways remain unclear.

While some of the thermosensors mentioned above regulate DREB2A at the transcriptional level, which may contribute to long-term adjustment, none directly link temperature sensing to DREB2A protein degradation, which drives acute responses. This suggests the existence of an additional thermosensor that controls DREB2A phosphorylation and degradation. In this study, I successfully reconstituted the temperature-dependent phosphorylation of DREB2A in vitro, suggesting that CK1 has the potential to function as a thermosensor. To analyze the phosphorylation in the simplest manner, GST-fused full length DREB2A (Tähtinen and Fujii 2025) and MBP-fused *Arabidopsis* CK1 (AtCK1), both produced in *E. coli* were incubated with [$\gamma$-$^{32}$P] ATP at 23 °C, 32 °C and 37 °C. DREB2A phosphorylation occurred at 23 °C and 32 °C while the phosphorylation was reduced at 37 °C (Fig. 1A). The value at 32 °C and 37 °C are 92 ± 58% and 9 ± 7% of that at 23 °C, respectively (means ± SD). This temperature dependency is consistent with in vivo data (Mizoi et al. 2019). Similar results were

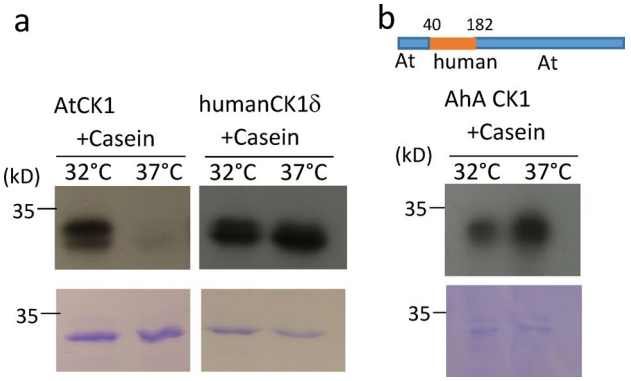


**Fig. 2** Domain responsible for thermosensing of AtCK1. **a** In vitro kinase assay with casein as a substrate. GST-AtCK1, humanCK1δ, and the chimeric AhA CK1 were incubated with casein in the presence of [$\gamma^{32}$P] ATP at the indicated temperatures. Phosphorylation of casein is shown in the autoradiograph (top panel), while protein levels are indicated by Coomassie staining (bottom panel). The signals for humanCK1δ and the chimeric AhA CK1 are comparable between at 32 °C and 37 °C, while the signal at 37 °C is decreased for AtCK1. The value for AhA CK1 at 37 °C is 137±45% of that at 32 °C (n=4), whereas that for WT is 26±29% (n=3, $P<0.05$). **b** Schematic illustration of AhA CK1, which consists of AtCK1-derived parts and a humanCK1δ-derived part. Amino acids 40–182 are replaced with the corresponding region from human CK1δ

obtained when tags were switched (GST-AtCK1 and MBP-DREB2A, Supplemental Fig. 1). A DREB2A mutant, with 5 major in-vivo phosphorylation sites in NRD (Mizoi et al. 2019) mutated to Ala (DREB2A-5A), showed reduced phosphorylation, confirming that in vitro phosphorylation sites align with those identified in vivo (Supplemental Fig. 1). These results indicate that the temperature-dependent phosphorylation of DREB2A is reconstituted in vitro with DREB2A and AtCK1.

In addition to DREB2A phosphorylation, AtCK1 autophosphorylation was reduced at 37 °C while the protein amount was not reduced (Fig. 1), suggesting that AtCK1 activity is temperature-sensitive, independent of substrate. To further assess AtCK1 activity, casein was used as a general substrate. AtCK1 phosphorylates casein in a similarly temperature-dependent manner (Fig. 2). Given that CK1is conserved across eukaryotes, including human, recombinant human CK1δ (Meng et al. 2019) was tested. As expected, based on the human body temperature and a previous report (Isojima et al. 2009), human CK1δ was active at 37 °C (Fig. 2). A BLAST search indicated 62% identity and 74% similarity between AtCK1 and human CK1δ (Supplemental Fig. 2). To identify the domain responsible for this temperature sensitivity, chimeric proteins were generated. The protein was divided into three parts for technical convenience, according to restriction enzyme sites (Supplemental Fig. 2). Replacing residues N41-M182 of AtCK1 with the corresponding human residues (C41-I182) resulted in a chimeric kinase active at 37 °C, indicating that N41-M182 plays a critical role in temperature sensitivity (Fig. 2). Notably, the key region for thermosensitivity lies in a relatively conserved segment.

Taken together, temperature-dependant phosphorylation of DREB2A can be reconstituted in vitro with AtCK1, which is sensitive to 37 °C (Fig. 1). Successful reconstitution of temperature-dependent DREB2A phosphorylation indicates that AtCK1 and DREB2A alone are potentially sufficient for thermosensing upstream of DREB2A stabilization. Switching between stress response and growth requires a mechanism for sensing environmental cues. Our findings demonstrate that AtCK1 is inherently temperature-sensitive and directly regulates DREB2A phosphorylation in response to temperature changes in vitro. To confirm its role as a thermosensor, in vivo experiments are required. The chimeric form of CK1 containing human C41-I182 can be used for in vivo experiments.

While AtCK1 can sense physiological temperature, not all members of CK1 family have similar dependency. Mammalian CKIε/δ-dependent phosphorylation is temperature-insensitive between 25 °C and 35 °C (Isojima et al. 2009), while temperature-sensitive binding of a substrate occurs at 20 °C to 45 °C (Shinohara et al. 2017). The sensitivity of AtCK1 is determined by relatively conserved region (Fig. 2, Supplemental Fig. 2), indicating that small amino acid substitution can change the sensitivity. Since the key domain surrounds the catalytic centre, including the catalytic and activation loop, temperature may cause subtle changes in its structure or mobility, resulting in significant differences in kinase activity. Some of 13 members in *Arabidopsis* and homologs in other plant species may have different sensitivities to critical temperatures. Further research is needed to explore whether similar temperature-sensitive mechanisms exist in other species.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s13562-025-01019-x.

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**Data availability** All data are included in the paper and supplementary files, or will be made available upon request.

## Declarations

**Conflict of interest** Nothing to declare except financial support from Turun Yliopistosäätiö and the Jane and Aatos Erkko Foundation.

**Ethical approval** Not applicable.

**Consent for publication** Not applicable.



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