

Early activation of CIPK3 in the cold-tolerant potato *Solanum commersonii*

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Abstract: *Solanum commersonii* shows tolerance to low temperatures, a key target trait for potato breeding. Calcium-dependent protein kinases (CIPKs) play a significant role in plant defence response to several stresses, including cold. In this study, we observed the expression of *ScCIPK1*, *ScCIPK3*, *ScCIPK23* and *ScCIPK24* in *S. commersonii* exposed to 4 °C at multiple time intervals. Initial findings revealed that these genes were under-expressed after 10 and 30 minutes of cold stress, except *ScCIPK3*. Notably, after 24 hours, all genes displayed higher expression levels compared to the non-stressed controls. These findings highlight the role of *ScCIPK3* in the early stages of cold response and indicate a coordinated regulatory mechanism across CIPKs that likely contributes to the cold stress tolerance observed in *S. commersonii*. In this work, we introduce a model to elucidate the signalling crosstalk under cold stress in *S. commersonii*, providing insights that could facilitate the development of cold-resistant potato cultivars.

Keywords: abiotic stress; gene expression; plant adaptation; signal transduction; wild potato

Plants, as sessile organisms, are constantly exposed to environmental stresses and have evolved complex mechanisms to respond effectively. Central to these responses is the rapid elevation of intracellular calcium (Ca^{2+}) levels, which initiates downstream signalling through calcium-binding proteins such as calmodulin-like proteins (CaMLs), calcium-dependent protein kinases (CDPKs), calcineurin B-like proteins (CBLs), and CBL-interacting protein kinases (CIPKs) (Ghosh et al. 2022). While CaMLs broadly interact with various targets and CDPKs act as direct Ca^{2+} -dependent kinases, CBLs specifically bind CIPKs, forming complexes that trigger phosphorylation cascades involved in modulating transcription factors and other regulatory proteins

(Huang et al. 2011; Tang et al. 2020). Genome-wide studies have identified CBL-CIPK networks across numerous species (e.g. Aslam et al. 2019; Du et al. 2021; Sun et al. 2021). In the wild potato, 27 CIPK genes have been linked to drought and osmotic stress, including interactions between *StCBL* and *StCIPK10* (Ma et al. 2021). The cold-tolerant wild species *Solanum commersonii*, which features 10 CBL and 26 CIPK genes (Aversano et al. 2015; Esposito et al. 2019), is a promising genetic resource for improving abiotic stress tolerance in cultivated potato (*S. tuberosum*) (Dong et al. 2023). In particular, phylogenetic analysis revealed the presence of two CIPK3 paralogs in *S. commersonii* compared to one in *S. tuberosum* (Aversano et al. 2015; Esposito et al.

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2019). Transcriptome profiling has further shown that *CIPKs* in *S. commersonii* exhibit differential expression patterns under various biotic and abiotic stresses (Esposito et al. 2019), highlighting their regulatory diversity and conservation across species. Among wild potatoes, *S. commersonii* is particularly suited to studying cold stress signalling due to its exceptionally low-temperature tolerance (Vega et al. 2000). To investigate the involvement of *CIPKs* in its cold response, we analysed the transcript profiles of four genes – *ScCIPK1*, *ScCIPK3*, *ScCIPK23*, and *ScCIPK24* – following exposure to 4 °C. These genes were selected as the most highly expressed intron-poor members in *S. commersonii* (Esposito et al. 2019), whose *Arabidopsis* orthologs are key regulators of abiotic stress and cold signalling (Qiu et al. 2002; Kim et al. 2003; D'Angelo et al. 2006; Li et al. 2006; Cheong et al. 2007). *In vitro*-grown plants were subjected to cold treatment for 10 min (CS10), 30 min (CS30), and 24 h (CS24), and expression levels were compared to unstressed controls. We selected these points to assess temporal dynamics in calcium-mediated signalling and classify chosen *CIPKs* in early and late-responsive genes involved in cold adaptation.

In vitro plantlets of *Solanum commersonii* clone cmm1T (accession PI243503), obtained from the Inter-Regional Potato Introduction Station (Sturgeon Bay, Wisconsin), were micropropagated. Plantlets were grown on Murashige and Skoog medium containing 2% (w/v) sucrose and 0.8% (w/v) agar, under controlled conditions (24 °C, 16/8 h light/

dark, 3 000–4 000 lux) for four weeks. Eighteen plants were subjected to cold treatment at 4 °C, and samples were collected from six biological replicates at each time point (CS10, CS30 and CS24). Six additional plants maintained at 24 °C served as controls. The sequences of the four *ScCIPKs* were retrieved from Esposito et al. (2019), and gene-specific primers were designed for RT-qPCR (*ScCIPK1_FW* 5'-AAAAGGGAATGCGAGTAGGG-3', *ScCIPK1_RV* 5'-CTTGGACGATAAAGAAATGGGCT-3'; *ScCIPK3_FW* 5'TGGAGCTAAAGTAACGAGAGAA-3', *ScCIPK3_RV* 5'CCTTTGACTTCCGCACCTC-3'; *ScCIPK23_FW* 5'-TGTAGCCAAGTCCCAGGTT-3'; *ScCIPK23_RV* 5'-CCGGTGGACAGGTTCTGTA-3'; *ScCIPK24_FW* 5'-CAATTCCCAGGCGACTTCTC-3', *ScCIPK24_RV* 5'-TGATGTAGCGTGCAAAG-3') for the expression analysis. For *ScCIPK3*, the primers were designed to amplify both paralogs (*ScCIPK3a* and *ScCIPK3b*), as their sequences are highly similar; this approach allowed us to evaluate the overall *CIPK3* transcriptional response rather than distinguish between the two allelic forms. Gene expression was normalised against elongation factor 1- α (EF1 α , Nicot et al. 2005) and analysed using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001), with results expressed as log₂ fold changes.

All *ScCIPK* genes were under-expressed after 10 min of cold stress. However, *ScCIPK3* transcripts (coming from all isoforms) were induced after 30 min of cold stress (CS30), showing a 2.0-fold increase compared to the control (Figure 1). Previous research identified *CIPK3* as a key mediator in the Ca^{2+} signalling

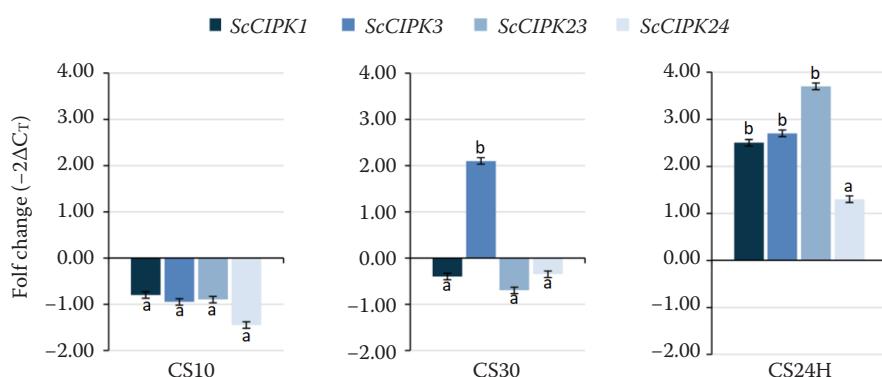


Figure 1. Gene expression profiles of *ScCIPK1*, *ScCIPK3*, *ScCIPK23* and *ScCIPK24* in *Solanum commersonii* (cmm1T clone) under a time-course experiment (CS10 – 10 min, CS30 – 30 min and CS24H – 24 h) determined by RT-qPCR. Expression levels are shown relative to control plants maintained at 24 °C and were normalised to the housekeeping gene elongation factor (EF1 α), according to the REST analysis; bars represent the mean \pm standard deviation (SD) of six biological replicates ($n = 6$); P -value ≤ 0.05 was considered statistically significant

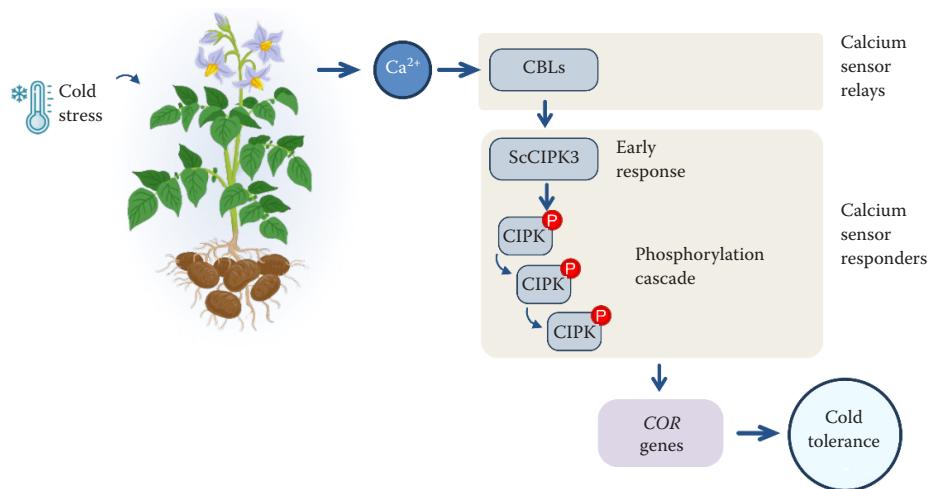


Figure 2. Schematic representation of the signal transduction mechanism in *Solanum commersonii* in response to cold stress, where early activation of *ScCIPK3* has been evidenced

pathway, acting as a hub for cross-talk during cold stress in *Arabidopsis*. In particular, it plays a pivotal role in the early response to stresses by phosphorylating transcription factors that are crucial for cold adaptation (e.g., DREB1A/CBF3), enabling the rapid activation of downstream stress-responsive genes such as *RD29A* and *KIN1/KIN2* (Kim et al. 2003).

Previous results have shown that CIPK3 from *Arabidopsis* works as molecular cross-talk between abscisic acid (ABA) and stress-related gene expression, and more recent studies that have an impact also on Mn homeostasis in cells (Kim et al. 2003). Latz et al. (2013) showed that AtCIPK3 is one of the earliest CIPK genes to be rapidly induced following stress exposure in *Arabidopsis*. Although their study did not demonstrate a direct role of AtCIPK3 in triggering the expression of other CIPK genes, its early induction suggests a potential involvement in initiating broader signalling cascades. A similar hierarchical activation pattern may occur in *S. commersonii*. Indeed, only after prolonged cold exposure (24 h, CS24H) did all *ScCIPK* genes exhibit increased expression, with fold-change values ranging from 1.8 for *ScCIPK24* to 3.5 for *ScCIPK23* (Figure 1). However, *S. commersonii* CIPK3 paralogs may possess different functionality, unrelated to those reported for AtCIPK3. Moreover, more recent results suggest that *Arabidopsis* CIPK3 has a more general function in manganese homeostasis and ABA signalling rather than direct in cold.

Initially, the abiotic stress (in this case, cold) triggers an increase in intracellular Ca^{2+} . This el-

evation is detected by CBL sensor relays. Indeed, upon binding calcium, they can interact with sensor responders (namely the CIPK proteins), activating them (Sánchez-Barrena et al. 2013). As reported by Chaves-Sanjuan et al. (2014), CIPKs are activated by phosphorylation from upstream kinases through conformational changes in the activation loop, which facilitate substrate interaction. Accordingly, the activation of *ScCIPK3* via CBL interaction may initiate a phosphorylation cascade involving other *ScCIPK* proteins. Such phosphorylation leads to the activation of the signal transduction mechanism and ultimately up-regulating cold stress-responsive genes (COR genes), potentially responsible for the cold tolerance observed in the wild potato *S. commersonii*. In conclusion, the transcriptional early activation observed after 30 min of stress exposure suggests a conserved mechanism shared with *Arabidopsis*, where CIPK3 mediates ABA-dependent cold responses (Kim et al. 2003; Huang et al. 2011) (Figure 2). According to the phylogenetic analysis of *S. commersonii* CIPKs (Esposito et al. 2019), *ScCIPK3a/b* cluster with AtCIPK3 within the intron-poor clade, supporting their evolutionary and functional relatedness. These findings underscore the prominent role of *ScCIPK3* in the signalling of *S. commersonii* during cold stress at 4 °C. As the genome of this wild potato species is available and is starting to be explored, our preliminary results can lay the foundation for future, more extensive studies aimed at defining the signalling network in *S. commersonii* and its remarkable cold tolerance.

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