

Salt Stress Triggers Phosphorylation of the *Arabidopsis* Vacuolar K⁺ Channel TPK1 by Calcium-Dependent Protein Kinases (CDPKs)

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ABSTRACT 14–3–3 proteins play an important role in the regulation of many cellular processes. The *Arabidopsis* vacuolar two-pore K⁺ channel 1 (TPK1) interacts with the 14–3–3 protein GRF6 (GF14-λ). Upon phosphorylation of the putative binding motif in the N-terminus of TPK1, GRF6 binds to TPK1 and activates the potassium channel. In order to gain a deeper understanding of this 14–3–3-mediated signal transduction, we set out to identify the respective kinases, which regulate the phosphorylation status of the 14–3–3 binding motif in TPK1. Here, we report that the calcium-dependent protein kinases (CDPKs) can phosphorylate and thereby activate the 14–3–3 binding motif in TPK1. Focusing on the stress-activated kinase CPK3, we visualized direct and specific interaction of TPK1 with the kinase at the tonoplast *in vivo*. In line with its proposed role in K⁺ homeostasis, TPK1 phosphorylation was found to be induced by salt stress *in planta*, and both *cpk3* and *tpk1* mutants displayed salt-sensitive phenotypes. Molecular modeling of the TPK1–CPK3 interaction domain provided mechanistic insights into TPK1 stress-regulated phosphorylation responses and pinpointed two arginine residues in the N-terminal 14–3–3 binding motif in TPK1 critical for kinase interaction. Taken together, our studies provide evidence for an essential role of the vacuolar potassium channel TPK1 in salt-stress adaptation as a target of calcium-regulated stress signaling pathways involving Ca²⁺, Ca²⁺-dependent kinases, and 14–3–3 proteins.

Key words: potassium channel; vacuole; calcium; calcium-dependent kinase; 14–3–3 protein; salt stress.

INTRODUCTION

Abiotic stress such as elevated salt levels affects plant growth and productivity. To survive salt stress and to reproduce, plants have to accommodate a stress physiology. A basic requirement for plants to adapt to salt stress is the maintenance of cellular potassium homeostasis. This is achieved by pumping out Na⁺ or dumping the toxic cation into the vacuole (Munns and Tester, 2008). Accordingly, ion transporters and channels in the plasma and vacuolar membrane represent key factors in determining salt and osmotic stress tolerance (Apse et al., 1999). To maintain electroneutrality, long-term sodium uptake requires counterfluxes of appropriate anions or efflux of potassium from the vacuole (Rodriguez-Navarro and Rubio, 2006). Salt stress, particularly sodium stress, activates the salt-overly-sensitive (SOS) system in plants leading to sodium sequestration from the cytosol (Zhu, 2003). In experiments, sudden exposure of plants to salt stress triggers a transient rise in free cytosolic calcium (Knight et al., 1997), which can be

perceived by the SOS system (Qiu et al., 2002). In general, these calcium signals could be sensed by calcium-binding proteins including calmodulin, the Calcineurin B-like sensors with their interacting kinases (CBLs/CIPKs), as well as calcium-dependent protein kinases (CDPKs or CPKs in *Arabidopsis*) (Dodd et al., 2010; Kudla et al., 2010). The latter two transmit the signal into phosphorylation cascades capable of modulating gene expression and target protein activity (Curran et al., 2011). In line with the existence of up to four calcium-binding EF-hands

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in CDPKs for intramolecular calcium perception (Wernimont et al., 2010), these kinases were found to be involved in calcium-mediated abiotic stress responses (Zhu et al., 2007; Mehler et al., 2010; Franz et al., 2011; Wurzing et al., 2011). In this context, it should be mentioned that CDPKs and CBL/CIPKs, through their interaction with ion channels and transporters, seem to represent part of membrane-delimited plant stress responses (Hedrich and Kudla, 2006; Wan et al., 2007; Geiger et al., 2009, 2011; Tsay et al., 2011; Hubbard et al., 2012). Like salt stress, osmotic stress adaption requires action on the level of the plasma membrane as well as the vacuolar membrane.

Arabidopsis TPK1 represents the founding member of potassium-selective vacuolar K⁺ (VK) channels (Ward and Schroeder, 1994; Allen and Sanders, 1996; Gobert et al., 2007; Isayenkov et al., 2010; Maathuis, 2011). The TPK1 channel is activated by elevated cytosolic calcium concentrations and, moreover, the open probability of this calcium-activated potassium channel is boosted by interaction with GRF6, a 14–3–3 protein (GF14-lambda; Bihler et al., 2005; Gobert et al., 2007; Latz et al., 2007). 14–3–3 proteins constitute important hubs within the *Arabidopsis* interactome and, as such, function not only as regulators of key metabolic points, but in addition mediate red light signaling, defense-related cell death, stomatal opening, or abiotic stress responses (Paul et al., 2012, and references therein; Tseng et al., 2012). Interaction of TPK1 with GRF6 is dependent on the phosphorylation of a serine residue in the cytosolic N-terminus of the vacuolar potassium channel. Here, we report that this residue is phosphorylated during salt-stress signaling by CDPKs. The interaction of TPK1 with CPK3 is stabilized by two conserved arginine residues within the channels' 14–3–3 binding motif. Phenotypes of *tpk1* and *cpk3* mutants underpinned the relevance of this vacuolar K⁺ channel kinase interaction for *Arabidopsis thaliana* in balancing cytosolic potassium homeostasis and thereby overcoming episodes of salt stress.

RESULTS

TPK1 Is Phosphorylated by Calcium-Dependent Protein Kinases

Phosphorylation of the tandem-pore potassium channel TPK1 at serine 42 in its N-terminal 14–3–3 consensus binding motif is a prerequisite for 14–3–3 protein binding (Latz et al., 2007). In order to identify the kinase in question, cellular extracts from *Arabidopsis* seedlings were tested for TPK1 phosphorylation capability. For this purpose, the N-terminus of TPK1 (amino acids 1–79) covering the 14–3–3 binding motif was recombinantly expressed in *Escherichia coli*, purified and subjected to *in vitro* phosphorylation by *Arabidopsis* leaf extracts after cell fractionation. Thereby, the cytoplasmic protein fraction, rather than the microsomal part, was identified to exhibit highest activity in phosphorylating the TPK1 N-terminus (Supplemental Figure 1). Interestingly, TPK1 substrate phosphorylation efficiency seemed to depend on the presence

of divalent ions such as calcium (Supplemental Figure 1). The presence of the chelator EDTA (Ethylenediaminetetraacetic acid) in the soluble kinase fraction dramatically reduced phosphorylation efficiency. These subcellular fractionation and phosphorylation properties suggested that a putative soluble, calcium-dependent protein kinase is capable of phosphorylating the 14–3–3 binding motif of TPK1.

The family of CDPKs in *A. thaliana* comprises 34 members. While some CDPKs have been shown to localize in the cytoplasm, others exhibit a more complex localization pattern including (partial) membrane localization (Lu and Hrabak, 2002; Dammann et al., 2003; Benetka et al., 2008; Mehler et al., 2010). Based on the localization characteristics determined for the as-yet unknown kinase that can phosphorylate TPK1, we thus focused on calcium-dependent kinases, which are located primarily in the cytoplasm and showed overlapping expression profiles with the TPK1 channel. Among the *Arabidopsis* CDPKs, CPK1, 3, 4, 5, 11, 12, and 29 met the search criteria (Hruz et al., 2008), and were thus further investigated for their ability to phosphorylate TPK1. To this end, the corresponding cDNAs were cloned, expressed in *E. coli*, and employed in *in vitro* phosphorylation assays using the TPK1 N-terminus as a substrate. Among the CDPKs tested, we found CPK3, CPK4, CPK5, and CPK11 capable of efficiently phosphorylating the purified, recombinant TPK1 N-terminal peptide in a calcium-dependent manner (Figure 1). Compared with CPK3, TPK1 phosphorylation by CPKs 4 and 11 was similarly effective, while CPK5 exhibited slightly lower efficiency. These results were obtained with at least three independent experiments and were corroborated in Western blots using a commercially available phosphorylation state-specific 14–3–3-binding site antibody (Supplemental Figure 2). In contrast, CPK12 did not phosphorylate the TPK1 peptide under conditions tested in this study (Supplemental Figure 3). Hence, CPK3, CPK4, CPK5, and CPK11 were assigned as candidate kinases responsible for the phosphorylation of TPK1 in *Arabidopsis*.

CPK3 Is Constitutively Co-Expressed with TPK1 and Activated by Calcium

Among the CDPKs investigated in this study, *Arabidopsis* CPK3 is well characterized in terms of biotic or abiotic stress responses (Mori et al., 2006; Kanchiswamy et al., 2010). Recently, CPK3 has also been shown to play an important role in salt-stress acclimation in *Arabidopsis* and a number of membrane proteins have been identified as potential CPK3 targets (Mehler et al., 2010). To test for the presence of CPK3 in TPK1-expressing tissues, we analyzed the transcriptional regulation of TPK1 and CPK3 in response to salt stress. In line with transcriptome data available (Kreps et al., 2002; Hruz et al., 2008), however, TPK1 and CPK3 under the given experimental settings appeared not to be transcriptionally regulated in 10-day-old seedlings of wild-type plants (Col0) as well as *cpk3* knockout mutants (Figure 2A). In contrast, CPK29, which was barely detectable under control conditions,

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