Short Communication

Constitutive activation of calcium-dependent protein kinase 3 confers a drought tolerance by inhibiting inward K⁺ channel KAT1 and stomatal opening in Arabidopsis

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The changes of turgor pressure in guard cells resulted from the massive fluxes of osmotic ions into and out of guard cells through ion channels and transporters are the main driving force for stomatal movement [1]. The massive efflux of diverse osmotic anions are mainly mediated by slow and rapid anion channels for stomatal closure, whereas the influx of K⁺, the main osmotic cation and the counter ion of the diverse anions in guard cells, are mainly through inward K⁺ (K⁺in) channels [1]. K⁺in channels in guard cells are homo- and/or hetero-tetramers assembled with a few members of Shaker family in Arabidopsis, including AKT1, AKT2, AtKC1, KAT1 and KAT2, out of which KAT1 is the main one [2]. Cytosolic Ca²⁺ is involved in stomatal movement as an important second messenger in diverse stimuli-induced stomatal movement, and also plays an essential role in the cross-talks between different signaling pathways. The elevation of cytosolic Ca²⁺ concentration ([Ca²⁺]cyt) in guard cells is capable of down-regulating the activity of K⁺in channels in guard cells [3], and Ca²⁺ sensing proteins are believed to be the transmitters linking upstream stimuli to the downstream K⁺in channels. Several calcium-dependent protein kinases (CPKs) have been reported to be involved in stomatal movement by regulating the activity of K⁺in and K⁺out channels. The inhibitory effects of Vicia faba CPKs on KAT1 were observed in a Ca²⁺-dependent manner in the early study [4]. CPK8 and CPK10 are involved in the K⁺in channel regulation in a Ca²⁺-dependent manner [5], and CPK13 specifically inhibits KAT2 and KAT1 [6]. A recent screening revealed that several CPKs are capable of modulating the activity of K⁺in channel KAT1 and K⁺out channel GORK in Xenopus oocytes [7]. But the molecular mechanisms of K⁺in channels by CPKs in guard cells are still elusive.

To address this scientific question, we conducted a screening by testing the activity of KAT1 in Xenopus oocytes using voltage clamp technique. Six CPKs, including CPK3, 4, 6, 11, 21, and 23, were selected as the candidate regulators of the main K⁺in channel KAT1 in guard cells, and the cRNA of those CPKs and KAT1 were pooled and co-injected for voltage clamp experiments. Large K⁺in currents of KAT1 were readily recorded in oocytes expressing KAT1 alone as reported (Fig. S1a, b) [8], and K⁺in currents was not obviously reduced by the co-expression of KAT1 and the native CPKs compared with negative control (Fig. S1a, b), indicating that KAT1 was not obviously inhibited by the group of native CPKs. Then we generated truncated versions of the six CPKs by deleting their EF hand domains, the Ca²⁺ binding sites, to render Ca²⁺-insensitive and constitutive active forms of the CPKs as reported [9], and designated the truncated CPKs CPKCAs. We found that the K⁺in currents of KAT1 were reduced significantly by approximately 50% by the co-expression of KAT1 and those CPKCAs compared with the oocytes expressing KAT1 alone (Fig. S1c, d), indicating that one or a few CPKCAs are capable of inhibiting KAT1. Further voltage clamp experimental results showed that the activity of KAT1 was reduced by approximately 65% by the co-expression of KAT1 with CPK3CA and CPK6CA, not obviously by the coexpression of KAT1 with either CPK4CA + CPK11CA or CPK21CA + CPK23CA (Fig. S2a, b), indicating that KAT1 was inhibited specifically by either CPK3CA and/or CPK6CA. We eventually found that KAT1

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was not obviously inhibited by CPK3, CPK6 and CPK6CA (Fig. S2c, d), whereas the K⁺ currents of KAT1 were significantly reduced by CPK3CA in a dose-dependent manner when the cRNA amount of CPK3CA injected was increased and that of KAT1 was retained relative to the oocytes expressing KAT1 alone (Fig. 1a, b). Thus we found that the constitutive and Ca²⁺-independent CPK3CA is a strong negative regulator of KAT1. We also analyzed the biophysical properties of CPK3CA-mediated inhibition of KAT1. The half-activating voltage of KAT1 was shifted to negative voltage direction by CPK3CA (Fig. 1c and Table S1), and the co-expression of CPK3CA and KAT1 reduced the maximal cord conductance (Gmax) in a CPK3CA [cRNA]-dependent manner (Fig. 1d and Table S1). The inhibitory effects of KAT1 by native CPK3 was observed recently [7], but our observation is different. 25 ng cRNA was injected into each oocyte for each gene [7], but we only injected 6 ng cRNA which is much less than 25 ng. This could be the main cause of the different observation. KAT1 and KAT2 are close homologues to each other. We then tested the effects of CPK3CA on KAT2, and did not observe obvious inhibition (Fig. S3a, b). We also tested the effects of CPK3CA on the chimeric K⁺ channels KAT1ds2 and KAT2ds1 as reported recently [8], and found that the activity of KAT2ds1 (Fig. 1e, f), not that of KAT1ds2 (Fig. 1e, g), was dramatically inhibited by CPK3CA relative to the
oocytes expressing either KAT1ds2 or KAT2ds1 alone (Fig. 1e, g). Thus we revealed that the C terminus of KAT1 is the main domain for the inhibition of KAT1 by CPK3CA.

Protein interaction experiments using the split-ubiquitin system and bimolecular fluorescence complementation technique showed clear protein-protein interaction between KAT1 and CPK3CA (Fig. S4a, b online). The conserved Aspartic acid residue at position 202 in CPK3CA was substituted by an Alanine acid residue to render an inactive form of CPK3CA as reported [6], which was termed CPK3CA-DN, and further voltage clamp experimental data showed that KAT1 was significantly inhibited by CPK3CA, not by CPK3CA-DN, compared with the oocytes expressing KAT1 alone (Figs. 1h, S5 online). In vitro phosphorylation assay using histone as the substrate showed that CPK3CA, not CPK3CA-DN, has kinase activity (Fig. S6 online). Those data indicate that CPK3CA-mediated inhibition of KAT1 is dependent on protein phosphorylation of KAT1 by CPK3CA.

To test whether the inhibition of KAT1 by CPK3CA had any physiological significance in vivo, we generated transgenic lines by introducing either CPK3CA or wild type CPK3 into Arabidopsis under a guard cell-specific promoter GC1 [10]. Two transgenic lines over-expressing CPK3CA were selected and designated CPK3CA-33 and CPK3CA-44 (Fig. S7a, b online), and two transgenic lines expressing wild type CPK3 were selected and designated CPK3-OE2 and CPK3-OE9 (Fig. S8a online). We conducted stomatal movement assay using epidermal peels, and observed a significant impairment of light-induced stomatal opening (Fig. 1i) and an ABA-hypersensitive phenotype in CPK3CA-33 and CPK3CA-44 (Fig. 1j) compared with wild type. But no stomatal movement phenotype was observed in CPK3-OE2 and CPK3-OE9 compared with wild type (Fig. S8b, c online). Water loss assay of detached leaves showed that CPK3CA-33 and CPK3CA-44 lost their fresh weight significantly slower than wild type (Fig. 1k), and the intact CPK3CA-33 and CPK3CA-44 seedlings showed a drought resistance phenotype relative to wild type (Fig. 1l). We also conducted light-induced stomatal opening in cpk3-1 mutant, and did not observe obvious difference between the mutant and wild type plants (Fig. S9 online). Thus this research reveals that the constitutive active CPK3 is involved in stomatal movement regulation by negatively regulating KAT1 in a Ca2+-dependent manner in Arabidopsis.

Conflict of interest

The authors declare that we have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.scib.2018.07.011.

References