**Allelic variation in the vacuolar TPK1 channel affects its calcium dependence and may impact on stomatal conductance**

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**SUMMARY**

Natural variation can be exploited to identify allelic variants of proteins. In this study patch clamp was used to determine transport properties of two AtTPK1 alleles from Landsberg and Kas-2 ecotypes. No difference in conductance or ion selectivity was observed but the Kas version of TPK1 showed different Ca2+ dependence in its open probability compared to Ler. Leaves from Kas showed lower rates of water loss than those of Ler, in either the absence or presence of ABA, an observation that is consistent with higher TPK1 channel activity at comparable cytoplasmic Ca2+ concentrations. A model that explains the results is presented.

**KEY WORDS**

Arabidopsis, TPK1, stomata, patch clamp, SNP, polymorphism, water loss

**HIGHLIGHTS**

-Kas and Ler TPK1 sequences differ in 5 SNPs

-The Kas TPK1 allele shows different Ca2+ dependent open probability

-Increased Kas TPK1 open probability may be caused by an SNP in the C-terminal EF hand

-Kas leaves show less water loss compared to Ler leaves which may result from lower stomatal conductance in Kas

**INTRODUCTION**

TPK (two pore K+) proteins are potassium selective ion channels encoded by small gene families. In Arabidopsis, four out of five TPK isoforms are expressed on the vacuolar membrane (tonoplast) whereas in rice at least two tonoplast isoforms have been reported. TPK activity is controlled by cytoplasmic factors such as Ca2+ and 14-3-3 proteins (Latz et al., 2007; Isayenkov et al., 2011) but also by mechano-stimuli (Maathuis, 2010). TPKs can catalyse both inward (into the cytoplasm) and outward (into the vacuole) current but, due to prevalent electrochemical gradients, are likely to mediate vacuolar K+ efflux in most cells.

The physiological role of most vacuolar TPKs is unclear but all four were able to complement *Escherichia coli* cells deficient in K+ uptake (Isayenkov and Maathuis, 2013). Out of these, AtTPK1 is the best characterised TPK channel. It was initially identified as the vacuolar K+ (VK) current by Ward and Schroeder (1994). Its expression is ubiquitous and a number of physiological functions has been ascribed to AtTPK1 and its close orthologues, including a role in stomatal closure (Ward and Schroeder, 1994; Gobert et al., 2007), salt tolerance (Latz et al., 2013) and general K homeostasis (Gobert et al., 2007; Hamamoto et al., 2008). AtTPK1 is very well characterised in terms of transport properties (e.g. conductance, voltage dependence, ion selectivity, pH dependence, Ca2+ dependence) but it is unknown if and how these properties are modulated by natural variation.

The ‘1001 genome sequencing’ project (http://1001genomes.org/datacenter/) has revealed a wealth of information regarding natural variation in both coding and non-coding sequences of the Arabidopsis genome. However, in the majority of cases it is completely obscure whether any of the identified polymorphisms has physiological relevance in the sense that they impact on plant phenotype. The AtTPK1 coding sequence is 363 amino-acids in length and, using the genome of the Col-0 ecotype as a reference, can contain up to 7 SNPs (single nucleotide polymorphisms). SNPs can drastically alter protein function. For example, a single SNP in an arsenate reductase rendered this protein virtually inactive in the Arabidopsis Kr-0 ecotype, leading to large changes in tissue arsenic contents (Chao et al., 2014).

With the publication of multiple Arabidopsis thaliana genomes we are now in a position to evaluate if and how allelic variation affects TPK protein function. The electrophysiological properties of two allelic variants of AtTPK1 derived from two Arabidopsis ecotypes (Landsberg, or Ler-0, and Kas-2) were compared. No difference in unitary conductance or ion selectivity was observed but a distinct difference in Ca2+ dependent channel open probability was recorded with the Kas variant consistently showing more channel activity at comparable cytoplasmic Ca2+ concentrations. A model that explains the results in the context of TPK1 channel gating is discussed.

**METHODS**

**Plant material and growth:** *Arabidopsis thaliana* (L) ecotypes Landsberg-0 (Ler) and Kas-2 (Kas) were grown hydroponically as described previously (Gobert et al., 2007) in a growth room under short day conditions (10 h light at 200 µmol.m-2.s-1 intensity, 14 h dark, 23°C day, 17°C night temperature). The growth medium contained 1.25 mM KNO3, 0.5 mM Ca(NO3)2, 0.5 mM MgSO4, 0.625 mM KH2PO4 as macronutrients and was renewed weekly.

**Protoplast and vacuole isolation:** Leaf protoplasts were isolated as described previously (Maathuis et al., 1998) and vacuoles were released by washing protoplasts with a solution containing 10 mM EDTA, 10 mM EGTA, pH 8 with an osmolarity of 350 mOsM. Patch clamp apparatus and methodology were as described (Maathuis, 2011; Maathuis et al., 1998). Briefly, current was recorded using an EPC7 (List , Darmstadt, Germany) amplifier and data acquisition occurred through a CED (Cambridge, UK) A/D converter at 1 to 3 kHz and data were filtered at 0.5 kHz. CED Patch v5.5 software was used for data analysis. Glass pipettes were pulled from Kimax (Kimble, Ohio, USA) glass using a List electrode puller. Standard experimental solutions for bath and pipette contained 100 mM KCl, 0.1 mM CaCl2, 5 mM MES/Tris pH 6.0 and sorbitol adjusted to 430 mOsm total osmolarity. To control free cytoplasmic Ca2+ concentrations, solutions containing 100 mM KCl, 1 mM CaCl2, 5 mM MES/Tris pH 7 were buffered with various concentrations EGTA to obtain free [Ca2+]cyt of 0, 0.01, 0.05, 0.1, 0.5, 1, 10 and 100 µM. Free [Ca2+]cyt was calculated using the ‘Calcium’ software (Foehr and Warchol, unpublished, Dept of Biophysics, Pozan, Poland). Open probability (Po) is expressed as Po = (t\_open/t\_total)/n \*100 where ‘n’ is the number of channels in the membrane patch (Gobert et al., 2007). Open probabilities were normalised to the maximum Po at saturating free [Ca2+]cyt of 1 mM. Recordings of 60 s duration at a membrane potential of 50 mV were used to determine Po. Open probability data are given as the average ± SD using vacuoles from 4 to 11 individual protoplasts for each ecotype and Cacyt concentration. Kd values for Ca2+ induced changes in Po were calculated by fitting the semilog data to a sigmoidal curve using the following equation: y=a-d/1+(x/c)^b.

**Protoplast transformation and TPK1 expression:** mEYFP cDNA was amplified using primers containing the BamHI site and inserted into pART7 (Isayenkov et al., 2011) to create pART7-EYFP. Ler-0 and Kas-2 specific TPK1 cDNAs were amplified from leaf RNA using the forward primer TPK\_Xho\_for GCCTCGAGATGTCGAGTGATGCAGCT and reverse primer TPK\_Sma\_rev GCCCCGGGCCTTTGAATCTGAGACGT and inserted into the pART7 vector to create pART7-EYFP-TPK1 under control of the CaMV 35S promoter. Mesophyll protoplasts isolated from a *tpk1* null mutant (Gobert et al., 2007) were transformed according to the protocol described by Abel and Theologis (1994). Fluorescent vacuoles were patch clamped 24-48h after transformation.

**TPK1 and EF-hand modelling:** Using a locally run version of ‘Deep View Swiss PdbViewer’ (v4.0.1. Guex et al., 1997), a model of the AtTPK1 monomer was created using the human two pore domain K+ channel TRAAK (KCNK4 or K2P4.1) and 4I9W (RCSB PDB: http://www.rcsb.org/pdb/explore.do?structureId=4I9W) as a template. An approximate dimer was generated by superimposition of the modelled monomers onto the two subunits of the TRAAK channel. The first TPK1 EF-hand motif (amino acid 292-313, containing the N295S SNP) was modelled on the parvalbumin EF-hand 1A75 (PDB entry domain 17216) and the effect of residue substitution was assessed using the ‘mutation’ facility in Deep View.

**Leaf Dehydration Assay**

The 4th-7th leaves of four week old plants were detached at midday and placed in petri dishes with 25 ml of stomatal opening buffer (50 mM KCl, 0.1 mM CaCl2 in Mes-KOH at pH 6.15) for 90 minutes. Three leaves from each genotype were then treated with 50 μM ABA for 30 minutes. Control leaves received ethanol only. Leaves were then blotted dry and weighed before being placed in a fume hood. Each leaf was then re-weighed every half hour for three hours. Experiments were repeated three times. Log transformation of data was carried out and gradient differences of linear regressions were tested (P < 0.05) between ecotypes and treatments using a mixed linear model with leaf identity added as a random effect to account for self-similarity between repeated measurements of leaves (Kuznetsova et al., 2013).

# Whole leaf conductance measurements

Leaves of mature plants were incubated in ‘opening’ buffer consisting of 10 mM KCl, 10 mM MES-KOH pH 6.15 for 2 h in the light to induce maximum opening. Subsequently, leaves were treated as described above and H2O gas exchange was determined using a Infrared Gas Analyser, Li-Cor 6400 (LI-COR, Cambridge, UK) at t=0, 30 and 90 minutes. A total of 9 leaves, derived from 3-4 different plants, per ecotype was used and data are presented as the mean±SE. Ecotype differences were statistically tested using Student’s t-test at P < 0.05.

**Stomatal Density Assays**

Three detached 4th-7th leaves of four week old plants of each genotype had clear nail polish applied to the abaxial side. Clear adhesive tape was used to remove the nail polish once dried and stomata were counted at three locations across the leaf surface, avoiding the mid-vein. Leaf impressions were viewed at 40x magnification and the number of stomata was determined in 3 different regions of mature leaves.

**RESULTS AND DISCUSSION**

*TPK1 proteins contain two non-synonymous SNPs*

Of the 7 identified SNPs in the TPK1 coding sequence, 5 are synonymous (N26; T91; A199; S243 and L269) and 2 (S261T and N295S) are non-synonymous (Fig. 1). Synonymous substitutions do not change protein sequence and therefore are unlikely to affect protein function (Ng and Henikoff, 2006). The non-synonymous SNPs in TPK1 are found in the 4th transmembrane domain (TMD) and in the 1st EF-hand which is present in the cytoplasmic C-terminus. Apart from very few exceptions, ecotypes either show the S261; N295 or the T261; S295 genotype in an approximate ratio of 5 to 1 across the 1001 SALK listed ecotypes. The S261; N295 or the T261; S295 genotypes from now on will be referred to as the ‘Ler’ and ‘Kas’ genotypes.



*Ler and Kas genotypes show different Ca2+ dependence.*

Both Ler and Kas vacuoles were subjected to extensive patch clamp analyses, predominantly using cytoplasmic side out excised tonoplast patches, to assess if there were any differences in channel properties between these ecotypes. Fig. 2 shows representative currents from a Ler and a Kas patch, showing increasing channel activity when the cytoplasmic Ca2+ concentration ([Ca]cyt) is raised.



The single channel conductance was the same for Ler and Kas with 53±3.4 and 26±2.1 pS for inward and outward current in the presence of 100 mM KCl for Ler and 57.6±5.5 and 28±2.5 pS for Kas. Similarly, the K:Na selectivity (calculated from the Nernst potential using 100/100 KCl/NaCl) did not differ and was 43±5 (n=3). One regulatory mechanism that modulates TPK1 open probability, and therefore protein activity, is [Ca]cyt (Gobert et al., 2007; Hamamoto et al., 2008). The impact of a range of [Ca]cyt (from nominally 0 to 0.5 mM) was therefore tested on both genotypes. Fig. 3 shows that TPK1 Ca2+ dependence in the Ler background is less steep than that for Kas. Fitting of the semilog data using a sigmoid function to calculate values for Ca2+ binding revealed Kd values of 8.3 (R2=0.984) and 23.2 µM (R2=0.991) respectively for Ler and Kas.

Although it has been shown that AtTPK1 most likely functions as a homodimer (Voelker et al., 2006) other TPK isoforms (TPK2, 3 and 5) also express at the tonoplast (Voelker et al., 2006) and SNPs in these could therefore in principle affect TPK1 current properties. Supplemental figure 1 shows that TPK2 and TPK5 do not contain any SNPs between Ler and Kas. In contrast, TPK3 has a serine at position 434 (Kas) or glycine (Ler) at the C terminus. Recently it was shown that TPK3 expresses at the thylakoid membrane (Carraretto et al., 2013) and therefore the S434G SNP is extremely unlikely to affect the data reported in figure 3.

To obtain more certainty that the differential [Ca]cyt effect is an innate TPK1 property, we repeated a number of [Ca]cyt values using transient expression of Ler and Kas TPK1 in the *tpk1* null mutant background (Gobert et al., 2007). The figure 3 inset shows that the difference in Ca2+ dependence between Ler and Kas is still apparent when using this homologous expression strategy. The similarity between native membrane properties and those of vacuoles that transiently express Ler or Kas TPK1, is strong evidence that the observed difference in Po calcium-dependence is linked to TPK1 protein properties and not to other, ecotype related phenotypes.

Figure 3 also shows that there is a residual open probability in the Kas background, even when the free [Ca]cyt is nominally zero (no added Ca2+ and 5 mM EGTA). In this sense Kas resembles rice (Isayenkov et al., 2011) and tobacco (Hamamoto et al., 2008) TPKs which also show residual TPK activity when [Ca]cyt is zero. Interestingly, both rice TPKa and TPKb contain a methionine at the corresponding position (Suppl. Fig 2). In contrast, the Ler Ca2+ dependence resembles that of Col-0 (Gobert et al., 2007) and *Vicia faba* (Ward and Schroeder, 1994). Thus, everything else equal, one could argue that at 'resting' levels of [Ca]cyt, which are typically around 200 nM, Kas



TPK1 activity would be greater than that of Ler TPK1 and this may also be the case in the presence of a Ca2+ signal (which typically remain below a few micromolar).

*Different TPK1 versions affect water loss*

The most significant role of TPK1 channels is probably in vacuolar K+ release during stomatal closure (Ward and Schroeder, 1994; Gobert et al., 2007). To assess whether the different Ca2+ dependencies of Ler and Kas TPK1 are translated into different stomatal phenotypes, we grew Ler and Kas ecotypes to maturity and recorded water loss from their rosette leaves. Figure 4 shows that Ler leaves have a higher rate of water loss than those of Kas. When leaves were treated with ABA before water loss measurements, the rate of evaporation was reduced in both ecotypes but remained significantly higher in Ler. The data suggest that leaf conductance in Ler is higher. We therefore measured whole leaf conductance using an infra red gas analyser. Fig 4c shows that, although slightly lower in Kas, initial leaf conductances are similar. During water loss assays, conductance drops in both ecotypes but more quickly in Kas, culminating in a significantly lower (p<0.02) value in this ecotype after 90 minutes. This difference was not due to stomatal density which is actually 15-20% lower in Ler than in Kas and as such would lead to less evaporation rather than more.



In combination our results therefore suggest that evaporation in Ler leaves exceeds that of Kas leaves because of greater stomatal apertures. At the same time, our transient expression data strongly suggest that the difference between Ler and Kas in calcium dependent Po is an intrinsic property of the various TPK1 isoforms. Although conclusive proof is not available from our data, it is tempting to relate these differences in channel properties to the altered phenotype that was found in the intact leaves. Thus, our hypothesis is that the reduced calcium responsiveness in Ler may translate in a higher steady state stomatal conductance and/or less responsiveness to ABA.

*Natural variation in the C-terminal EF-hand could alter gating properties*

The non-synonymous SNPs are at positions 261 and 295. Position S261T is approximately in the middle of TMD4. Threonine is slightly less polar and has an extra methyl group compared to serine. These minor differences are unlikely to affect either the membrane spanning helix or channel function at large. It is even less likely to affect channel Ca2+ kinetics. In contrast, the second SNP is in the first EF-hand motif where it changes the bulky asparagine side group for the much smaller serine side group. N295S could therefore interfere with Ca2+ binding or it might affect the interaction of the EF domains with the channel gate. EF hands typically consist of a 9 residue helix, followed by a loop stretch of 9 residues that is involved in Ca2+ coordination, and a second 11 residue helix (Fig 5a). N295S is located in the first helix at the 3rd residue position. Since it is not in the Ca2+ coordination loop, it is unlikely that N295S directly impacts on Ca2+ binding. However, N295S could alter the interaction between EF-motif and channel gate. How Ca2+ binding translates into changes in TPK1 open probability is unknown. Somehow, conformational changes in the EF-hand lead to interaction of the EF domain with the channel gate. For example, during the transition of an EF domain with bound Ca2+ bound to an empty EF-hand, the helices move from a more antiparallel arrangement in the non binding configuration, to being nearly perpendicular when Ca2+ is fully coordinated (Gifford et al., 2007).



Such a structural rearrangement could trigger association of the EF-hand with the channel gate which is typically located near the cytoplasmic side of the membrane (Fig. 6a). In analogy with K2P type mammalian channels (Lolicato et al., 2014), gating may rely on the position of the cytoplasmic C-termini; when these are suspended in the cytoplasm they force membrane helices 2 and 4 to buckle and occlude the pore (Fig. 6b). When the C-termini ‘swing apart’ mechanical strain in the membrane coils is relieved allowing the protein to attain the open conformation (Fig. 6c). In an analogous model for TPK type channels, C-terminal movement would be facilitated by Ca2+ binding, after which contact between the EF-hand helices and other parts of the channel protein may be important to maintain the open position. Thus, in this model the smaller side group of the Ser in the Kas genotype could promote stabilisation of the interaction between EF-hand and TMD spans.



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**Figure legends**

**Figure 1:** Protein sequence of *A thaliana* cvs Landsberg 0 and Kas-2 isoforms of AtTPK1. Dark green and red highlights identify synonymous and non-synonymous polymorphisms (relative to the reference sequence of *A thaliana* cv Col-0 ). Turquoise indicates EF hand domains and green sequence denotes transmembrane spans with GYGD pore region in bold.

**Figure 2:** Representative patch clamp recordings of cytoplasmic side out, excised patches from mesophyll vacuoles derived from Landsberg-0 (a) and Kas-2 (b). Arrows on the left denote closing levels at 50 mV holding potential. Numbers on the right indicate cytoplasmic [Ca2+] in µM. The I/V (current/voltage) plot (c) shows no difference in channel conductance between cultivars (bars denote SE and n=3).

**Figure 3:** **Figure 3:** The sensitivity of TPK1 open probability to cytoplasmic Ca2+ is different between Ler-0 and Kas-2 cultivars. Cytoplasmic Ca2+ was varied from 1 nM to 0.5 mM and open probability measured of native Kas and Ler tonoplasts. Kas cultivar shows more open probability at low [Ca2+] and a steeper increase, especially in the range of around 0.5 to 10 µM. Inset shows similar results using homologous expression of either Ler or Kas TPK1 in a *tpk1* null mutant. Data were fitted to a sigmoidal function and yielded a Kd value of 8.3 µM (R2 =0.984) and 23.2 µM (R2 =0.991) for Ler and Kas respectively.

**Figure 4:** Dehydration assay carried out on Kas and Ler leaves. The main plot (a) shows how mass declined for three hours in control and ABA treated leaves. The bar chart (b) shows the average rate of water loss over the first 30 minutes in control and ABA treated leaves. (c) Leaf conductance in the two ecotypes during the first 90 minutes of dehydration assays. (d) Relative stomatal density of Kas and Ler ecotypes. Points and bars are the mean of 9 leaves from 3 experiments and error bars indicate the S.E.M.

**Figure 5:** **(a)** Generalised Ca2+ coordination and topology of EF Ca2+ binding motif with the Ca2+ ion depicted as green sphere. In **(b)** and **(c)** the first helix of the EF 'helix-loop-helix' configuration is shown for respectively Ler-0 and Kas-2. Note the presence of the much larger side chain of asparagine in Ler-0 compared to the serine at the same position in Kas-2.

**Figure 6:** **(a)** Topographic model of TPK1 based on mammalian TRAAK K+ channel (code K2P4.1) showing position of K+ pore on the luminal side and the C-terminal EF hands with coordinated calcium on the cytoplasmic side. A hypothetical model of how EF hand Ca2+ binding could lead to conformational changes in transmembrane domains is based on that proposed by Lolicato et al., 2014 for K2P channels. **(b)** The absence of Ca2+ keeps transmembrane domains 2 and 4 in a kinked configuration which occludes the pore. **(c)** Ca2+ binding releases strain on transmembrane domains and allows ion conduction.

**Supplementary figures**

**Figure S1:** Alignments of other TPK isoforms from Arabidopsis. Red highlights identify non-synonymous polymorphisms (relative to the reference sequence of A thaliana cv Col-0 ). Turquoise indicates EF hand domains. TPK4 expresses in the plasma membrane specifically in pollen tissue (Becker et al., 2001) whereas TPK3 expresses in thylakoid membranes (Carraretto et al., 2013).

**Figure S2:** Alignment of C terminal section with non-synonymous SNPs in Arabidopsis (Col-0 ecotype) and the rice OsTPKa and TPKb.