Loss-of-Function of Constitutive Expresser of Pathogenesis Related Genes5 Affects Potassium Homeostasis in Arabidopsis thaliana

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Abstract

Here, we demonstrate that the reduction in leaf K⁺ observed in a mutant previously identified in an ionic screen of fast neutron mutagenized Arabidopsis thaliana is caused by a loss-of-function allele of CPR5, which we name cpr5-3. This observation establishes low leaf K⁺ as a new phenotype for loss-of-function alleles of CPR5. We investigate the factors affecting this low leaf K⁺ in cpr5 using double mutants defective in salicylic acid (SA) and jasmonic acid (JA) signalling, and by gene expression analysis of various channels and transporters. Reciprocal grafting between cpr5 and Col-0 was used to determine the relative importance of the shoot and root in causing the low leaf K⁺ phenotype of cpr5. Our data show that loss-of-function of CPR5 in shoots primarily determines the low leaf K⁺ phenotype of cpr5, though the roots also contribute to a lesser degree. The low leaf K⁺ phenotype of cpr5 is independent of the elevated SA and JA known to occur in cpr5. In cpr5 expression of genes encoding various Cyclic Nucleotide Gated Channels (CNGCs) are uniquely elevated in leaves. Further, expression of HAK5, encoding the high affinity K⁺ uptake transporter, is reduced in roots of cpr5 grown with high or low K⁺ supply. We suggest a model in which low leaf K⁺ in cpr5 is driven primarily by enhanced shoot-to-root K⁺ export caused by a constitutive activation of the expression of various CNGCs. This activation may enhance K⁺ efflux, either indirectly via enhanced cytosolic Ca²⁺ and/or directly by increased K⁺ transport activity. Enhanced shoot-to-root K⁺ export may also cause the reduced expression of HAK5 observed in roots of cpr5, leading to a reduction in uptake of K⁺. All ionic data presented is publically available at www.ionomicshub.org.

Introduction

Potassium (K⁺) is a macro nutrient essential for normal plant growth and development. It participates in numerous physiological processes including regulation of enzyme activity, cell expansion, stomata movement and defence towards pathogens [1,2]. Numerous mechanisms are known to be involved in K⁺ homeostasis, and many K⁺ channels and transporters have been identified. In plants K⁺ is highly mobile. Its movement into the xylem is driven by transpiration, and in the phloem by the specific requirements of tissues and organs [3,4]. Environmental conditions also regulate the movement of K⁺. For example, under drought stress the concentration of K⁺ in the xylem decreases, possibly because of the closure of stomatal pores induced by abscisic acid (ABA) [5].

The effects of low K⁺ have been investigated in some depth, and it has emerged that jasmonic acid (JA) plays a central role in the response of plants to K⁺ shortage [6,7]. Nevertheless, whether the application of K⁺ is beneficial or not in conferring resistance towards pathogens is controversial [8]. At the molecular level the role of K⁺ is clearer. Plant-pathogen interactions cause an increase in cytosolic Ca²⁺ triggering anion channel activation, plasma membrane depolarization, activation of K⁺ permeable efflux channels leading to enhanced K⁺ efflux and the initiation of the hypersensitive response (HR) [9,10].

In this study, we describe the characterization of an A. thaliana mutant with a 10–30% reduction in leaf K⁺ which was previously identified in an ionic screen of fast neutron mutagenized plants [11]. Genetic analysis revealed this mutant to be a new null allele of CPR5, a gene originally identified in two independent screens for altered response to pathogens [12,13]. cpr5 has a high content of salicylic acid (SA) and shows constitutive expression of pathogenesis related genes (PR), as well as plant defensin PDF1.2 which is a marker of the JA-dependent pathway. Therefore, it has been suggested the CPR5 is a negative regulator of local defence response to pathogens [14]. CPR5 is also implicated in cell senescence [15–17], cell proliferation and trichome development [18], cell wall biosynthesis [19] redox balance [20], and water relations via enhanced ABA sensitivity [21]. Here, we show that CPR5 is also associated with K⁺ homeostasis possibly via modulation of expression of various CNGCs and HAK5.

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Materials and Methods

Plant growth and mutant screening

Fast neutron-mutagenized M2 A. thaliana seeds were purchased from Lehle Seeds (Round Rock, TX) and plants screened for their leaf elemental profile by ICP-MS [11]. Seeds of Col-0 (CS6000) and cp3-2 (CS3770) were provided by the Arabidopsis Biological Resource Center (The Ohio State University).

For non-axenic conditions, plants were grown in pots containing moist soil (Scotts Potting Medium, Scotts-Sierra Horticultural Products Company, Marysville, OH) in a climate-controlled room (temperature 19–22°C, day-night; humidity 60%; photoperiod 10–14 hours light-dark; light intensity 100 μmol m−2 sec−1) and bottom watered at regular intervals with a solution containing 0.25× Hoagland’s macro and micronutrients [11].

For plants grown in axenic conditions, surface sterilized seeds were stratified at 4°C in the dark for five days before sowing. To measure the expression of HAK3 and AKT1 plants were grown for 2 weeks on solidified medium containing 1/20th MS salts accordingly to Cheong et al., [22] and containing 20 mM or 100 μM of KCl, and in a second experiment on a minimal medium without NH4Cl [23] with 10 g L−1 UltraPure sucrose (Sigma) and solidified with 10 g L−1 pure agarose (Molecular Biology Grade, Research Products International Corp.), which contains negligible amounts of K+ (approximately 8 μg Kg−1 as determined by ICP-MS analysis). K+ was added as KCl at the final concentration of 10, 50 and 100 μM and the pH adjusted to 5.8 with Ca(OH)2. K+ content in root and shoot was measured in plants grown for 2 weeks on solidified medium containing 1/20th MS salts accordingly to Cheong et al., [22] and containing 20 mM of KCl.

Genetic analysis

cpr3-2 was outcrossed to A. thaliana Landsberg erecta (Ler-0) accession, F1 seeds were planted on 0.5× MS medium solidified with 10 g L−1 of agar and seedlings visually screened for hyponastic and early yellowing cotyledons. All seedlings of F1 generation were selected for small size and yellow early senescing leaves and used to map the mutation with a positional cloning approach with single strand length polymorphism (SSLP) markers.

Quantitative real-time PCR

Total RNA was extracted with the Qiagen RNasyPlant Mini Kit (http://www.qiagen.com) from five weeks old plants grown on soil or from two week old plants grown on plates. DNase digestion on column was performed to eliminate possible contamination with DNA. Two micrograms of total RNA were used as a template to synthesize first-strand cDNA with SuperScript VILO cDNA Synthesis Kit (InvitrogenLife Technologies, http://www.invitrogen.com). Quantitative real-time PCR was performed with the SYBR Green reagent mix in a StepOnePlus instrument according to the manufacturer’s instructions (Applied Biosystems, California, USA). The expression of K+ channel transporter genes was detected with the following set of primers: HAK3, 147 (5′-TGCGTGATCAGGTCATCTTGATCTTGTC-3′) and 148 (5′-AAAGCAGGATATTGGCAAACAT-3′); AKT1, 170 (5′-TCTAANATTGTTTCTCTTCTCTGTAAGA-3′) and 171 (5′-CCCTCCTGCG-GTCCTGCTGCAA-3′); CPR5, 139 (5′-TTTCTCATTGTTGCGAT-3′) and 138 (5′-AATGCCCTTTTTGCTGGCAT-3′); CNGC10, 196 (5′-GATGAACGATGCTTATCATGAC-3′) and 197 (5′-CTAACCAGATGCTTATCATGAC-3′). As an internal reference the expression of PP2A (At1g13320) was used since it showed stable expression throughout the experimental series of development, shoot and root abiotic stress, hormones, nutrient stress, light and biotic stress [24], all of which are affected by loss-of-function of CPR3. In addition to PP2A the expression of HAK3 and AKT1 was also normalized to UBQ10 (At4g05320). UBQ10 also shows stable expression in cp3-2. The average value from real-time PCR measurements from at least three independent biological replicates was used to evaluate transcript abundance. Biological replicates were composed of tissues harvested from between 5 and 10 plants for analysis of shoot tissue, and between 25 and 30 plants for analysis of root tissue, from plants grown on plates. For plants grown in soil 2–3 leaves from at least 3 individual plants were used. Steady state mRNA levels were calculated relative to a reference gene and presented based on the 2−ΔΔCt method [25].

Determination of the elemental content of plant tissues

Plants grown in soil were non-destructively sampled by removing 1–2 leaves (approximately 3 mg dry weight), rinsed with 18 MΩ water, placed into Pyrex digestion tubes and dried at 92°C for 20 hours. Alternatively, shoots and roots were harvested from plants grown on 1/20th MS medium modified accordingly to Cheong et al. [22] as previously described. After cooling, all samples were digested with 0.7 mL concentrated nitric acid (OmniTrace, VWR) and diluted to 6.0 mL with 18 MΩ water. Acid used for digestion was spiked with gallium (Ga) to act as an internal standard to control for errors in dilution, variations in sample introduction, and plasma stability in the ICP-MS instrument. Sample sets also contained analytical blanks, standard reference material (NIST SRM 1547) digested in the same manner as the plant samples, and quantitative calibration standards. Calibration standards and standard reference material samples were included at the beginning and end of the sample sets to control for drift during the analysis. Samples were introduced into an inductively coupled plasma mass spectrometer (ICP-MS) (Elan DRCe, PerkinElmer) and analyzed for Li, B, Na, Mg, P, K, Ca, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Mo and Cd. All samples were normalized to calculated weights, as determined with an iterative algorithm using the best-measured elements, the weights of the seven weighed samples, and the solution concentrations, detailed at www.ionomicshub.org.

Determination of SA content

Total SA content was quantified in leaves of five weeks old plants using a Waters Alliance HPLC system equipped with Millenium software, 2695 Separation Module, 2475 Fluorescence Detector, and 2996 Photodiode array detector. A Nova-Pack C-18 column was used with a flow rate and methanol gradient as described previously [26]. SA (Sigma; catalogue no. S-6271) was used to develop the standard curve for quantification.

Grafting

cpr3-2 and Col-0 seeds were germinated in the dark on 0.5× MS plates containing 1 mL L−1 of MS Vitamins (Caisson Laboratories, Inc.), 3 mg L−1 Benomyl (methyl 1-(butylcarbamoyl)-2-benzimidazolcarbamate; Sigma), 0.04 mg L−1 BA (6-benzylaminopurine; Sigma), 0.02 mg L−1 IAA (indole acetic acid; Sigma), and 12 g L−1 agar. Plates were held vertically and after seven days
seedlings were grafted as previously described [27] and then grown for an additional seven days on plates before transfer into soil. Plants were grown for a further four weeks in soil before being analyzed for their elemental content. Plants that from a visual inspection showed adventitious roots coming from the shoot above the graft were excluded from the experiment.

Statistical analysis

ANOVA was conducted using the software CoStat 6.2 (CoHort Software, CA, USA). Separation of means was performed using LSD test at P = 0.05 significance level.

Results

Mutant identification

Fast neutron mutagenized A. thaliana Col-0 plants were previously screened for altered leaf elemental composition [11]. In this screen 12645 was identified as a low K+ mutant with a reduction in leaf K+ of approximately 20% compared to wild-type Col-0 (raw data are available at www.ionomicshub.org, experimental tray 229) (Fig. 1). In addition, 12645 was also smaller in size than wild-type Col-0 and developed symptoms of hypersenescence in cotyledons and mature leaves. Moreover, cotyledons of mutant plants were hyponastic when seeds were germinated in soil, as well as in plates.

Genetic analysis

To map and define the Mendelian character of the mutation, 12645 was out-crossed to Ler-0, and F1 and F2 populations scored for mutant like plants. All F1 plants from this cross looked wild-type, while in the F2 small plants with early senescent leaves segregated with a ratio of 3:1 (wild-type to mutant phenotype), confirming to be the case for a single recessive nuclear mutation. Small hypersenescent plants from the F2 population also showed reduced K+ content (38.3 μg g⁻¹ dry weight) compared to those that looked wild-type (42.8 μg g⁻¹ dry weight), indicating that the traits of early senescence and small size co-segregate with reduced leaf K+ (P<0.001).

The chromosomal position of the 12645 mutation was determined using a positional cloning approach with SSLP markers in 171 F2 plants from the outcross with Ler-0. The recombinant population scored for markers in the region between nucleotides 23,500,000 and 26,100,000 on chromosome 5 revealed that only one plant had Ler-0 alleles on the BAC clone MUB3 and two plants on F15O5, which placed the mutation on the BAC clone MXK3 (Fig. 2A). DNA sequencing of this region revealed that 12645 contains a 972 base pair insertion at nucleotide 1474 in the fourth exon of the At5g64930 gene (Fig. 2B). This insertion originated from nucleotides 77,802–78,764 on chromosome 1, a region that does not contain any annotated loci. A. thaliana mutants carrying recessive loss-of-function alleles of At5g64930 have been previously and independently identified in screens for constitutive expression of pathogen resistance, and the early appearance of hypersenescence symptoms, and named cpr5-1 [13], cpr5-2 [12] and hys1 [17]. Based on the previous nomenclature we renamed 12645 as cpr5-3. As expected CPR5 expression is lost in cpr5-3 (Fig. S1A). All cpr5 mutants (cpr5-1, cpr5-2 and cpr5-3) were grown together, along with the wild-type Col-0 and the concentration of Li, B, Na, Mg, P, S, K, Ca, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Mo, Cd in leaves of each genotype determined by ICP-MS (n = 10–22 replicate plants per genotype). Of all the elements measured only K+ was found to be significantly different between the wild-type Col-0 and all the cpr5 alleles (P<0.005 after Bonferroni correction for multiple testing). Wild-type Col-0 had a leaf K+ concentration of 40.067±6222 μg g⁻¹ dry weight, compared to 27.490±3575 μg g⁻¹ dry weight for cpr5-1, 29.751±2616 μg g⁻¹ dry weight for cpr5-2, and 27.324±2502 μg g⁻¹ dry for cpr5-3 (raw data are available at www.ionomicshub.org, experimental tray 1020).

Figure 1. Leaf ionomic profile of cpr5-3 (12645). The concentration of each element was measured in leaves of five weeks old plants of both 12645 (M3 generation) (black lines; N = 21) and wild type Col-0 (blue lines; N = 12) plants. For each element given on the abscissa the correspondent z-score value is given on the ordinate. The z-score represents the number of standard deviations of the wild-type Col-0 each plant differs from the mean of the wild-type Col-0 (raw data are available at www.ionomicshub.org, experimental tray 743). doi:10.1371/journal.pone.0026360.g001
question, whether low leaf K\(^+\) in cpr5 results from the constitutively high SA in this mutant. To address this we measured K\(^+\) content in three different cpr5 alleles, in the single eds5-1 mutant, and in the double mutant cpr5eds5 prepared from cpr5-1 and eds5-1 [14]. The mutation in eds5-1 suppresses the biosynthesis of SA and in the double mutant cpr5eds5 the elevated SA content of cpr5-1 is returned to the level of the wild-type [14,28,29]. As shown in Fig. 3A, K\(^+\) content in eds5-1 leaves is slightly reduced but not significantly different from Col-0 whereas in cpr5eds5, which has low SA but carries the cpr5-1 mutation, the content of K\(^+\) is similar to that of single cpr5 alleles (raw data are available at www.ionomicshub.org, experimental tray 1020).

We also followed a similar approach to determine if the low leaf K\(^+\) of cpr5 is dependent on the elevated JA in this mutant [16]. The jasmonate resistant 1 (jar1) mutant is unable to conjugate JA with isoleucine to form the active jasmonoyl-L-isoleucine form of JA that is required to elicit the JA response [30], and therefore this mutant is insensitive to JA [31]. Fig. 3B shows that the double mutant cpr5jar1, prepared from cpr5-1 and jar1-1 [14], and single allelic mutants cpr5-1, cpr5-2 and cpr5-3 all share a similar reduction of leaf K\(^+\) which is not observed in the single jar1-1 mutant (raw data are available at www.ionomicshub.org; experimental tray 1840). From this we conclude that the low leaf K\(^+\) of cpr5 is not dependent on JA signalling. Taken together these experiments support the conclusion that the reduced leaf K\(^+\) of cpr5 is independent of both SA and JA.

cpr5 is easily distinguishable from wild-type plants from the presence of necrotic and chlorotic spots on the leaves, a trait which characterizes plants infected by bacteria and/or fungi, as well as mutants with a constitutively active response to pathogens. Hypothesizing that low leaf K\(^+\) was associated with the response to pathogens, we measured K\(^+\) content in leaves of lesion mimic mutants (LMM) selected from the two major classes of initiation (dni1, dni2, ago2, aco6, lsd6) and propagation (acd1, acd2, rad1) of lesion mutants [32]. None of the lesion mimic mutants tested showed reduced leaf K\(^+\) (Fig. 4A, B). From this we conclude that the low leaf K\(^+\) observed in cpr5 is not a result of the presence of lesions.

Low leaf K\(^+\) in cpr5 is primarily driven by the shoot but roots also play a role

Low K\(^+\) in cpr5 leaves could be caused by reduced uptake from roots, impaired root-to-shoot translocation, or enhanced shoot-to-root circulation through the phloem. CPR5 is equally expressed in both roots and leaves (Fig. S1B) making it possible that CPR5 could contribute to K\(^+\) homeostasis in either organ. Therefore, we performed reciprocal grafting of cpr5 and wild-type Col-0 to determine in which tissue cpr5 exerts its influence. Shoots of cpr5-2 were grafted onto wild-type Col-0 roots and vice versa, grafted plants allowed to grow for four weeks in soil and the K\(^+\) content measured in leaves. When cpr5-2 shoots were grafted on wild-type Col-0 roots we observed the K\(^+\) content in leaves to be significantly reduced by 43% compared to self-grafted Col-0 (Fig. 5A).

Moreover, plants with cpr5-2 shoot and wild-type Col-0 root also retained the hypersenescence phenotype of chlorotic and necrotic
spots observed on leaves of non-grafted cpr5-2 plants. In contrast, plants with wild-type Col-0 shoots grafted on cpr5-2 roots showed only an 11% reduction in leaf K⁺ and were indistinguishable from Col-0 in respect to symptoms of hypersenescence. Interestingly, self-grafted cpr5-2 plants showed the lowest leaf K⁺ of all the grafting types tested, with a reduction in leaf K of 58%. These results suggest that loss-of-function of CPR5 in shoots plays a primary role in the reduced leaf K⁺ phenotype of cpr5, but loss-of-function of CPR5 in roots also exerts a lesser yet significant influence.

To further understand the role of roots versus shoots in the low leaf K⁺ phenotype of cpr5 we measured the K⁺ content of shoot and root tissue of plants grown on solidified MS medium in plates. This experiment revealed no significant difference in the root concentration of K⁺ between cpr5 and Col-0, while cpr5 shoots retained the low K⁺ phenotype observed in plants grown in soil (Fig. 5B).

Expression of Cyclic Nucleotide Gated Channels is elevated in shoots of cpr5

Based on the importance of the shoot in driving the reduced leaf K⁺ in cpr5 we investigated expression of Cyclic Nucleotide Gated Channels (CNGCs) that may be directly or indirectly involved in K⁺ efflux during the response to pathogens in A. thaliana. An initial
survey of transcriptional data publically available in experiment 175 on Geneinvestigator [33] revealed that the expression of numerous CNGCs was up-regulated in cpr5 leaves. We confirmed the differences initially observed in the database using qRT-PCR, and performed similar measurements in root tissue. Our analysis revealed that steady state levels of CNGC10, CNGC11, CNGC12, CNGC19, and CNGC20 mRNA are all elevated in leaves of both cpr5-2 and cpr5-3 compared to wild-type Col-0 (Fig. 6). Analogous measurements performed in roots did not revealed any substantial differences from wild-type Col-0 for the cpr5 mutants (Fig. 6).

Expression of HAK5 encoding a high affinity $K^+$ transporter is reduced in roots of cpr5

The $K^+$ transporter HAK5 is known to contribute to $K^+$ uptake in A. thaliana primarily at low $K^+$ supply (0–0.25 mM) [23,34–37], whereas the $K^+$ channel AKT1 [38,39] contributes to $K^+$ uptake at both low and intermediate $K^+$ supply (0.01–5 mM) [35–37,40]. Above 5 mM external $K^+$ the transport processes involved in $K^+$ uptake are currently undefined [36]. Given the importance of both HAK5 and AKT1 in $K^+$ uptake in A. thaliana we used qRT-PCR to quantify the steady state levels of HAK5 mRNA in cpr5 roots to test if altered expression of these genes may be involved in the low leaf $K^+$ phenotype we observe in cpr5. Steady state levels of AKT1 mRNA in cpr5 where found to be the same as wild-type Col-0 after growth on medium supplemented with either high (20 mM) or low (100 µM) $K^+$ (Fig. 7C). A slight increase in AKT1 mRNA was observed in wild-type Col-0 plants grown on medium supplemented with 100 µM $K^+$ compared to 20 mM $K^+$. Enhanced expression of AKT1 was not previously observed [41], though this is possibly due to the fact that the previous authors used RT-PCR to determine expression of AKT1. Interestingly, steady state levels of HAK5 mRNA were observed to be reduced in cpr5 compared to wild-type Col-0 grown in medium with either high and low $K^+$ supply (Fig. 7B). Further, the increase in HAK5 mRNA observed in wild-type Col-0 grown on medium containing 100 µM $K^+$ was essentially abolished in cpr5 (Fig. 7B). Interestingly, root growth of cpr5 on medium supplemented with 100 µM $K^+$ was found to be reduced compared to wild-type (Fig. 7A). However, in medium supplemented with 20 mM $K^+$ growth of cpr5 and wild-type was similar (Fig. 7A).

To confirm and extend these results we performed a more detailed dose response experiment with solidified medium supplemented with 10, 50, and 100 µM KCl lacking NH$_4^+$, given that NH$_4^+$ is known to suppress activity [38,39] and expression [23] of HAK5. Pure agarose was used to solidify the growth medium to avoid any extra $K^+$ supply [42]. In wild-type Col-0 root steady state levels of HAK5 mRNA were observed to increase as external $K^+$ was reduced (Fig. 8A), as expected [34]. In roots of cpr5-2 and cpr5-3 steady state levels of HAK5 mRNA were also increased as the external $K^+$ concentration was reduced (Fig. 8A). However, steady state levels of HAK5 mRNA in cpr5-2 and cpr5-3 were consistently lower then wild-type Col-0 at 10, 50 and 100 µM $K^+$ in the growth medium (Fig. 8A). In agreement with our previous observations no consistent differences in expression of AKT1 between wild-type Col-0 and cpr5 were observed (Fig. 8B).

Figure 6. Expression of CNGCs in cpr5. Steady state levels of CNGC10, CNGC11, CNGC12, CNGC19, CNGC20 mRNA in leaves and roots of cpr5-2, cpr5-3 and wild-type Col-0 evaluated by qRT-PCR. Seedlings were germinated and grown on 1/20th MS medium supplemented with 20 mM KCl. After two-week of growth shoot and root tissue was harvested and RNA extract for qRT-PCR. PP2A (At1g13320) was used as an endogenous reference gene for normalization across samples, and data presented as 2$^{-\Delta Ct}$. Error bars represent standard deviations calculated following [29].

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Discussion

The data presented here establishes that the wild-type allele of CPR5 is required to maintain normal $K^+$ homeostasis in A. thaliana Col-0. We observed that loss-of-function alleles of CPR5 show a consistent and specific reduction in leaf $K^+$ of 10–30% in plants grown in both soil or on solidified MS medium with high $K^+$ supply. Further, this defect leads to reduced growth at low $K^+$ supply. Genetic analysis confirmed that this reduction in leaf $K^+$ is not dependent on the elevated SA or JA known to occur in cpr5. Further, genetic analysis confirmed that reduced leaf $K^+$ is not a feature of lesion mimic mutants in general. Through reciprocal grafting we establish that the reduced leaf $K^+$ observed in cpr5 is primarily driven by the shoot (74%), with the root playing a
significant but smaller role (19%). Interestingly, the presence of the cpr5 allele in both roots and shoots is required to produce the full low leaf K+ phenotype, suggesting that feedback between both organs is needed. In investigating the factors that in cpr5 cause the reduction of leaf K+, we surveyed the experiment AT-175 performed on cpr5 shoots (www.genevestigator.ethz.ch) and this revealed that genes belonging to the CNGC family are highly expressed in cpr5. Using qRT-PCR we confirmed that CNGC10, CNGC11, CNGC12, CNGC19, CNGC20 are indeed highly expressed in leaves, but not roots, of cpr5. The expression of CNGC1 and CNGC13 which showed a milder increase in the AT-175 array did not show any difference when expression was analyzed by qRT-PCR. CNGC2 and CNGC4, null mutants of which (dnd1 and dnd2) have enhanced disease resistance, also did not show any misregulation when analyzed by qRT-PCR. In the interaction between plant and pathogens the recognition of factors of avirulence by the host plant induces fluxes of Ca2+ that initiate the immune response and leads to enhanced K+ efflux [10]. CNGCs are believed to be the channels that deliver the Ca2+ signal required for pathogen recognition [43], and dnd1, dnd2 and the gain of function chimeric CNGC11/12 mutant cpr22 strongly support this conclusion [44–50]. Moreover, heterologous expression of specific CNGCs provides direct evidence of Ca2+ transport activity [51,52]. It is therefore possible that in cpr5 the constitutively high level of expression of CNGCs we observe leads to a persistent activation of this Ca2+ initiated signalling cascade that in turn leads to constitutively enhanced K+ efflux [10]. Such an enhanced K+ efflux could increase shoot-to-root K+ export, and may explain the leaf-driven portion of the reduced leaf K+ observed in cpr5. The transporters/channels involved in this K+ efflux are currently unknown. However, these is evidence that CNGC10 transports K+ [53], and steady state levels of CNGC10 mRNA are elevated in cpr5, making it possible that CNGC10 could play a role in the proposed enhanced K+ efflux and shoot-to-root export in cpr5. HAK5 encodes the primary high affinity root K+ transporter in A. thaliana [23,34–37], and its expression in wild-type Col-0 roots is induced at low K+ supply [34]. Significantly, cpr5 roots show a consistent reduction in the steady state levels of HAK5 mRNA under both high and low K+ supply. Though HAK5 primarily plays a role in K+ uptake at low K+ supply, it is possible that the reduced steady state levels of HAK5 mRNA in cpr5 roots we observe even at high K+ supply is responsible for the small (18%) but significant contribution of roots to the reduced leaf K+.

Figure 7. Expression of HAK5 and AKT1 in roots of cpr5. Seeds were germinated on solidified 0.5× MS salts, and after five days seedlings transferred to solidified 1/20th MS medium supplemented with either 100 µM (A) or 20 mM KCl (B). Pictures were taken after two weeks of growth. Steady state levels of HAK5 (C) and AKT1 (D) mRNA were quantified using qRT-PCR in roots of wild-type Col-0 (white bars) and cpr5-2 (gray bar). RNA was extracted from roots of three independent samples generated from between 25 and 30 plants per plates. UBQ10 (At4g05320) was used as an endogenous reference gene for normalization across the samples, and data presented as 2^−ΔΔCt. Error bars represent standard deviations calculated following [29].
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phenotype of cpr5. It is also interesting to speculate that expression of HAK5 in cpr5 roots is suppressed by an increased flux of K+ arriving in the roots from the shoots driven by elevated expression of CNGCs in shoots.

Based on sequence analysis CPR5 encode a membrane protein with five transmembrane domains and a nuclear localization signal (NLS). CPR5 appears to localize to the nucleus [54] where it has been proposed to be targeted to the inner nuclear membrane [19,54]. It has been further suggested that proteolytic cleavage can release the nucleosolic domain of CPR5 from the membrane to allow its participation in transcriptional processes [19,54]. Recent studies have identified the transcription factors TPR1 and EDS1 that direct repression of expression of CNGC2 and CNGC4 [55,56], and we speculate that CPR5 may play a similar role in the nucleus to negatively regulate the expression of the various CNGCs affected on the cpr5 mutant.

In summary, we have identified a new low leaf K+ phenotype for cpr5 that is primarily driven by the shoot, and is independent of the elevated levels of SA and JA found in this mutant. We suggest that the reduced leaf K+ of cpr5 is caused by the elevated expression of various CNGCs in shoots and the reduced expression of HAK5 in roots, driving both an enhanced K+ export from shoots and a reduced K+ uptake in roots. Our observation of altered K+ homeostasis in cpr5 may also be an important piece of evidence linking the function of CPR5 as a negative regulator of local defence responses to pathogens with the role K+ efflux plays in these responses; likely through the direct or indirect regulation of CNGCs by CPR5.

Supporting Information

Figure S1  CPR5 expression and salicylic acid (SA) levels in A. thaliana leaves and roots of wild-type Col-0 and cpr5. A. Steady state levels of CPR5 mRNA in wild-type Col-0 and cpr5-3 quantified using qRT-PCR. RNA was extracted from leaves of five-week old plants grown in soil. Data represents the mean of measurements from at least three independent biological replicates. Errors bars represent standard deviation. B. Steady state levels of CPR5 mRNA in root and shoots of wild-type Col-0 quantified using qRT-PCR. RNA was extracted from shoots and roots of two week old wild-type Col-0 plants grown on 0.5× MS media solidified with 1% agar (w/v). Values represent mean of measurements from at least three independent replicates. Errors bars represent standard deviation. Steady state mRNA levels (A & B) are presented as 2^{ΔΔCT}. UBQ10 (At4g05320) was used as an endogenous reference gene for normalization across samples. C. SA content (mg g^{-1} of fresh weight) in leaves of wild-type Col-0, cpr5-2 and cpr5-3. Data represents the mean of three independent leaf samples harvested from individual plants grown in soil for five weeks. Error bars represent the standard error. (TIF)

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Author Contributions

Conceived and designed the experiments: MB AR DES. Performed the experiments: MB AR. Analyzed the data: MB AR DES. Wrote the paper: MB DES.
1. Marschner H (2002) Mineral nutrition of higher plants. New York: Academic Press; pp 299–312.
2. Ashley MK, Grant M, Grabov A (2006) Plant responses to potassium deficiencies: a role for potassium transport proteins. J Exp Bot 57: 423–436.
3. Grignon C, Sentenac H (1991) pH and ionic conditions in the apoplasm. Ann Rev Plant Biol 42: 103–128.
4. White P (1997) The regulation of K+ influx into roots of (Saccul colu L) seedlings by negative feedback via the K+ flux from shoot to root in the phloem. J Exp Bot 48: 2063–2073.
5. Shahaba S (2007) Transport from root to shoot. In: Yeo A, Flowers T, eds. Plant solute transport Blackwell Publishers, pp 214–234.
6. Armengaud P, Breitling R, Ammann A (2004) The potassium-dependent transcription of Arabidopsis reveals a prominent role of jasmonic acid in nutrient signaling. Plant Physiol 136: 2556–2576.
7. Armengaud P, Sulprice R, Miller AJ, Smit M, Ammann A, et al. (2009) Mutations in the Arabidopsis primary metabolism provides new insights into the role of potassium nutrition for glycosin and nitrogen assimilation in Arabidopsis roots. Plant Physiol 150: 772–785.
8. Ammann A, Troufflard A, Armentaud P (2008) The effect of potassium nutrition on pest and disease resistance in plants. Plant Physiol Plantarum 133: 692–699.
9. Jabs T, Tischké M, Colling C, Hahnbrock K, Scheel D (1997) Elicitor-stimulated ion fluxes and O2- from the oxidative burst are essential components in triggering defense gene activation and phytoalexin synthesis in parsley. Plant and Cell Physiol 38: 4800–4805.
10. Jaworutzki E, Roelfsema MRG, Anschutz U, Krol E, Elzenga JTM, et al. (2010) Arabidopsis: AtHKT1;5, AtHAK5, and AKT1 are vital for seedling establishment and postgermination growth under low-potassium conditions. Plant Physiol 155: 863–875.
11. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 25: 402–408.
12. Freeman JL, Garcia D, Kim D, Hopf A, Salt DE (2005) Constitutively elevated potassium uptake supporting plant growth in the absence of AKT1 channel activity: Inhibition by ammonium and stimulation by sodium. J Gen Physiol 117: 909–921.
13. Ali R, Zielinski R, Berkowitz G (2006) Expression of plant cyclic nucleotide-gated cation channel. Plant Physiol 140: 915–203.
14. Jain A, Poling MD, Smith AP, Nagarajan VK, Lahner B, et al. (2009) Variations in the composition of gelling agents affect morphophysiologic and molecular responses to deficiencies of phosphate and other nutrients. Plant Physiol 150: 1033–1049.
15. Moeder W, Urquhart W, Ung H, Yoshioka K (2010) The role of cyclic nucleotide-gated ion channels in plant immunity. Mol Plant 4: 442–452.
16. Balague C, Lin B, Alcon C, Hottes M, Galmstrom S, et al. (2003) HLM1, an Arabidopsis cyclic nucleotide-gated channel, AtCNGC10, influences salt tolerance in Arabidopsis. Plant J 34: 175–185.
17. Jurkowski GI, Smith RK, Jr., Yu IC, Ham JH, Sharma SB, et al. (2004) Arabidopsis AtCNGC10 rescues potassium channel mutants of E. coli, yeast and Arabidopsis. PLoS ONE 9 October 2011 | Volume 6 | Issue 10 | e26360
18. Navrath C, Metraux J (1999) Salicylic acid induction-deficient mutants of Arabidopsis express PR-2 and PR-5 and accumulate high levels of camalexin and pathogenesis-related proteins. Plant Cell 11: 1399–1409.
19. Rogers E, Ausabel F (1997) Arabidopsis enhanced disease susceptibility mutants exhibit enhanced susceptibility to several bacterial pathogens and alterations in PR-1 gene expression. Plant Cell 9: 305–316.
20. Stawiski PF, Wei Su, House SH (1992) Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an Arabidopsis thaliana mutant. NAPR 89: 6517–6804.
21. Lorrain S, Vialleau F, Balague, Roby D (2003) Lesion mimic mutants for determining cell death and defense pathways in plants? Trends Plant Sci 8: 263–271.
22. Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W (2004) GENVESTIGATOR. Arabidopsis Microarray Database and Analysis Tool-Box. Plant Physiol 136: 2621–2632.
54. Perazza D, Laporte F, Balague C, Chevalier F, Reno S, et al. (2011) GeBP/GPL transcription factors regulate a subset of CPR5-dependent processes. Plant Physiol doi: 10.1104/111.179804.

55. Zhu Z, Xu F, Zhang Y, Cheng YT, Wiermer M, et al. (2010) Arabidopsis resistance protein SNC1 activates immune responses through association with a transcriptional corepressor. Proc Natl Acad Sci U S A 107: 13960–13965.

56. García AV, Blanvillain-Baumont S, Huibers RP, Wiermer M, Li G, et al. (2010) Balanced nuclear and cytoplasmic activities of EDS1 are required for a complete plant innate immune response. PLoS Pathog 6: e1000970.