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Potassium content diminishes in infected cells of Medicago truncatula nodules due to the mislocation of channels MtAKT1 and MtSKOR/GORK

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Abstract

Rhizobia establish a symbiotic relationship with legumes that results in the formation of root nodules, where bacteria encapsulated by a membrane of plant origin (symbiosomes), convert atmospheric nitrogen into ammonia. Nodules are more sensitive to ionic stresses than the host plant itself. We hypothesize that such a high vulnerability might be due to defects in ion balance in the infected tissue. Low temperature SEM (LTSEM) and X-ray microanalysis of Medicago truncatula nodules revealed a potassium (K+) decrease in symbiosomes and vacuoles during the life span of infected cells. To clarify K+ homeostasis in the nodule, we performed phylogenetic and gene expression analyses, and confocal and electron microscopy localization of two key plant Shaker K+ channels, AKT1 and SKOR/GORK. Phylogenetic analyses showed that the genome of some legume species, including the Medicago genus, contained one SKOR/GORK and one AKT1 gene copy, while other species contained more than one copy of each gene. Localization studies revealed mistargeting and partial depletion of both channels from the plasma membrane of M. truncatula mature nodule-infected cells that might compromise ion transport. We propose that root nodule-infected cells have defects in K+ balance due to mislocation of some plant ion channels, as compared with non-infected cells. The putative consequences are discussed.

Keywords: AKT1, GORK, Medicago truncatula, potassium channels, SKOR, symbiosis, symbiosome, X-ray microanalysis

Introduction

The symbiotic relationship of soil bacteria, collectively known as rhizobia, with leguminous plants is the source of the ammonia produced from atmospheric nitrogen by the symbiosomes (Roth and Stacey, 1989), the form of rhizobia housed in infected cells of root nodules.

Nodules are more sensitive to stresses, such as salt and heavy metals, in comparison with the roots of the host plant and the plant itself. These stresses are detrimental for nodule formation and nitrogen-fixing activity (Zahran, 1999; Tsyanov et al., 2007; Shvaleva et al., 2010; Coba de la Peña and Pueyo, ...
2012; Bertrand et al., 2016). Although such abiotic stresses have an effect on the plant ionome, and the high vulnerability of the nodule might be due to defects in ion balance in the infected tissue, a comprehensive model of plant–microsymbiont ion homeostasis in legume nodules has not yet been established.

One of the essential ions involved in plant adaptive responses to abiotic stresses is potassium (K⁺) (Wu et al., 2018; Rubio et al., 2020). It is a macronutrient involved in the control of osmotic status and turgor, cation/anion balance, control of membrane polarization, cytoplasmic pH regulation, cell expansion, and organ movements, such as stomatal aperture (Karkim et al., 2017; Ragel et al., 2019).

The availability of K⁺ for the cell depends on transport, ion voltage, and concentration of K⁺ on both sides of the membrane. K⁺ transport is expected to oscillate between uptake and loss over periods of tens of seconds to many minutes (Wang and Wu, 2013; Shabala and Potosin, 2014; Ragel et al., 2019). Plant K⁺ transporters have defined functions in K⁺ uptake or release, storage in vacuoles, and ion translocation between tissues and organs (Wang and Wu, 2013; Ragel et al., 2019). Regarding legume nodule, some K⁺ transporters were identified in root hair transcriptomes of Lotus japonicus, Glycine max, and Medicago truncatula, and have been proposed to be involved in the early stages of nodulation (Desbrosses et al., 2004; Damiani et al., 2016; Rehman et al., 2017). However, information about K⁺ transport in nodules is rarely scarce (Benedito et al., 2010; Udvardi and Poole, 2013; Drain et al., 2020).

AKT1 is one of the main K⁺-permeable channels (Coskun and Kronzucker, 2013; Véry et al., 2014). In Arabidopsis thaliana, the Shaker-type channel AtAKT1, located in the plasma membrane, has been shown to mediate both high- and low-affinity K⁺ acquisition in roots (Sharma et al., 2013). The so-called plant Shaker family is a group of voltage-gated K⁺ channels. The structure of a Shaker-type K⁺ protein subunit consists of six transmembrane domains close to the N-terminus, a pore domain (involved in voltage sensing) located between the fifth and sixth transmembrane domains, an acyclic nucleotide monophosphate-(cNMP) binding domain involved in channel activation potential, several repeated ankyrin domains involved in protein–protein interactions and signal transduction, and a dimerization domain (KHA) located at the C-terminal end. Functional K⁺ channels are tetramers of this protein subunit (Wang and Wu, 2013; Nieves-Cordones et al., 2014; Véry et al., 2014).

Outward-rectifying channels mediate K⁺ release and open at depolarized membrane potentials; this group is composed of SKOR and GORK channels in A. thaliana (Ragel et al., 2019). SKOR is involved in K⁺ transport from root stellar cells into the xylem and from roots to shoots, while GORK is expressed in the epidermis and functions in K⁺ release (Gaymard et al., 1998; Demidchik, 2014). According to the data of Long-Tang et al. (2018), constitutively overexpressed melon SKOR has a positive effect on growth and improves saline tolerance in A. thaliana. Recently, it was shown that only one gene of an outward Shaker K⁺ channel is present in the genome of M. truncatula (Drain et al., 2020). These authors analysed the functional activity of this channel. They have shown that this channel poorly contributed to K⁺ translocation towards the shoots, but was quite important in the membrane outward K⁺-permeable conductance in leaf guard cells. Hence, this channel showed the functional features of a GORK channel, and it was designated by Drain et al. (2020) as MtGORK. However, this channel is annotated as MtSKOR in the NCBI database, so we are using a double name MtSKOR/GORK to prevent confusion.

In the present study, to investigate the ion compartmentalization in the symbiotic tissue, we have performed an ion distribution analysis (IDA) of M. truncatula root nodules by low temperature SEM (LTSEM) and X-ray microanalysis. The analysis was mainly focused on vacuoles of the nodule tissues and symbiosomes. In the nodule-infected zone, K⁺ content decreased in the vacuoles of infected cells and in symbiosomes during the life span of the infected cell. This suggests that K⁺ homeostasis could be impaired in infected cells.

To clarify the decrease of K⁺ content observed during the life span of the infected cells, we analysed two key plant Shaker K⁺ channels with opposite K⁺ transport vectors: the inwardly rectifying K⁺ channel AKT1 (MtAKT1) and the Stellar K⁺ outward rectifier channel SKOR/GORK (MtSKOR/GORK). Differences in the location of both channels were found between infected and non-infected nodule cells. In infected cells, mistargeting and partial depletion of both channels in the plasma membrane occurred before K⁺ decrease in the vacuole. We propose that the maintenance of the intracellular bacterial colony might have an effect on the K⁺ availability for the host cell. Root nodule-infected cells might have defects in K⁺ balance due to the differences/flaws in the location of MtAKT1 and MtSKOR/GORK, as compared with non-infected cells. Our results provide new insights into infected cell development and location of the K⁺ channels MtAKT1 and MtSKOR/GORK in nodules.

Materials and methods

Plants, bacteria, and growth conditions

Medicago truncatula Gaertn. cv. Jemalong A17 plants were grown according to Limpens et al. (2004). Agrobacterium rhizogenes strain MSU440 was used for hairy root transformation (Limpens et al., 2004). Sinorhizobium meliloti strain Sm2011 or S. meliloti Sm2011–mRFP expressing the monomeric red fluorescent protein (mRFP) were used for nodulation (Snuit et al., 2005).

Ion distribution analysis (IDA)

IDA was performed with a Zeiss DSM-962low temperature scanning electron microscope with energy-dispersive spectrometry and X-ray microanalysis (Pentaflet, Oxford, UK). Twenty-eight days post-inoculation (dpi), nodules were collected and immediately frozen. The nodules were fractured via the long axis to reveal all the developmental zones, and immediately analysed. For the IDA, an accelerating voltage of 20 kV, a resolution of 133 eV, and a spot acquisition method were used. Quantitative element analysis was obtained by using standard ZAF (atomic number, absorption, and fluorescence) correction procedures with Link Isis, version 3.2 (Oxford, UK). The high resolution of LTSEM permits the estimation
of the ion content in individual plant organs and cell structures (Leidi et al., 2010). We targeted the symbiosomes and vacuoles in the developmental zones that were distinguished by their position inside the nodules. The comparison was done between the same structures (symbiosomes or vacuoles) from different nodule developmental zones. For each analysis, 10 nodules were used, 10–15 cells were analysed per nodule, and 7–12 symbiosomes and vacuoles were examined.

Accession numbers or gene identifiers
MtAKT1 (MTR_4g113530) and MtSKOR/GORK (MTR_5g077770). The homologue M. truncatula genes MtAKT1 and MtSKOR/GORK were identified using the available genomic and cDNA sequence databases utilizing the A. thaliana sequences for BLAST search.

Phylogenetic and protein sequence analyses
MtSKOR/GORK and MtAKT1 nucleotide and protein sequences and homologues in other legume species were retrieved from several databases and plant genomic resources: National Centre for Biotechnology Information (https://www.ncbi.nlm.nih.gov/), Phytozone (https://www.phytozone.jgi.doe.gov/pz/portal.html), the Legume Information System (http://legumefo.org/), the White Lupin Genome site (https://www.whiteupln.fr/), the pea genome project (https://urgi.versailles.inra.fr/Species/Pisum), and the Lotus japonicus genome assembly site (http://www.kazusa.or.jp/lotus). Arabidopsis thaliana AKT1, GORK, and SKOR sequences were used as queries.

Phylogenetic analyses of AKT1 and SKOR/GORK homologue genes of several legume species were performed by using the maximum likelihood method. The Tamura–Nei model (Tamura and Kumar, 2000) and general time reversible model (Nei and Kumar, 2000) were used for AKT1 and SKOR/GORK analyses, respectively. Node robustness was assessed by the bootstrap method (v=1000 pseudoreplicates). Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

Protein domains were predicted with the SMART tool (http://smart.embl-heidelberg.de/). Putative protein transmembrane domains were predicted with the Phyre2 Web portal prediction and analysis tool (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgt?id=index; Kelley et al., 2015). Multiple protein sequence alignment was performed with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Plasmid constructs and plant transformation
The MtAKT1 ORF and its 2.5 kb regulatory sequence were amplified via PCR from nodule cDNA and genomic DNA, respectively, using Phusion high-fidelity polymerase (Finnzymes).

MtAKT1 and MtAKT1 putative promoters were directionally cloned into a pENTR-D-TOPO plasmid (Invitrogen). The Gateway cloning system (Invitrogen) was used to create genetic constructs for promoter:GUS (β-glucuronidase) and GFP (green fluorescent protein) fusions. pENTR-D-TOPO carrying MtAKT1 was recombined using Gateway LR Clonase II (Invitrogen) into the Gateway destination vector pKGW-GGRK by LR reaction to create the ProMtAKT1:GUS fusion. To make the ProMtAKT1:MtAKT1:GFP translational fusion, MtAKT1 was cut out from pENTR-D-TOPO with restriction enzymes NcoI and Ascl, purified from a gel, and ligated into pENTR 4, 1 digested with Bsal. Afterwards, pENTR constructions containing ProMtAKT1 and MtAKT1 and pENTR 2, 3 containing GFP were introduced by multistep LR recombination into the Gateway destination vector pKGW-MGW. All primers used are listed in Supplementary Table S1.

RNA extraction and gene expression analysis
Nodules at 28 dpi were collected and dissected into three parts: the white apical zone containing meristem (M) and the infection zone (z2), the medial red part (young and mature nitrogen fixation zone) (z3), and the basal part, which included the senescent zone (z4).

Total RNA was extracted from nodules using the E.Z.N.A. RNA Plant Mini Kit (Omega Bio-Tek, Norcross, GA, USA) and retro-transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). Expression analysis of MtAKT1 and MtSKOR/GORK was performed by real-time quantitative PCR (qPCR). The constitutively expressed Mt27 and MtGAPDH genes were used as endogenous controls (Coba de la Peña et al., 2008; Verdier et al., 2008). Gene-specific primers were designed using the PRIMER 3-PLUS software (Untergasser et al., 2007) and with Primer Express v3.0 software (Applied Biosystems, Foster City, CA, USA). The real-time PCRs were performed on a 7300 Real Time PCR System (PE Applied Biosystems) using SYBR Green Supermix (Bio-Rad). The comparative Ct method (Pfaffl, 2001) or the standard curve method (Torres et al., 2014) were applied for relative quantification. All primers used are listed in Supplementary Table S1.

Localization of gene expression by GUS staining
Transgenic roots and nodules were collected, washed in 0.1 M sodium phosphate buffer, pH 7.2, incubated in GUS buffer under vacuum at room temperature for 30 min, and incubated at 37 °C for 2 h for the enzymatic reaction. Roots and hand-sectioned nodules were photographed using a Nikon Optiphot-2 microscope.

Confocal microscopy
A Leica MZFLIII fluorescence microscope with filter cubes for GFP (excitation, 470/40 nm; dichroic, 495 nm; emission, 525/50 nm) and DsRed (excitation, 545/30 nm; emission, 620/60 nm) was used for the selection of transgenic tissue.

Confocal imaging was done on hand-sectioned nodules with a Zeiss LSM 5 Pascal confocal laser–scanning microscope (Carl Zeiss) and a Meta LSM 510 microscope in a single image mode.

Nodules were fixed in 1% formaldehyde, cut along the longitudinal axis, and blocked with 2% BSA. The GFP signal in the sections carrying ProMtAKT1:MtAKT1:GFP constructs was enhanced by using an antibody (Ab) against GFP developed in rabbit (Molecular Probes) at 1:50 dilution.

For double immunolabelling of MtAKT1 and Rab7, anti-GFP rabbit Ab was mixed with anti-Rab7 Ab developed in mouse (Limpens et al., 2009), followed by addition of the secondary Abs anti-rabbit C13 Ab (excitation maximum 548 nm, emission maximum 561 nm; Molecular Probes) and anti-mouse Alexa 488 (excitation maximum 490 nm, emission maximum 525 nm; Molecular Probes), respectively. For immunolocalization of MtSKOR/GORK, a custom-made Ab developed in rabbit (GenScript) from the peptide sequence HPWDPEQRRNG [peptide–keyhole limpet haemocyanin (KLH) conjugate] was used in a 1:100 dilution. An anti-rabbit Alexa 488 Ab (excitation maximum 490 nm, emission maximum 525 nm; Molecular Probes), diluted 1:200, was used as secondary Ab.

The comparative estimation of the relative intensity of the fluorescence signal was performed by ImageJ multipoint analysis of brightness (Schneider et al., 2012). Analysis of the signal for MtAKT1 and MtSKOR/GORK in infected and non-infected cells was performed on 10 cells for each group on the same set of green channel Tiff images obtained by confocal microscopy; each sampling contained 45 points.

Electron microscopy
Transgenic nodules carrying the ProMtAKT1:MtAKT1:GFP construct were fixed and embedded in Lowicryl K4M (Fedorova et al., 1999). EM grids with the samples were blocked with 2% BSA and incubated overnight at 4 °C with the primary Ab anti-GFP developed in rabbit.
Potassium and the channels MtAKT1 and MtSKOR/GORK in nodules | 1339

(Molecular Probes) at 1:50 dilution, followed by colloidal 10 nm gold-conjugated goat anti-rabbit Ab (BioCell).

For the localization of MtSKOR/GORK, wild-type nodules fixed and embedded as described above were used. The immunolocalization was carried out with the custom-made Ab developed in rabbit (GenScript) at a dilution of 1:50, followed by colloidal 10 nm gold-conjugated goat anti-rabbit Ab (BioCell).

Samples were analysed using a LIBRA120 electron microscope (Carl Zeiss).

**Purification of plasma membrane**

Purification of plasma membrane from the roots of 5-day-old *M. truncatula* seedlings was performed by the method of aqueous two-phase partitioning (Hodges and Mills, 1986).

The seedling roots were homogenized in 100 mM Tris–HCl buffer at pH 8 in the presence of 300 mM sucrose, 10 mM EDTA, 5 mM K$_2$S$_2$O$_5$, 5 mM DTT, and 1 mM phenylmethylsulfonyl fluoride (PMSF; 1/2 w/v) at 4 °C, filtered through a cotton cloth, and centrifuged at 10 000 g for 15 min. The obtained supernatant was centrifuged at 186 000 g for 40 min, the precipitate was resuspended in buffer consisting of dextran T500 and polyethylene glycol 3350 supplemented with 0.3 M sucrose, 3 mM KCl, 0.1 mM EDTA, and 1 mM DTT, and centrifuged at 2500 rpm for 5 min. The upper phase of the phase separation gradient, which contains the plasma membrane, was centrifuged at 186 000 g for 40 min. The precipitate containing the plasma membrane proteins was used for western blot analysis.

**Western blot analysis**

The proteins from the precipitate of the plasma membrane were resuspended in 0.025 M Tris–HCl buffer containing 1 mM EDTA, 1 mM DTT, and protease inhibitor cocktail (Roche). A 75 μg aliquot of protein was loaded per well. The proteins were separated by 12% SDS–PAGE and were blotted to a nitrocellulose membrane (Bio-Rad). The membrane was incubated in 3% BSA, as a blocking agent, followed by primary anti-SKOR-specific Ab, at a 1:50 dilution, followed by the secondary Ab anti-rabbit immunoglobulin G peroxidase produced in goat (Sigma), at a 1:5000 dilution. The immunosignal was revealed by incubation with the Immuno-Star Western chemiluminescent kit (Bio-Rad). Blots were photographed with a Molecular Imager Chemi Doc XRS+, using Image Lab software in Chemi mode. The pre-stained protein ladder was photographed in normal light.

**Statistical methods**

IDA was performed twice from two separate experiments. Ten nodules from different plants per experiment were used. Measurements were performed for infected and non-infected cells; 10–15 cells from each nodule were analysed. Gene expression analysis was performed twice from two separate experiments. The Student test was used to determine the significance of the difference between the means of data sets.

**Results**

**Ion compartmentalization in nodule cells**

IDA was performed on root nodules fractured along the long axis to reveal all developmental zones (Fig. 1A). *Medicago truncatula* nodules have a developmental gradient of cells in an apical–basal direction from the apical meristem to the zone of senescence (Vasse et al., 1990). Proximal to the nodule meristem (M) is positioned the infection zone (z2), where bacteria are released from infection threads. Next to z2 is the nitrogen fixation zone (z3), which consists of large infected cells and small non–infected cells. The basal part of the nodule (z4) contains senescent infected cells. The cortex surrounding the central part of the nodule consists of non–infected cells only. High magnification was used to distinguish individual symbiosomes and vacuoles (Fig. 1B).

Distribution of K$^+$, sodium, magnesium, phosphorus, chlorine, calcium, iron, copper, and molybdenum was analysed (Fig. 1C; Supplementary Table S2). The major difference detected in the cells of the infected zone, according to the IDA, was in K$^+$ content, which significantly diminished during the life span of infected cells (Fig. 1C). The level of K$^+$ was lower in symbiosomes in mature and senescent infected cells than in young infected cells. In the young symbiosomes, the K$^+$/Na$^+$ ratio was ~5, while in mature and senescent symbiosomes, this ratio diminished until it reached 1. Vacuoles of infected cells in the senescent zone also showed a lower score for K$^+$ ions than those in young and mature infected cells. The vacuoles of non–infected cells from the inner and outer cortex and from the central part of the nodule did not show significant differences in K$^+$ content (Fig. 1C). Vacuoles of nodule cortex cells showed differences in sodium, chlorine, and calcium content compared with vacuoles of non–infected cells situated in the infected zone (Supplementary Table S2).

Our results suggest that K$^+$ transport may be altered during the life span of infected cells; therefore, we decided to study two of the main K$^+$ channels in the plant cell plasma membrane, an inwardly rectifying K$^+$ channel (AKT1) and an outwardly rectifying channel that mediates K$^+$ release from cells (SKOR).

**Phylogenetic analyses**

A phylogenetic analysis of *A. thaliana* AKT1 putative homologues of several legumes was performed. Legume homologues that formed a clade with *A. thaliana* AKT1 are shown in Fig. 2A. It was observed that some legume species (*M. truncatula*, *Pisum sativum*, *Trifolium pratense*, *Cicer arietinum*, *Lotus japonicus*, *Cajanus cajan*, *Vigna sp.*, *Phaseolus vulgaris*, and *Anachis hypogaea*) have just one *ATK1* homologue in a single locus in their genomes, whilst the genomes of other species (*Glycine sp.*, *Lupinus sp.*, and *Anachis hypogaea*) contain two homologues, in two different loci (Fig. 2A).

Phylogenetic analysis of putative SKOR/GORK gene homologues of several legumes was also performed. The genomes of *A. thaliana* and other Brassicaceae (*Brassica napus* and *Capsella rubella*) contain one SKOR gene homologue and one GORK gene homologue, that were included in the phylogenetic inference. The obtained phylogenetic tree displayed two differentiated clades of legume SKOR/GORK-like homologues (designated as ‘legume homologues clade I’ and ‘legume homologues clade II’ in Fig. 2B). Legume homologues in clade I clustered with Brassicales SKOR, and GORK genes. SKOR/GORK legume homologues clade I contained one or
two genes (corresponding to one or two loci) per genome, with the same relationship between species and number of loci observed for AKT1 homologues. An exception was Lupinus albus, as three genes were identified in its genome; two of them (Lalb_Chr17g0346031 and Lalb_17g0346021) were located next to each other in the same chromosome and putatively code for shorter proteins (Fig. 2B).

SKOR/GORK legume homologues clade II contains other SKOR/GORK-like genes identified in C. cajan, Vigna sp., Glycine sp., and Arachis sp., but not found in the genomes of the other studied legume species (Fig. 2B).

Sequence analyses of legume homologues of potassium channels AKT1, SKOR, and GORK

Sequence analysis showed that both M. truncatula homolog proteins MtAKT1 and MtSKOR/GORK contained a voltage-dependent K+ channel domain, a cNMP-binding
domain, several repeated ankyrin (ANK) domains involved in signal transduction, and a dimerization domain rich in hydrophobic and acidic residues (KHA) located at the C-terminus (Supplementary Fig. S1A, B). Both proteins also contained six transmembrane domains (S1–S6), which are also characteristic of these Shaker K⁺ channel proteins (Supplementary Figs S2A, S3A). MtAKT1, MtSKOR/GORK, and their corresponding homologues identified in other legume species also have the pore loop domain (P) between S5 and S6, harboring the GYGD hallmark motif, present in P domains of highly selective K⁺ channels (Supplementary Figs S2B, S3B) (Lebaudy et al., 2007; Drain et al., 2020). Both AtSKOR and AtGORK legume homologues also display two conserved amino acid residues (M, V) of the P loop and two residues (D, M) in S6 that are involved in the dependency of the channel voltage-sensitive gating on the external K⁺ concentration (Fig. S3B) (Johansson et al., 2006; Drain et al., 2020). A particular case is that of L. albus, with three SKOR/GORK homologues. Two of them (Lab_Chr17g0346031 and Labb_17g0346021) are shorter proteins, one of them containing the transmembrane domain and voltage-dependent K⁺ channel domain, and the other containing the cyclicNMP, ANK, and KHA domains.

Expression analysis of potassium channels in nodules

An analysis was performed by qPCR from RNA extracted from 28 dpi nodules dissected in three parts: white meristem plus infection zone (M/z2), red middle nitrogen-fixing zone (z3), and basal senescence zone (z4) (Fig. 3A, B). The expression levels of MtAKT1 and MtSKOR/GORK showed no significant differences among the analysed nodule zones.

Promoter:GUS expression analysis of AKT1 in root showed its expression in meristematic cells, cortex, and vascular bundles (Fig. 3C). In nodule primordia, expression was detected over the whole organ (Fig. 3D). Mature nodules showed a uniform distribution of expression throughout the nodule (Fig. 2).
3E) and, in senescent nodules, expression was mainly detected in vascular bundles (Fig. 3F). Promoter:GUS expression analysis of MtSKOR:GORK was recently reported by Drain et al. (2020). The analysis showed gene expression throughout the nodule and in vascular bundles of the root.

Localization of MtAKT1 and MtSKOR/GORK proteins in infected and non-infected cells using confocal and electron microscopy

MtAKT1 was located in the plasma membrane of meristematic cells, non-infected cells along the nodule developmental zones, and infected cells in the infection zone (Fig. 4A, B). In mature infected cells, packed with symbiosomes, the plasma membrane labelling was quite low, in contrast to non-infected cells (Fig. 4C). MtAKT1 signal was mainly detected in the cytoplasm in a dotted pattern and was partly depleted from the plasma membrane (Fig. 4C; Table 1; Supplementary Fig. S4).

The specificity of the anti-MtSKOR/GORK Ab was tested by western blot. In plasma membrane isolated from M. truncatula roots, anti-SKOR Ab recognized a band of 95.99 kDa, the expected size for MtSKOR/GORK protein (Supplementary Fig. S5). In nodule cells, MtSKOR/GORK signal was detected in both the plasma membrane and the symbiosome membrane (Fig. 4D, F; Supplementary Fig. S4). In mature infected cells, the protein was depleted from the plasma membrane similarly to MtAKT1 (Fig. 4E; Table 1).

With the aim to characterize the observed dot-like signal, we performed a double localization analysis of MtAKT1 and the small GTPase Rab7, which is a marker for late endosomes/vacuoles (Limpens et al., 2009). According to the analysis, the MtAKT1 signal partially (24%) co-localized with Rab7 (Fig. 4G). Hence, this result indicated that part of MtAKT1 protein was internalized from the plasma membrane by endosomes similar to the cycles of turnover of the Shaker channel KAT1 in stomatal cells (Meckel et al., 2004), but most of the dot-like signal might be located in other organelles.

To resolve the dot pattern observed by confocal microscopy, immunogold EM was performed. According to the analysis, the immunogold signal for MtAKT1 and MtSKOR/GORK was detected in the endoplasmic reticulum and plasma membrane (Fig. 5A, B). The immunogold EM analysis of MtSKOR/GORK confirmed the data of confocal microscopy and showed that MtSKOR/GORK was also associated with the symbiosome membrane (Fig. 5B).

The comparative estimation of fluorescence intensity over the plasma membrane regions of infected and non-infected cells demonstrated the partial depletion of both channels from the plasma membrane of infected cells (Table 1).

Discussion

The root nodule is a temporary organ with a short life span. The co-existence of symbionts is usually described as mutually beneficial for both of them (Wang et al., 2018). However, the infected cells have a short life span in comparison with non-infected root cells (Puppo et al., 2005), and the inevitable scenario of symbiosome lysis and death of the host cell after 12–18 d of existence has been well described (Kijne, 1975; Yang et al., 2017).
It is a reasonable assumption that the maintenance of an intracellular bacterial colony has its cost, and may have a detrimental effect on the host cell. Based on the high sensitivity of the root nodule to ionic stresses, we hypothesized that the infected nodule cells might have physiological defects in maintaining their ionic balance. In the present study, we describe a new aspect of the development of symbiosis: the eventual decrease of K⁺ during the infected cell’s life span and the putative reasons for such loss.

K⁺ is one of the main players in maintaining osmotic pressure, water potential, and turgor in plant cells (Lebaudy et al., 2007; Honsbein et al., 2011; Shabala and Shabala, 2011). Drastic loss of K⁺ is one of the symptoms of stress caused by pathogen elicitors, heavy metals, reactive oxygen species (ROS), salinity, and drought (Demidchik et al., 2014; Shabala and Potosin, 2014). The loss of K⁺ leads to the activation of cytosolic endonucleases that in other conditions are inhibited by this ion (Seon and J-Eun, 2002; Demidchik et al., 2010). We should
Table 1. Estimation of the fluorescence signal levels for MtAKT1 and MtSKOR/GORK by ImageJ multipoint analysis of brightness on the plasma membrane region.

|          | MtAKT1 | MtSKOR/ GORK | Level of autofluorescence |
|----------|--------|--------------|---------------------------|
| Non-infected cell | 94.2±5.3* | 93.5±1.6* | 30±2.9                    |
| Infected cell     | 33.7±3.4* | 35.6±2.9* |                           |

Data represent means ±SD (n=10 cells, 45 dots per cell). The difference in the level of signal over the plasma membrane region of infected and non-infected cells is significant with "P<0.01.

point out that stress-induced K+ leakage reaction is quite rapid, induced in minutes, lasts around 1 h, and terminates in programmed cell death (PCD; Atkinson et al., 1990; Demidchik, 2014).

However, according to our results (Fig. 1C), in contrast to the stress reaction described above, in root nodules the decrease of K+ happens gradually within 12–15 d after the release of rhizobia into the host cell, as observed in infected cells. During the life span of infected cells, K+ diminished 3–5 times in vacuoles and symbiosomes. The reasons for such a drastic loss are complex. The pool of K+ in infected cells should be shared between two partners, the host plant cell and several thousand bacteria that reside inside host cells. Rhizobia need K+ for the maintenance of the osmotic status and turgor, cation/anion balancing, and control of membrane polarization (Domínguez-Ferreras et al., 2009). According to our data, the level of K+ diminishes within 6–7 cell layers (6–7 d after release from infection threads) in the nitrogen fixation zone (z3), as is shown by the low level of K+ in mature symbiosomes. The vacuolar pool of K+ becomes mostly exhausted in the senescence zone, situated in 12–15 cell layers. In nodules, infected cells display the earliest signs of senescence, and infected cells degenerate before non-infected cells (Pérez Guerra et al., 2010). The loss of K+ may cause the activation of cytosolic endonucleases in mature infected cells and eventually promote the so-called ‘senescence’ of symbiosomes and rapid termination of symbiosis. In non-infected cells, the level of K+ was stable and they do not undergo premature senescence. The reasons for such a scenario may depend on the functional status, expression, and localization of the proteins involved in the inward or outward transport of K+ in legume nodules. As a first study in this context, we analysed the homologues of two of the main K+ channels in plants, the inwardly rectifying K+ channel AKT1 and the outward-rectifying K+ channel SKOR, in M. truncatula.

In this work, gene homologues of the channels AtAKT1, AtSKOR, and AtGORK were identified in the genomes of M. truncatula and other sequenced legumes. Phylogenetic analyses allowed us to characterize these homologues (Fig. 2). Legume AKT1 homologues clustered in one clade. Legume SKOR/GORK homologues clustered in two clades (I and II). Only one AKT1 and one SKOR/GORK homologue were identified in the genome of M. truncatula, as was previously reported (Damiani et al., 2016; Drain et al., 2020). We observed the same event in other legumes (P. vulgaris, L. japonicus, and P. sativum, among others). In the case of A. hypogaea, L. angustifolius, and Glycine sp., two AKT1 homologues and two SKOR/GORK homologues of clade I were identified. Rehman et al. (2017) identified these AKT1 and SKOR/GORK homologues in G. max. These results are consistent with the documented genome evolution of these species. The presence of two gene loci in A. hypogaea is in agreement with the recent hybrid origin of this allotetraploid species (Bertioli et al., 2019). On the other hand, additional whole-genome duplications took place during the evolution of the Lupinus and Glycine lineages, not shared by other Papilionoideae (Schmutz et al., 2010; Wang et al., 2015; Hane et al., 2017; Rehman et al., 2017). Interestingly, Rehman et al. (2017) proposed that one AKT1 homologue and the two soybean SKOR/GORK homologues of clade I are involved in nodule development.

In addition, our phylogenetic analysis showed a second clade of SKOR/GORK-like homologues in legumes (clade II, Fig. 2). These homologues were present in the genomes of the studied tropical legumes (Arachis, Vigna, Cajanus, and Glycine), with the exception of Phaseolus. These homologues were not found in the genomes of the studied temperate legumes (Medicago, Lupinus, Pisum, Trifolium, Cicer, and Lotus). These observations suggest that gene loss events of SKOR/GORK-like homologues from clade II occurred during the evolution of different lineage lineages. Lineage-specific gene loss events during plant evolution have been reported, and some putative evolutionary implications for symbiotic relations have been suggested (Lin et al., 2014; Gu et al., 2016; van Velzen et al., 2018). There is little information on the functional characterization of any of these legume homologues of clade II. It is interesting to note that the G. max SKOR/GORK homologue of clade II is not apparently involved in nodule development, as...
Rehman et al. (2017) did not report its differential expression linked to nodulation.

The expression of both channels, MtAKT1 and MtSKOR/GORK, was detected (Fig. 3) in M. truncatula nodules (this work; Drain et al., 2020), which is explained by the fact that the root developmental programme is co- opted for nodule formation (Fransen et al., 2015). However, as our data showed, the expression of channels MtAKT1 and MtSKOR/GORK is not a guarantee that their predicted functional role will be performed. According to the analysis of protein localization (Figs 4, 5; Table 1), both channel proteins were mistargeted in infected cells and partly depleted from the plasma membrane, and MtSKOR/GORK was retargeted towards the symbiosome membrane. Previously, we reported the retargeting towards the symbiosome membrane of several proteins (Limpens et al., 2009; Ivanov et al., 2012; Gavrin et al., 2014, 2016, 2017; Coba de la Peña et al., 2018). These changes were found to be positive for the maintenance and the propagation of bacterial symbionts, but the consequences for the host cell itself were not estimated. However, in the present study when analysing the ion distribution in both infected and non-infected cells, we came across some effects, probably caused by the presence of bacteria in host cells, which resulted in the drastic changes in K+ accessibility for the host cell housing mature nitrogen-fixing bacteria. The partial depletion of the inward channel MtAKT1 from the plasma membrane of infected cells cannot but adversely affect its functional activity and inward K+ transport. In fact, the decrease of the AKT1 labelling signal in the plasma membrane can be observed prior to the decrease of K+ content in the vacuoles, as these two events occur in z2 and z3, and in z4, respectively. We can speculate that the low level of the outward channel MtSKOR/GORK in the plasma membrane in infected cells, in contrast to that of MtAKT1, may have a compensatory effect for host cell survival in such harsh situations, because it can prevent the further loss of K+ from the cell. The AtGORK mutant of A. thaliana, lacking a functional K+ efflux channel, showed less damage under salinity stress conditions than the wild-type control (Demidchik et al., 2010). However, the location of MtSKOR/GORK in the symbiosome membrane may contribute to the K+ decrease observed in symbiosomes. The goal for a further study should be to find out the reasons for the mistargeting and the depletion of these channels in the plasma membrane in mature infected cells. One of the reasons for the incorrect incorporation of proteins into the plasma membrane might be the drastic changes in the actin cytoskeleton conformation found in nodule-infected cells as compared with non-infected cells, that may negatively affect the correct targeting of the vesicles (Fedorova et al., 2007; Gavrin et al., 2015). The existence of a mechanism coordinating SNARE-mediated vesicle trafficking and ion transport engaging directly with a subset of K+ channels, which has been identified in A. thaliana (Honsbein et al., 2009; Karnik et al., 2017), provides direction for further research of the process.

Nodule development is the result of selection, evolution, and also gene duplication (van Velzen et al., 2018). Evolution has given to legumes and rhizobia the ability to form an integral unit, the root nodule. However, the intracellular lifestyle of symbiotic bacteria affects the host cell. Symbiotic infected cells sustain a huge bacterial colony, and several physiological differences/changes in the infected cell have to be produced to allow it, as compared with nodule-non-infected cells. The changes in the host cell include, among others, membrane traffic and protein retargeting, as we have previously reported, such as the relocation of proteins of the SNARE group and small GTPases, which regulate the membrane fusion (Limpens et al., 2009; Ivanov et al., 2012; Gavrin et al., 2016), changes in the vacuolar system of infected cells and the relocation of aquaporins (Gavrin et al., 2014), rearrangements of the cytoskeleton conformation, and relocation of proteins involved in actin formation to the symbiosome membrane (Fedorova et al., 2007; Gavrin et al., 2015). Infected cell membrane trafficking is one of the main factors for the maintenance of symbiotic bacteria in the host cell.

Now, in this work, we have reported a new aspect in membrane trafficking and protein targeting in nodule-infected cells: mistargeting and partial depletion of the K+ channels MtAKT1 and MtSKOR/GORK from the plasma membrane. A clear mislocation of both channels was found in infected cells, as compared with non-infected cells. The mislocation of the channels is an event that might depend on changes in the cytoskeleton pattern, which in turn depends on the presence of bacteria in the symplast of infected cells.

Apart from the role of these channels in K+ transport, they may have important functions in stress response, because not only a cytosolic K+/Na+ ratio, but also absolute concentrations of K+ are essential to confer stress tolerance (Sharma et al., 2013; Wu et al., 2018; Adem et al., 2020). The outward-rectifying channel GORK is involved in stress-induced K+ loss from the cytosol (Adem et al., 2020), and modulates plant responses to abscisic acid (Lim et al., 2019). ROS are able to activate GORK and increase the loss of K+ (Demidchik et al., 2010). In the case of salinity stress, the deficiency of K+ in root cells can be compensated from the vacuolar pool, but, after this source is exhausted, cytosolic K+ declines below a certain threshold, and triggers PCi by activating caspase-like proteases and endonucleases (Adem et al., 2020). The data on the progressive decrease in K+ content in the vacuoles of infected cells support this scenario. According to IDA, in the senescence zone, the level of K+ in the vacuoles drastically diminished. It should be noted that hypoxia negatively affects K+ availability in plant roots (Gill et al., 2017; Wang et al., 2017), and the root nodule is forming a hypoxic environment. Nodules also show the presence of ROS (Andrio et al., 2013; Puppo et al., 2013; Montiel et al., 2016), which are major activators of K+ efflux by SKOR (Demidchik, 2018). Nodules need a fine-tuning of the K+ balance, and our results suggest that this ion homeostasis might differ between infected and non-infected cells and it might contribute to the short life span of infected cells.
In conclusion, in this work we have reported remarkable differences in K⁺ content and location of the channels MtAKT1 and MtSKOR/GORK between infected and uninfected nodule cells, which might compromise K⁺ availability and have physiological consequences for the host cell. We propose that *M. truncatula* nodule–infected cells do have alterations in K⁺ availability, as compared with non-infected cells, probably due to the high demand of intracellular rhizobia and the flaws in the location of the proteins of the host plant K⁺ channels MtAKT1 and MtSKOR/GORK. The reduction of K⁺ levels in the host cell, and in the microsymbiont itself, might negatively affect the life span of the infected cells and the ability of the symbiotic tissue to withstand stress. Analyses of *M. truncatula* lines overexpressing or silencing these channel genes might provide further insight into the mechanisms related to the events described here and their putative role in senescence, ionic stress responses, and other nodule physiological processes. Therefore, the special features of infected cell K⁺ transport need to be taken into consideration in studies aimed to improve the stress tolerance of root nodules.

**Supplementary data**

The following supplementary data are available at JXB online.

Table S1. List of primers used in this research.

Table S2. Relative distribution of elements in *Medicago truncatula* nodules.

Fig. S1. Putative functional domains of MtAK1 and MtSKOR/GORK proteins, predicted with the SMART tool.

Fig. S2. Structural features of the N–terminal region of MtAKT1.

Fig. S3. Structural features of the N–terminal region of MtSKOR/GORK.

Fig. S4. Single channel confocal images of the immunolocalization of MtAKT1 and MtSKOR/MtGORK shown in Fig. 4C and F.

Fig. S5. Western blot analysis of MtSKOR/GORK antigens in plasma membrane of *M. truncatula* roots with anti-MtSKOR antibody.

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**Author contributions**

EEF, TCP, and MML designed the research; EEF, VL-D, TCP, NAT, OK, JJP, and MML performed experiments and analysed data; EEF, TCP, and MML wrote the manuscript; EEF, TCP, OK, JJP, and MML edited the manuscript. The authors have no conflict of interest to declare.

**Data availability**

The raw data supporting the results reported in this article will be made available from the corresponding authors on request, without reservation.

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Potassium and the channels MIAKT1 and MTSKOR/GORK in nodules | 1347

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