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KT/HAK/KUP High-affinity Transporters in Plants
Vysokoafinitní přenašeče KT/HAK/KUP v rostlinách

Dissertation thesis

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RNDr. Edita Tylová, Ph.D.

List of Abbreviations

Aa	*Arabidopsis arenosa*
ABA	Abscisic acid
AIP1	Abscisic acid induced phosphatase 1
AKT1, 2	K^+ channel (*Arabidopsis* K^+ transport 1, 2)
APC	Superfamily of transporters (Acid-polyamine-organocation)
ARF2	Auxin response factor 2
At	*Arabidopsis thaliana*
bHLH121	Basic helix-loop-helix 121
CBL1, 8, 9, 10	Ca^{2+} binding regulatory protein (Calcineurin B-like 1, 8, 9, 10)
CIPK1, 4, 6, 9, 23	CBL-interacting protein kinase 1, 4, 6, 9, 23
CML9	Calmodulin-like 9
CPA	Family of K^+ transporters (Cation proton antiporter)
CTR1	Ethylene signalisation repressor (Constitutional triple response 1)
DDF2	Dwarf and delayed flowering 2
EIN2	Transducer of ethylene response (Ethylene insensitive 2)
ER	Endoplasmic reticulum
GFP	Green fluorescent protein
Gh	*Gosypium hirsutum*
GORK	K^+ channel (Guard cell outward rectifying)
HAK1, 2, 3, 5, 10, 21	K^+ transporters (High-affinity K^+ 1, 2, 3, 5, 10, 21)
HATS	High-affinity transport system
Hv	*Hordeum vulgare*
CHX	Subfamily of K^+ transporters (Cation/H^+ exchanger)
IAA	Indole-3-acetic acid
IAM	Indole-3-acetamide
ILK1	Integrin-linked kinase 1
JLO	Jagged lateral organs
KC1	K^+ channel 1
KCO/TPK	K^+ channel outward-rectifying/Two pore K^+
KEA	Subfamily of K^+ transporters (K^+ efflux antiporter)
KT/HAK/KUP	Family of K^+ transporters (K^+ transport/high-affinity K^+/K^+ uptake)
KT1	K^+ transport 1
KUP1, 2, 4, 5, 6, 7, 8, 9, 12	K^+ transporters (K^+ uptake 1, 2, 4, 5, 6, 7, 8, 9, 12)
LATS	Low-affinity transport system
Le	*Lycopersicon esculentum* (syn. *Solanum lycopersicum*)
Lj	*Lotus japonicus*
MRH1	Morphogenesis of root hair 1
MS	Growth medium (Murashige-Skoog)
MYB	Myeloblastosis

NHX	Subfamily of Na^+ transporters (Na^+/H^+ exchanger)
NRT1.5	Nitrate transporter 1.5
Os	*Oryza sativa*
PIN1, 2	Auxin transporter (Pin-formed 1, 2)
Pp	*Physcomitrella patens*
PP2C	Protein phosphatase 2C
PP2CA	Protein phosphatase 2CA
Ppe	*Prunus persica*
QC	Quiescent centre
qRT-PCR	Quantitative real-time polymerase chain reaction
RAM	Root apical meristem
RAP2.11	Related to AP 2.11
RbohD	Respiratory boost oxidase homolog D
RCI3	Rare cold inducible 3
RDR6	RNA dependent RNA polymerase 6
RHD2/RbohC	Root hair defective 2/Respiratory boost oxidase homolog C
ROS	Reactive oxygen species
RSA	Root system architecture
SGN1/SGN3	Schengen 1/Schengen 3
SKOR	K^+ channel (Stelar K^+ outward rectifier)
SNARE	Synaptosomal-associated protein receptor
SRAS1	Salt-responsive alternatively spliced 1
St	*Solanum tuberosum*
SUT1, 4	Sucrose transport 1, 4
SYP121	Syntaxin protein 121
T-DNA	Transfer-DNA
TEA	Tetraethyl ammonium
TFII_A	Transcription factor II_A
TPC1	Ca^{2+} channel (Two-pore channel 1)
TPCs	Members of TPC family (Two pore channel)
TPK1	K^+ channel (Two-pore K^+ 1)
TRH1	K^+ transporter (Tiny root hair 1)
TRK/HKT	Family of K^+ and Na^+ transporters (Transport of K^+/High-affinity K^+
Vv	*Vitis vinifera*

Abstract

Due to essential role of potassium in plant metabolism and its often low availability in the soil, sufficient potassium uptake and its management are among the challenges plants face to survive in different environments. Since all potassium functions are related to the transport of the monovalent cation K^+, research focuses on the transporters responsible for K^+ uptake and allocation.

In addition to playing an essential role in potassium nutrition, K^+ transporters also mediate the uptake of pollutants such as Cs. Radioisotopes ^{134}Cs and ^{137}Cs released from nuclear bomb tests and nuclear power plant accidents remain in the environment and their accumulation in plants is being studied to reveal the risks of crop production in contaminated soils as well as the possibility to remediate contaminated soil through plants. Ion transport also seems to be a major driver of plant adaptation to unfavourable environments. Several ion transporters appear to be involved in the adaptation of *Arabidopsis arenosa* populations to serpentine soils, including the potassium transporter AaKUP9.

This thesis summarises my effort to characterise two transporters from KT/HAK/KUP family, AtKUP7 and AtKUP9, in *Arabidopsis thaliana*, and related projects. For AtKUP9, I have significantly expanded the knowledge of its involvement in physiological processes. I studied the root growth phenotype of *atkup9* and showed that under K^+ deficient conditions, growth is unevenly distributed in favour of the primary root over the lateral roots and this phenotype cannot be reversed by auxin application. I detected *AtKUP9* expression throughout the plant body and showed that it is significantly increased and shifted under low-K conditions. I found an altered carbohydrate allocation in *atkup9* plants. *Atkup9* retained large amounts of soluble carbohydrates in shoot as well as it contained more starch in K^+ deficiency. All these results suggest the participation of AtKUP9 in various physiological processes that remain to be investigated in future. It is obvious that AtKUP9 affects pathways that control the growth of root system and its architecture.

I further summarised the relationship between K^+ availability and the growth and development of the root system in a review paper. I examined how and why root growth is reduced under K^+ deficiency in general, including the low root-to-shoot ratio and root system architecture changes. Emphasis was placed on the associated signalling and nutrient localisation. The effect of K^+ availability on stress tolerance and cell growth was also summarised.

Characterisation of AtKUP7 focused on its role in Cs translocation in the plant body and I showed that *atkup7* plants take up and allocate to the shoot less $^{134}Cs^+$ than wild type. Although this do not translate into increased Cs tolerance in *atkup7*, it suggests that AtKUP7 is a transporter that mediate Cs accumulation in plants and Cs translocation to shoot, which is of ecological importance.

Finally, within a project related to a role of AaKUP9 in serpentine adaptation, I took part in a characterisation of multiple colonisations of serpentine habitats by *Arabidopsis arenosa*. I analysed root system traits of three *A. arenosa* population pairs *in vitro* on medium simulating low Ca/Mg ratio typical for serpentine soils. These results help to describe parallel adaptations as well as to discern interesting non-parallel adaptations among the populations, such as the relatively low Ca/Mg ratio in the tissue of one of the studied populations. It is clear that while two populations manage their uptake of Mg^{2+} and Ca^{2+}, third population is able to withstand unfavourable Ca/Mg ratio in its tissue.

Keywords: Potassium, membrane transporters, osmoregulation, root system, caesium

Abstrakt

Vzhledem k esenciální roli draslíku v rostlinném metabolizmu a jeho omezené dostupnosti v půdě, patří zajištění dostatečného příjmu draslíku a regulace jeho koncentrace v rostlině k jedněm z výzev, kterým rostliny čelí při snaze přežít v různých prostředích. Protože všechny funkce draslíku jsou vázány k transportu jeho monovalentní formy K^+, výzkum je soustředěn na transportéry zodpovědné za příjem a transport tohoto iontu.

Kromě své esenciální role ve výživě draslíkem, transportéry K^+ také umožňují příjem polutantů jako například cesia. Radioisotopy ^{134}Cs a ^{137}Cs se do prostředí dostaly při testech jaderných bomb a při haváriích jaderných elektráren a zůstávají v něm dodnes. Jejich akumulace v rostlinách je studována za účelem odhalení možného rizika pěstování plodin na znečištěných půdách a pro možnou remediaci kontaminovaných půd rostlinami. Iontové transportéry jsou zřejmě také zásadní pro přizpůsobení rostlin k nehostinnému prostředí. Několik transportérů je zapojeno do adaptace populací *Arabidopsis arenosa* k hadcovým půdám, včetně draselného transportéru AaKUP9.

Tato práce shrnuje mé úsilí o charakterizaci dvou transportérů z rodiny KT/HAK/KUP, AtKUP7 a AtKUP9, v *Arabidopsis thaliana* a mou práci na přidružených projektech. Pro AtKUP9 jsem významně rozšířil poznání o jeho zapojení do fyziologických procesů. Studoval jsem růstový fenotyp kořenů *atkup9* a prokázal jsem, že za podmínek nedostatku K^+ je růst nerovnoměrně rozdělen ve prospěch hlavního kořene oproti postranním kořenům a tento fenotyp nemůže být zvrácen aplikací auxinu. Lokalizoval jsem expresi *AtKUP9* napříč rostlinným tělem a ukázal jsem, že při nedostatku K^+ se exprese signifikantně mění. Také jsem zjistil, že alokace sacharidů je změněná v *atkup9*. *Atkup9* akumuluje velké množství rozpustných sacharidů v prýtu a krom toho také vytváří více škrobu při nedostatku K^+. Mé výsledky ukazují na zapojení AtKUP9 do řady fyziologických procesů, které v této souvislosti doposud nebyly zkoumány. Je zřejmé, že AtKUP9 ovlivňuje dráhy, které řídí růst kořenového systému a utváření jeho architektury.

V přehledovém článku jsem se ještě více zaměřil na vztah mezi dostupností K^+ a růstem a vývojem kořenového systému. V článku shrnuji, proč a jak je růst kořenů omezován při deficienci K^+, což vede až ke sníženému poměru kořenů k prýtu a ke změnám v architektuře kořenového systému. Především jsem se zaměřil na zapojené signalizační dráhy a na alokaci živin při tomto procesu. Vliv dostupnosti K^+ na růst buněk a na odolnost ke stresu byl také diskutován.

Charakterizace AtKUP7 byla zaměřena na jeho roli v translokaci Cs v rostlinném těle a podařilo se mi ukázat, že v *atkup7* je snížen příjem Cs i transport do prýtu oproti divokému genotypu. Ačkoli se tento fenotyp nepromítá do odolnosti *atkup7* k Cs, moje výsledky naznačují, že AtKUP7 je zapojen do transportu Cs.

V neposlední řadě, jsem se, v rámci projektu zabývajícího se rolí AaKUP9 v adaptaci na hadcové půdy, zapojil do charakterizace kolonizací hadcových půd *Arabidopsis arenosa.* Analyzoval jsem kořenové systémy tří párů populací *A. arenosa in vitro* pěstovaných na médiu simulujícím nízký poměr Ca/Mg typický pro hadcové půdy. Tato kultivace pomohla určit, že se jedná o paralelní kolonizace, a navíc odhalila neparalelní znaky mezi jednotlivými adaptacemi, jako například relativně nízký Ca/Mg poměr v pletivech jedné ze studovaných populací. Z výsledků je zřejmě, že zatímco dvě studované populace výrazně regulují příjem Mg^{2+} a Ca^{2+}, třetí populace je schopna překonat nepříznivý Ca/Mg poměr ve svých pletivech.

Klíčová slova: Draslík, membránové transportéry, osmoregulace, kořenový systém, cesium

1. Introduction

Plants have to cope with changing conditions of different environments. One of the challenges they face is to get optimal amount of essential mineral nutrients despite their highly variable availability in soils. Potassium is no exception. Although it is the seventh most abundant element in lithosphere, concentration of plant-accessible K^+ varies in soils mostly due to K^+ immobilization on clay particles. Steady uptake of K^+ by plant roots may also develop depletion zones around the roots where concentration of available K^+ in soil solution might be in micromolar range (Römheld and Kirkby, 2010), while K^+ concentration in cytoplasm is maintained around 100 mM (Leigh and Wyn Jones, 1984). K^+ serves as a major osmotic of plant cell and in this role, it drives the volume growth of plant cell (Takahashi and Kinoshita, 2016) and participates in stomata closure (Blatt, 2016). K^+ homeostasis in cytoplasm is also important for proteosynthesis and enzyme activity (Walker et al., 1998). Large array of mechanisms has evolved in order to maintain K^+ levels in plant tissue enabling growth and reproduction.

K^+ has to be taken up into the symplast of root cells, then it may be transported to other plant parts. In cells, K^+ is either used to establish homeostasis in the cytoplasm or stored in the vacuole, where it also enables water accumulation during volume growth. As the monovalent cation is the only chemical form of potassium used by plants, research is focused on the transporters responsible for K^+ uptake and allocation. Novel transporters are usually characterized in the model plant *Arabidopsis thaliana* and this knowledge is later applied to crops such as barley (*Hordeum vulgare*), rice (*Oryza sativa*) or tomato (*Solanum lycopersicum*).

The major aim of this thesis is to contribute to the understanding of K^+ transport mechanisms in plants and plant response to potassium deficiency by characterizing KT/HAK/KUP (K^+ transport/high-affinity K^+/K^+ uptake) family transporters AtKUP7 and AtKUP9, whose functions in *Arabidopsis thaliana* are not fully understood. The relationship between K^+ supply and root growth were examined in the experiments as well as thoroughly reviewed in the review paper included in this thesis.

In addition to their essential role in potassium nutrition, K^+ transporters also enable the uptake and transport of other alkali ions such as Na^+, Rb^+ and Cs^+. On one hand, this enables usage of Rb^+ and Cs^+, which are mostly missing in plant tissue, as K^+ tracers in experiments. On the other hand, it also provides a pathway by which pollutants enter plant tissues. Caesium occurs naturally in the soil in very low concentrations of around 3 ppm, although radioactive isotopes ^{134}Cs and ^{137}Cs were released into the environment during atomic bomb tests and nuclear power plant accidents; especially in Chernobyl in 1986 and Fukushima in 2011. These isotopes are the first relatively stable products (half-life of 2 and 30 years) of enriched uranium fission in power plants and bombs and remain in the environment to this day. Their accumulation in plants is studied to reveal risks of crop production in contaminated soils as well as the potential to remediate contaminated soil via plants. Dynamics of Cs^+ accumulation in plants lacking K^+ transporter AtKUP7 is studied in one of the papers included in this thesis.

Ion transport also seems to be an important driver in the adaptation of plant populations to hostile environments, including serpentine soils. Several ion transporters were recruited in multiple separate adaptations of *Arabidopsis arenosa* to serpentine soils including AaKUP9 and AaTPC1 (Konečná et al., 2021). In a paper included in this thesis, I contributed to the characterization of growth response of serpentine and non-serpentine populations of *Arabidopsis arenosa* by *in vitro* simulation of serpentine conditions.

2. Scientific background

2.1. Important families of K^+ transport proteins

In the beginning, I want to introduce the families of K^+ transport proteins with an emphasis on KT/HAK/KUP transporters, which are the core of this thesis.

2.1.1. Voltage gated K^+ channels

Plant voltage-gated K^+ channels are related to animal Shaker channels, although they make up a distinct branch and they include more functional domains (Jegla et al., 2018). They consist of four alfa subunits surrounding a transmembrane aqueous pore. Each subunit contains six transmembrane domains (S1 – S6) and a large C-terminal domain. First four transmembrane domains form a voltage sensor, which is responsible for opening and closing of the channel. Fourth transmembrane domain contain multiple conserved positively charged amino acids. Domains S5 and S6 together with the pore loop participate in the formation of the pore in the functional tetramer. C-terminal domain is responsible for tetramerization of subunits in the membrane and it may be involved in binding with interacting proteins and targeting to the membrane (Nieves-Cordones et al., 2014, Jegla et al., 2018).

Voltage-gated K^+ channels are divided into three subfamilies according to their response to changes in membrane potential. 1) Inward-rectifying channels open upon membrane hyperpolarization and enable K^+ uptake. 2) Outward rectifying channels activate upon membrane depolarization and mediate K^+ release. 3) Week rectifying channels mediate K^+ currents in and out of the cell. In addition, some subunits are silent and do not form functional homotetramer channels. These subunits regulate channel activity by making heterotetramers with other subunits (Sharma et al., 2013).

Voltage gated K^+ channels participate in many physiological processes such as K^+ uptake under K^+ rich conditions (Hirsch et al., 1998, Li et al., 2014), xylem loading (Gaymard et al., 1998), transport of K^+ and carbohydrates into a phloem sap (Gajdanowicz et al., 2011) and stomatal movements (Hosy et al., 2003).

2.1.2. KCO/TPK channels

KCO/TPK (K^+ channel outward-rectifying/Two pore K^+) are voltage insensitive channels that are localized mostly on the tonoplast (Dabravolski and Isayenkov, 2021). This family is largely uncharacterized with the exception of AtTPK1. This channel is involved in K^+ homeostasis between cytoplasm and vacuole and it participates in stomata closing in ABA (abscisic acid) dependent manner (Gobert et al., 2007, Isner et al., 2018).

2.1.3. Two-pore channels

Two pore channels (TPCs) are localized on the tonoplast where they participate in so called slow vacuolar currents of K^+ (Gutla et al., 2012). These channels are activated by an increase in cytoplasmic Ca^{2+} concentration, and, apart from previously mentioned families, TPCs are less selective for K^+ over Na^+ and Cs^+ (Pottosin and Dobrovinskaya, 2022).

2.1.4. KT/HAK/KUP transporters

KT/HAK/KUP family belongs to APC (Acid-polyamine-organocation) superfamily of transporters (Vastermark et al., 2014). It is diverse in plants where 10 to 30 KT/HAK/KUP genes per genome are routinely reported. Genomes of fungi and bacteria usually contain 1 – 5 KT/HAK/KUP genes (Nieves-Cordones et al., 2016a), while these genes are missing in animals (Grabov, 2007). KT/HAK/KUP transporters of terrestrial plants are divided into five clusters that diverged after the colonization of land, further emphasizing the importance of KT/HAK/KUP transporters for coping with conditions of challenging ion uptake (Nieves-Cordones et al., 2016b, Santa-María et al., 2018). Detailed characterization of individual KT/HAK/KUP transporters is provided in 2.4. and other relevant chapters.

KT/HAK/KUP transporters consist of a variable number (10 – 14) of transmembrane helices and three cytoplasmic domains – the N- and C-terminal domains and a large loop between the second and third transmembrane helices (Sato et al., 2014, Santa-María et al., 2018). The N-terminal cytoplasmic domain is a target of activating phosphorylation, which was shown for AtHAK5, the key K^+ transporter of *A. thaliana* in low-K availability, as well as for its orthologues in *Chenopodium quinoa* and *Dionaea muscipula* (Ragel et al., 2015, Scherzer et al., 2015, Böhm et al., 2018). A similar mechanism was also reported for AtKUP7 (Han et al., 2016).

Plant KT/HAK/KUP transporters have the C-terminal domain enlarged compared to their bacterial and fungal relatives (Nieves-Cordones et al., 2016b). In AtHAK5, the C-terminal domain consists of an inhibitory and an activation domain, which participate in the activation of AtHAK5 by interaction with the phosphorylated N-terminus (Ródenas et al., 2021). In AtKUP6 and AtKUP7, phosphorylation sites crucial for the activation of these transporters were found in the C-terminal domain (Osakabe et al., 2013, Han et al., 2016).

Last but not at least, amino acids crucial for K^+ transport in the AtHAK5 pore were determined. The first transmembrane domain contains GVVYGD motif, which is very similar to the GYGD motif for K^+ selectivity in Shaker-like channels (Doyle et al., 1998). Mutations in G67, Y70, G71 and D72 all lead to loss of function in heterologous expression. In the same manner, mutation in D201 in the 3rd transmembrane domain and E312 in the 6th transmembrane domain results in loss of function (Ródenas et al., 2021).

2.1.5. TRK/HKT transporters

TRK/HKT (Transport of K^+/High-affinity K^+ transporter) transporters facilitate transport of K^+ and Na^+ (Corratgé-Faillie et al., 2010). TRK/HKT family members were identified as key players in K^+ and Na^+ homeostasis in *A. thaliana* (Berthomieu et al., 2003) and *O. sativa* (Hussain et al., 2022). TRK/HKT transporters are more diversified in plant species that are able to compensate K^+ scarcity by Na^+ uptake such as *O. sativa*, where they have specific functions in Na^+ uptake and allocation (Hussain et al., 2022).

2.1.6. CPA transporters

CPA (Cation proton antiporter) family is diverse and relatively little characterized. It consists of three subfamilies: NHX (Na^+/H^+ exchanger), CHX (Cation/H^+ exchanger) and KEA (K^+ efflux antiporter). KEA transporters are involved in K^+ homeostasis in chloroplasts. AtKEA1 and AtKEA2 localize to inner envelope membrane of chloroplast, while AtKEA3 is in thylakoid membrane. These three transporters play a role in the function of chloroplasts and the maintenance of their proper ultrastructure (Kunz et al., 2014).

NHX transporters are vital for salt stress tolerance and are involved in maintaining pH and osmotic potential as shown for AtNHX1 and AtNHX2 (Bassil et al., 2011).

2.2. K^+ uptake

Efficient uptake of K^+ from the soil is a prerequisite for successful plant K^+ management, therefore, understanding K^+ uptake by plant roots and the role of the relevant K^+ transport proteins has received considerable attention. This chapter summarizes the mechanisms by which plants obtain sufficient K^+ from the soil, focusing on the molecular mechanisms of K^+ uptake.

2.2.1 Kinetics of K^+ uptake

Studies on the rate and kinetics of K^+ uptake provided the first important insights into the relationship between K^+ availability and uptake into root cells before genetic models were available in plant experimental biology. The pioneering work of Epstein et al. (1963) established that K^+ uptake in barley is biphasic and consists of a high-affinity and a low-affinity system. Similar mechanisms were later found in other model plants, and due to the different sensitivity to inhibitors, it was proposed that the high-affinity and low-affinity systems are mediated by different transport proteins (Kochian and Lucas, 1982).

The low-affinity transport system (LATS) operates at higher extracellular K^+ concentrations and is energized by a negative membrane potential, making it energetically passive (Gassmann and Schroeder, 1994). In some studies, saturation of LATS was not observed even at very high K^+ external concentration (Kochian and Lucas, 1982), although these studies did not distinguish between apoplastic and cellular uptake (Coskun et al., 2016).

This system is inhibited by TEA (tetraethyl ammonium), an inhibitor of animal shaker channels (Kochian and Lucas, 1982), and in later studies the participation of plant shaker-like channels in LATS was proven. Among them, the involvement of AtAKT1 (*Arabidopsis* K^+ transport 1) channel in K^+ uptake is well described (Hirsch et al., 1998, Rubio et al., 2008).

For the high-affinity transport system (HATS), K_M values between 10 and 20 μM were found and its saturation was detected to be between 0,1 – 0,4 mM external K^+ (Epstein et al., 1963, Maathuis and Sanders, 1997, Martínez-Cordero et al., 2004). In *A. thalian* and *O. sativa*, energization of high-affinity transport was examined electro-physiologically and the transport of K^+ was proposed to be mediated by symport with 1 H^+ (Maathuis and Sanders, 1994, Nieves-Cordones et al., 2017). The sensitivity of HATS to NH_4^+ was found in various model plants (Spalding et al., 1999, Bañuelos et al., 2002, Martínez-Cordero et al., 2005, Ragel et al., 2019), suggesting that HATS transport was mediated by related transporters. Later KT/HAK/KUP transporters were isolated from different plant species and these transporters had the characteristics of respective HATS in heterologous expression (Santa-Maria et al., 1997, Martínez-Cordero et al., 2004, Nieves-Cordones et al., 2007).

2.2.2. Transporters and signalling elements involved in low-affinity K^+ uptake in roots

Involvement in low-affinity K^+ transport was first documented for the voltage gated K^+ channel AtAKT1 based on its transport properties and *AtAKT1* expression pattern (Hirsch et al., 1998). Later, similar roles were assigned to AtAKT1 orthologues in other plant models, e.g. in rice (Li et al., 2014) and barley (Vallejo et al., 2005). Nowadays it is generally accepted, that voltage-gated K^+ channels are involved in low-affinity K^+ uptake in most plant species.

AKT1 belongs to inward rectifying K^+ selective channels. These channels open when the membrane potential is low enough to allow K^+ to enter the cell. On the other hand, it was

discovered, that homotetramers of AtAKT1 are prone to K^+ leakage from the cells under physiological conditions (Geiger et al., 2009). In *A. thaliana*, this is compensated by the silent subunit KC1 (K^+ channel 1). AtKC1 does not form homotetramers *in vivo* but its incorporation into the channel complex with AtAKT1 subunits shift the voltage dependence of the complex to more negative potentials (-70 mV), thus preventing the K^+ leakage (Geiger et al., 2009, Wang et al., 2010).

AtAKT1 interacts with and is positively regulated by the AtCIPK23 (CBL-interacting protein kinase 23) kinase in the complex with one of the two AtCBL1 and AtCBL9 (Calcineurin B-like 1, 9) regulatory calcium binding proteins (Li et al., 2006, Xu et al., 2006). It was also shown that AtAKT1 is deactivated by PP2C-type (Protein phosphatase 2C) phosphatase AIP1 (Abscisic acid induced phosphatase 1) in *Xenopus* oocytes (Lee et al., 2007). The currently accepted model of AtAKT1 regulation is the molecular complex of CIPKs, CBLs and API1 whose exact composition and modulation affects activity of AtAKT1 (Lan et al., 2011).

2.2.3. Transporters and signalling elements involved in high-affinity K^+ uptake in roots

HvHAK1 (High-affinity K^+ 1) transporter from barley was described as the first transporter that is likely involved in high-affinity K^+ uptake (Santa-Maria et al., 1997). Later its orthologues AtHAK5 and OsHAK5 were assigned the same functions in *A. thaliana* and *O. sativa* (Gierth et al., 2005, Pyo et al., 2010, Yang et al., 2014). These transporters belong to the KT/HAK/KUP family. The high-affinity transport system is typically inducible and the expression of genes encoding involved transporters is rapidly increased when plants are exposed to low K^+ availability (Véry et al., 2014). The transcriptional regulation was intensively studied in *A. thaliana* on its dominant inducible K^+ transporter AtHAK5.

After the onset of K^+ limitation, plant roots produce large amounts of ethylene, which enables the plant response to low K^+ availability through its canonical effectors AtCTR1 (Constitutional triple response 1) and AtEIN2 (Ethylene insensitive 2), as well as through some other unknown pathways (Jung et al., 2009). Moreover, a K^+ sensing niche was identified inside *A. thaliana* root tip, which exhibits active K^+ depletion and a peak in cytoplasmic Ca^{2+} concentration within one minute after K^+ removal (Behera et al., 2017, Wang et al., 2021). Downstream of ethylene signalization, the production of ROS (reactive oxygen species) is upregulated through the action of NADPH oxidases AtRHD2/RbohC (Root hair defective 2/Respiratory boost oxidase homolog C)(Shin and Schachtman, 2004), AtRbohD (Respiratory boost oxidase homolog D)(Wang et al., 2021), which are activated through phosphorylation by AtSGN1/SGN3 (Schengen 1/Schengen 3) complex in Ca^{2+} dependent manner, and the peroxidase AtRCI3 (Rare cold inducible 3)(Kim et al., 2010). Subsequently, elevated ROS levels enhance the production of the transcription factor AtRAP2.11 (Related to AP 2.11), which binds to the ethylene responsive element and the GCC-box in the *AtHAK5* promoter (Kim et al., 2012).

Other transcription factors were also found to interact with *AtHAK5* promoter, although their upstream regulation is not known. Transcription factors AtDDF2 (Dwarf and delayed flowering 2), AtJLO (Jagged lateral organs), AtbHLH121 (Basic helix-loop-helix 121), and AtTFII_A (Transcription factor II_A) enhance *AtHAK5* transcription (Hong et al., 2013), while AtARF2 (Auxin response factor 2) binds to auxin responsive elements in *AtHAK5* promoter and shuts down transcription under K^+ sufficient conditions. In K^+ shortage, AtARF2 is rapidly phosphorylated by unknown kinase and released from *AtHAK5* promoter, enabling the transcription of *AtHAK5* (Zhao et al., 2016).

This system of transcriptional regulation is not strictly K^+ specific as *AtHAK5* expression is also induced in nitrogen and phosphorus deficiencies in *A. thaliana* and *Solanum lycopersicum* (Rubio et al., 2014). Interestingly, high-affinity K^+ uptake was detected only in

K^+ depleted plants suggesting a post-transcriptional regulation (Rubio et al., 2014). In accordance with this, AtHAK5 protein was localized mostly to endoplasmic reticulum in K^+-sufficient plants, while K^+ starvation triggered the enrichment of AtHAK5 protein in the plasma membrane (Qi et al., 2008). The activation of AtHAK5-mediated high-affinity K^+ transport was discovered in *A. thaliana* (Ragel et al., 2015), *Dionaea muscipula* (Scherzer et al., 2015) and *Chenopodium quinoa* (Böhm et al., 2018) to be mediated by the CIPK23/CBL1/9 interacting complex. This complex also participates in the regulation of the K^+ channel AtAKT1 as discussed earlier (Xu et al., 2006). Both AtCIPK23 kinase and AtCBL1/9 Ca^{2+} sensors were necessary and sufficient for AtHAK5 activation in yeast (Ragel et al., 2015). Besides AtCBL1/9, AtCBL8 and 10 are able to bind to AtCIPK23 and activate AtHAK5 in yeast (Ragel et al., 2015). The mechanism of AtHAK5 activation seems to be a modification of transport properties (increase in affinity (lower K_M) and increase in maximal rate of transport (V_M)) likely through conformational changes after phosphorylation. Since both phosphorylation by CIPK/CBL and physical interaction between AtHAK5 and CIPK/CBL complex is required for full activation in yeast, second mechanism of AtHAK5 regulation by CIPK/CBL complex was proposed to be the localization of AtHAK5 to the plasma membrane (Ragel et al., 2015, Ragel et al., 2019). Similar mechanism was discovered for Raf-like kinase AtILK1 (Integrin-linked kinase 1). The kinase together with calmodulin-like protein AtCML9 binds to AtHAK5 and when these three proteins are co-expressed in *Nicothiana benthamiana* leaves, the AtHAK5 is recruited to the plasma membrane (Brauer et al., 2016). AtHAK5 was also activated in heterologous expression by other members of the CIPK family: AtCIPK1 and AtCIPK9, although relevance of these interactions has not been examined yet (Lara et al., 2020).

2.2.4. Interplay of K^+ transport proteins under changing environmental conditions

Plant roots face heterogenous soil conditions that vary in K^+ availability in space and time. In addition, K^+ uptake by plants can lead to the formation of depletion zones around the roots, and plants must adapt to the decreased local K^+ concentration. The interplay of low-affinity and high-affinity transport systems is involved in fine-tuning of K^+ uptake within the root system to support plant growth.

When external K^+ is sufficient, it is taken up into the root cells mostly via AtAKT1 channel (Rubio et al., 2010), which remains active by interaction with AtCIPK23 kinase (Lee et al., 2007). This uptake is energized by negative membrane potential, which is created by the plasma membrane H^+ ATPases. At a common steady membrane potential of -120 to -180 mV, plant cells can sustain 100 – 1000x inward-directed K^+ gradient (Ragel et al., 2019). On the other hand, active uptake by symport with H^+ through KT/HAK/KUP transporters is not involved when external K^+ concentrations are relatively high. Under these conditions K^+/H^+ symport is not efficient enough to offset the increased energy cost of H^+ gradient restoration through H^+ ATPases.

When the concentration of K^+ in the extracellular environment decreases, the transport capacity of AtAKT1 is hampered, and Ca^{2+} dependent activation pathway is activated. First, the AtCIPK9/CBL2/3 complex is activated, which releases vacuolar K^+ reserves and enhances the accumulation of AtCIPK23, AtCBL1 and AtCBL9 proteins. AtCIPK23 is phosphorylated by unknown kinase and it phosphorylates AtCBL1 and AtCBL9 (Li et al., 2023). This AtCIPK23/CBL1/9 complex fully activates AtAKT1 while AtKC1 subunit is incorporated into the tetrameric channel to change channel transport properties and prevent K^+ leakage from the cells (Wang et al., 2016). On top of this, the plasma membrane is hyperpolarized during K^+ shortage, enabling K^+ transport along the electric potential gradient. In *A. thaliana*, AtAKT1 is significantly involved in K^+ uptake in low-K environment (Rubio et al., 2008) and was active in root protoplasts at K^+ concentrations as low as 10 μM (Hirsch et al., 1998). Moreover, the

transcription of *AtHAK5* is considerably upregulated in K^+ scarcity and this high-affinity K^+ transporter is post-transcriptionally activated by AtCIPK23/CBL1/9 (Ragel et al., 2015) and AtILK1 (Brauer et al., 2016) as described earlier. It was estimated that AtHAK5 mediated currents account for the vast majority of K^+ uptake (Gierth and Maser, 2007) The processes involved in response to low K^+ availability are summarized in Fig 1.

2.2.5. How plants sense the availability of K^+?

In this thesis, K^+ uptake proteins have so far been placed in the context of signalling pathways, where the crucial link between K^+ availability and plant response has been omitted. Until today, the search for a sensor for either K^+ availability at the plant-soil interface or K^+ level in plant cells has not been resolved. Here I present the fragmented information that has been obtained so far.

A. thaliana atakt1 seedlings lack the root growth cessation under low K^+ availability, observed in the wild type. In the wild type, acropetal auxin transport and localization of AtPIN1 (Pin-formed 1) transporter in the root stele are disrupted under K^+ shortage, whereas auxin homeostasis is maintained in *atakt1*, leading to sustained growth. These results suggest that AtAKT1 may have a sensory role in the growth response, although its relevance in regulating transport systems is unresolved (Li et al., 2017). Similarly, the canonical nitrate transporter AtNRT1.5 (Nitrate transporter 1.5) regulates the growth response to K^+ shortage through accumulation or degradation of AtPIN2 (Pin-formed 2) transporter (Wang et al., 2022). NRT1.5 is also involved in K^+ xylem loading as discussed later in this thesis, however its involvement in the regulation of K^+ uptake has not been yet studied. The transporters AtKUP5 and AtKUP7 could also hypothetically serve as K^+ sensors as both possess AMP-cyclase activity and the ability to transport K^+ (Al-Younis et al., 2015, Al-Younis et al., 2018). Cyclic AMP is a signalling molecule whose role in plants is still poorly understood. This AMP-cyclase activity may be coupled with the rate of K^+ transport, although this possibility remains to be studied.

Although the molecular sensor of K^+ availability has not been determined with certainty, there are discoveries, that suggest a link between environmental signals and the regulation of transport systems.

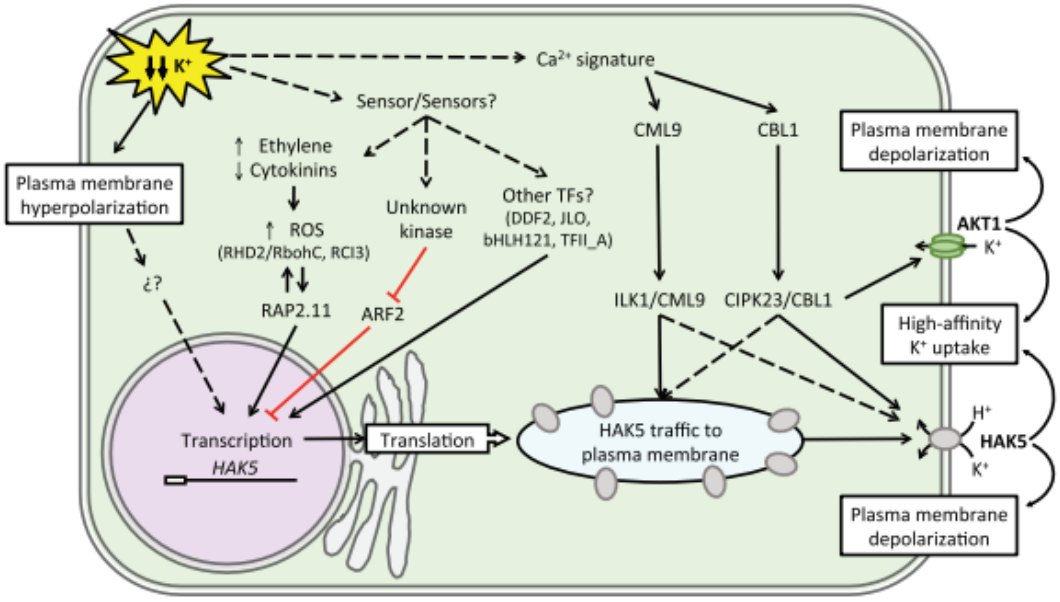

Fig 1: Summary of signalling involved in the response to K^+ scarcity in the root cell. Continuous arrows mark experimentally proven pathways, whereas discontinuous arrows indicate missing links in these pathways. Adapted from (Ragel et al., 2019).

Plasma membrane is hyperpolarized upon K^+ withdrawal as well as in a number of other nutrition stresses. Enhanced expression of *AtHAK5* as well as *LeHAK5* from *Solanum lycopersicum* was observed in other nutrient deficiencies such as N and P and was correlated to plasma membrane hyperpolarization (Nieves-Cordones et al., 2008, Rubio et al., 2014). Based on these observations, plasma membrane hyperpolarization was implicated by some authors into the transcriptional regulation of *AtHAK5*, although its inclusion among other systems is uncertain (Yang et al., 2014, Ragel et al., 2019). It is also apparent that this pathway is not specific for K^+ unlike the Ca^{2+} dependent pathways (Rubio et al., 2014).

A K^+-specific response was discovered in the apex of *A. thaliana* roots. Cells of a specific K^+-sensing niche located in the central part of the transition zone rapidly decrease their K^+ content upon K^+ withdrawal, which appears to be an active process (Wang et al., 2021), although its trigger remains to be found. The decrease in cytoplasmic K^+ concentration is followed by a short and intense spike in cytoplasmic Ca^{2+} concentration, which may activate the Ca^{2+} dependent CIPK23/CBL1,9 system in a K^+-dependent manner (Wang et al., 2021).

2.3. Long distance transport and partitioning of K^+

K^+ ions acquired by the roots have to be translocated to other organs of the plant body. This is managed via the xylem and phloem long distance transport together with other nutrients. The following chapters review xylem and phloem transport of K^+ with emphasis on the transporters and channels involved (for summary of transport proteins involved in K^+ partitioning see Fig. 2).

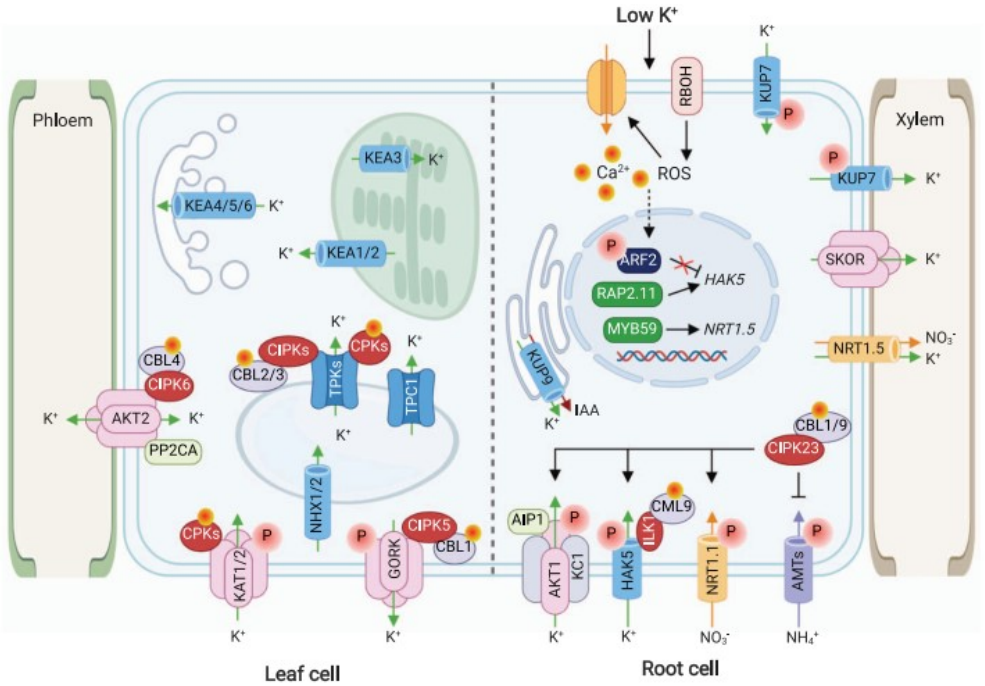

Fig. 2: Diagram showing major contributors to K^+ transport in *Arabidopsis thaliana* and some of the signalling elements involved. Adapted from (Wang et al., 2021).

2.3.1. Xylem transport

K^+ is taken up in the peripheral root cells, which are represented by the rhizodermis and cortex. K^+ is then either locally sequestered into vacuoles or transported to the upper parts of the plant via the xylem. Xylem transport is critical for K^+ allocation to the shoot and is mostly driven by negative hydrostatic pressure generated by shoot transpiration. Water uptake in roots stimulated by the accumulation of osmotically active nutrients such as K^+ in the xylem in the root stele also generates a positive force (positive hydrostatic pressure) known as root pressure. Long-distance transport through the xylem takes place in the apoplast of xylem tracheary elements, vessels or tracheids. To prevent uncontrolled leakage of nutrients from the stele to the root cortex, the stele is surrounded by specialized endodermal cells that block the apoplast laterally. This leads to the need for xylem loading. Nutrients are transported symplastically through the endodermis to the stele and then exported from the parenchyma of xylem to the apoplast of tracheary elements (Barberon, 2016). Several transporters involved in this process have been characterized.

AtSKOR (Stelar K^+ outward rectifier) voltage-gated K^+-selective channel was the first characterized xylem loader. *AtSKOR* is expressed in the pericycle and xylem parenchyma of the root stele, where this channel mediates K^+ efflux into xylem vessels (Gaymard et al., 1998). AtSKOR opens upon plasma membrane depolarization and its gating is sensitive to external K^+ concentration. At K^+ concentrations above 10 mM, AtSKOR opens at less negative membrane potentials, minimizing the possibility of becoming a K^+ influx pathway (Johansson et al., 2006).

Expression of *AtSKOR* is negatively affected by ABA. ABA is synthesized under drought stress condition, so the regulation of AtSKOR may suggest that K^+ retention in roots during drought helps to establish the osmotic adjustments in the roots (Gaymard et al., 1998).

AtSKOR is post-translationally regulated by ROS in voltage-dependent manner through oxidation of Cys168. This mechanism probably optimizes K^+ allocation to the shoot under saline conditions (Garcia-Mata et al., 2010).

In addition to AtSKOR, other transporters participate in the loading of K^+ into xylem. KT/HAK/KUP transporter AtKUP7 is involved in both K^+ acquisition and xylem loading under K^+ limiting conditions (Han et al., 2016). The mechanism by which AtKUP7 participates in xylem loading is not exactly clear. It was hypothesized that AtKUP7 may switch to a K^+ exporter in xylem parenchyma cells (Han et al., 2016) or may take up additional K^+ into these cells (Yang et al., 2014, Ragel et al., 2019). In rice, several KT/HAK/KUP transporters are involved in xylem loading. OsHAK1 is important for root-to-shoot transport of K^+ and its function affects grain yield (Chen et al., 2015). In K^+ deficiency, OsHAK5 appears to be the most important transporter for xylem loading, whereas OsHAK21 was proposed to be a major contributor under saline conditions (Shen et al., 2015).

Mutation of nitrate transporter *AtNRT1.5* surprisingly resulted in lowered content of both N and K^+ in shoot upon NO_3^- deficiency (Lin et al., 2008). Analysis of *atnrt1.5* and *atskor* lines showed that both transporters participate in K^+ xylem loading. AtNRT1.5 is dominant under low nitrate conditions, while AtSKOR is crucial in K^+ deficiency (Drechsler et al., 2015, Li et al., 2017a). In heterologous expression of *AtNRT1.5* it was also shown, that it is K^+ exporter and K^+/H^+ antiport was suggested (Li et al., 2017a). From these results it is clear that AtNRT1.5 is another contributor to K^+ xylem loading, which also plays a role in regulation of K^+/NO_3^- balance (Ragel et al., 2019).

2.3.2. Phloem transport

K^+ is also the major inorganic cation in phloem sap, where it acts as an osmotic agent, as a counter ion to NO_3^- and it is also vital for efficient loading of carbohydrates. In fact, the large amount of K^+ that is transported by the xylem to the shoot is subsequently recirculated through the phloem to the roots (Dieter Jeschke et al., 1985). The phenomenon of K^+ partitioning by the phloem and the phenomenon of diminished carbohydrate supply to roots under K^+ shortage is reviewed in (Šustr et al., 2019), and thoroughly discussed in 5.2., so this chapter is more focused on the molecular mechanisms of K^+ transport in the phloem.

So far, there is only one voltage-gated K^+ channel, AtAKT2 (*Arabidopsis* K^+ transport 2), which was shown to be important for K^+ transport in the phloem. In *A. thaliana*, *AtAKT2* is expressed in phloem tissue in both shoot and roots and the transporter probably facilitates K^+ loading as well as the energization of carbohydrate loading in the shoot, while it also participates in K^+ unloading in the roots (Deeken et al., 2002). AtAKT2 possess very interesting transport capabilities that are characterized by two gating modes, between which it switches by phosphorylation provided by cAMP-dependent protein kinase A (Michard et al., 2005a, Michard et al., 2005b). Mode 1 is characterized by inward-rectifying currents through the dephosphorylated channel. In this mode, AtAKT2 enables K^+ uptake into the sieve/companion cell complex if the plasma membrane is sufficiently polarized. In mode 2, phosphorylated AtAKT2 is permanently open and allows K^+ to flow inward or outward (Michard et al., 2005a). Switch between these modes is regulated both by the voltage sensor and by phosphorylation of two serine residues S210 and S329 in the pore region (Michard et al., 2005a, Sandmann et al., 2011). Exact protein kinase targeting AtAKT2 remains to be discovered, while a functional interaction between AtAKT2 and protein phosphatase AtPP2CA (Protein phosphatase 2CA) was proven (Chérel et al., 2002). A physical interaction between AtAKT2 and the AtCIPK6/CBL4 complex was also discovered, but no phosphorylation event was detected *in vitro*. Instead, it was proposed that the AtCIPK/CBL complex facilitates AtAKT2 recruitment to the plasma membrane in a Ca^{2+}-dependent manner (Held et al., 2011). Moreover, AtAKT2 interacts with AtMRH1 (Morphogenesis of root hair 1) receptor-like pseudokinase. Although AtMRH1 is crucial for AtAKT2 function, as shown by *atmrh1* characterization, it lacks functional kinase domains and its function probably depends on the recruitment of other signalling proteins (Sklodowski et al., 2017).

Apart from to the apparent switch between K^+ loading in the shoot and its unloading in the roots, this modulation of transport properties also serves a role in carbohydrate loading. Under conditions of ATP shortage to power H^+ ATPases, which is not rare in the sieve/companion cell complex, AtAKT2 switches to mode 2 and releases K^+ into the apoplast. Efflux of positively charged K^+ ions maintain membrane potential low enough and serve as an additional energy source for transport processes enabling a steady rate of sucrose loading (Gajdanowicz et al., 2011, Dreyer et al., 2017).

2.4. Functional characterization of KT/HAK/KUP transporters

In the previous chapters, K^+ uptake and long-distance transport was reviewed, focusing on involved transporters, channels and signalling elements. K^+ transporters and channels also play roles in cellular K^+ homeostasis (Osakabe et al., 2013), in plant development (Rigas et al., 2001) and may also participate in processes unrelated to K^+ transport such as production of cAMP and adaptation to heavy metals in environment (Al-Younis et al., 2015, Sanz-Fernández et al., 2021). In the following chapter, the diverse functions of KT/HAK/KUP transporters will be presented with the exception of K^+ uptake and long-distance transport, which were meticulously summarised in previous chapters.

2.4.1. Role in cell expansion and stomata movements

AtKUP6 gene expression is enhanced under water stress (Maruyama et al., 2009) and in response to ABA treatment in *A. thaliana in vitro* suspension cells (Böhmer and Schroeder, 2011). The single *atkup6* knock-out mutant plants did not show any phenotype, but multiple mutants with related KT/HAK/KUP transporters (*kup6xkup8*, *kup2xkup6xkup8*) or outward-rectifying channel GORK (Guard cell outward rectifying) (*kup6xkup8xgork*) showed higher fresh weights and enhanced cell expansion compered to wild type. These findings suggest that these transporters are involved in K^+ efflux from cells, thereby regulating cell turgor and volume growth. In addition, *AtKUP6* expression was localized, besides other tissues, in the guard cells of stomata. When the multiple mutants (*kup6xkup8*, *kup2xkup6xkup8*) were tested, they showed impaired ABA-mediated stomata closure and decreased tolerance to drought stress (Osakabe et al., 2013). These discoveries further confirmed the role of AtKUP2, 6 and 8 in the regulation of cell turgor and their ability to export K^+ from cells.

In other species, enhanced expression of genes encoding KT/HAK/KUP transporters was linked to rapid growth of plant organs or cells. Elongation of cotton fibres in *Gossypium hirsutum* is coordinated beside other factors by expression of *GhKT1* (Ruan et al., 2001). During nodule development in *Lotus japonicus*, the LjKUP gene is expressed at the late stages characterized by rapid growth, suggesting its role in volume growth (Desbrosses et al., 2004). In peach (*Prunus persica*) and grape wine (*Vitis vinifera*), the expression of *PpeKUP1*, *VvKUP1* and *VvKUP2* was correlated with fruit growth and volume growth of fruit cells (Davies et al., 2006, Song et al., 2015b). Further characterization of PpeKUP1 showed that it affected cell growth by K^+ intake (Song et al., 2015b). Presented studies convincingly show in multiple model plants, that some KT/HAK/KUP transporters are involved in regulation of cell expansion.

2.4.2. Involvement in root hair development and root gravitropic growth

Transporter AtKUP4 was named AtTRH1 (Tiny root hair 1) according to the phenotype of *atkup4/trh1* mutant plants. In these plants, trichoblasts are normally developed, but root hairs form only small bulks on the trichoblasts and are not capable of tip growth under low-K conditions (Rigas et al., 2001). It was later proven that *AtKUP4/TRH1* is not allelic to any other known gene affecting root hair development, nor does it interact at the transcriptional level with any other gene which can cause root hair disruption (Daras et al., 2015).

Surprisingly, this phenotype was not reverted by K^+ surplus (Rigas et al., 2001, Desbrosses et al., 2003). Moreover, roots of *atkup4/trh1* plants display an agravitropic growth. These findings imply a disruption in signalling, and both *atkup4*/*trh1* phenotypes were indeed reverted by exogenous auxin (Vicente-Agullo et al., 2004). In experiments with yeast expressing *AtKUP4/TRH1*, auxin efflux through this transporter was proven (Vicente-Agullo et al., 2004). *Atkup4/trh1* plants were found to accumulate more auxin in the root stele than wild type when exogenous auxin was applied. AtKUP4/TRH1 localizes to the basal part of the plasma membrane in the cells of stele in the root differentiation zone. Here it colocalizes with the AtPIN1 transporter. When *AtKUP4/TRH1* expression is disrupted, AtPIN1 localization is also disturbed (Rigas et al., 2013), which corresponds well with its putative role in auxin transport. AtKUP4/TRH1 is also present in protoderm and cortex of meristematic zone where it localizes to endomembrane structures (Rigas et al., 2013). According to its localization to basal membrane and its involvement in auxin efflux, AtKUP4/TRH1 participates in acropetal auxin transport. Moreover, due to its localization in endomembranes of lateral root cap and protoderm it is believed to be involved in basipetal auxin transport as well. Through its

involvement in acropetal and basipetal auxin transport AtKUP4/TRH1 may cause auxin deficiency in the rhizodermis, where root hairs ontogeny is disrupted.

The predicted structure for auxin transport is a pore formed by dimerization of the AtKUP4/TRH1 transporter. Dimerization *in planta* was demonstrated by bi-molecular fluorescence complementation with involvement of C-term domains, which was shown in yeast two-hybrid system (Daras et al., 2015).

2.4.3. Functional characterization of AtKUP9

The AtKUP9 transporter has recently received attention in several publications. Since the characterization of AtKUP9 is one of the main objectives of this dissertation thesis, the accumulated knowledge will be reviewed in detail.

In the earliest report, *AtKUP9* was identified as one of the genes positively affecting seed size. Its possible role in the delivery of K^+ and/or carbohydrates to seeds was discussed, although no further characterization was carried out (Tenorio-Berrio et al., 2018).

In a later report, the role of AtKUP9 in the root apical meristem (RAM) was described and analysed in detail by Zhang et al. (2020). When *atkup9* seedlings were grown on K^+-depleted medium, their primary root grew significantly slower compared to the wild type. It was revealed that this decline of growth was caused by a decrease in the number of cell divisions in RAM. The meristematic zone of *atkup9* plants was smaller compared with the wild type under low-K conditions, and the quiescent centre (QC) cells, essential for RAM organization, began to lose their cellular identity, as shown by the expression of key genes and the increased number of cell divisions in QC cells. This phenotype was reversed when bacterial amidase expressed in the QC supplied additional IAA (indole-3-acetic acid) by converting exogenous IAM (indole-3-acetamide) and partially also by exogenous auxin application. These findings, combined with *AtKUP9* expression in the QC, led researchers to test the possibility of IAA transport by the AtKUP9 transporter.

In subsequent experiments, *AtKUP9* was expressed in *Xenopus* oocytes, where this transporter mediated export of IAA and K^+. Based on these data and the localization of AtKUP9 to the endoplasmic reticulum (ER), the hypothesis was formed that AtKUP9 transports IAA from its storage in the ER to maintain auxin signalling in the quiescent centre during K^+ deficiency (Zhang et al., 2020).

In very recent studies, expression of *AtKUP9* throughout the plant body has been documented. *AtKUP9* is expressed in shoot in leaf blades as well as in vascular tissues. In the roots, it is expressed predominantly in vascular tissues under K^+ sufficient conditions. In K^+ deficiency, the localization of expression is shifted toward the root tips, this is accompanied by the stimulation of *AtKUP9* expression (Genies et al., 2021, Yamanashi et al., 2022).

Possible involvement of AtKUP9 in ion transport and homeostasis has been studied intensively, but results are inconsistent. Firstly, AtKUP9 was characterized in heterologous expression in *Escherichia coli* as a Cs^+ importer. This heterologous expression also rescued growth of *E. coli* strain defective in K^+ uptake on low-K medium. These findings supported the hypothesis that AtKUP9 is a K^+ importer *in vivo* and it is not selective for K^+ over other alkali metal ions (Kobayashi et al., 2010). These findings were challenged by experiments in *Xenopus* oocytes were outward currents of K^+ were documented (Zhang et al., 2020). While heterologous experiments remain contradictory, the ability of *atkup9* plants to take up alkali ions from the medium was tested. During incubations lasting 7 to 20 h, no differences in uptake of K^+ (Zhang et al., 2020) or Rb^+ (Genies et al., 2021) were detected between *atkup9* and wild type. On the other hand, uptake of Cs^+ was clearly enhanced in *atkup9* (Genies et al., 2021). These results question the involvement of AtKUP9 in K^+ uptake, but on the other hand, different uptake system with affinity to Cs^+ might be upregulated in *atkup9*. This hypothesis was further

supported by the enhanced accumulation of Cs^+ in *atkup9* in 7-day experiments (Adams et al., 2019). So far, it was reported that the transcription of genes encoding the major K^+ uptake facilitators, *AtAKT1* and *AtHAK5*, is not affected by *AtKUP9* knock-out (Zhang et al., 2020).

On top of this, long-term accumulation and partitioning of K^+ was studied in *atkup9*. First studies agreed that *atkup9* accumulate similar amounts of K^+ in roots and shoot as the wild-type irrespective of K^+ supply (Zhang et al., 2020, Genies et al., 2021). Recently, elevated levels of K^+ in roots of *atkup9* in K^+ scarcity were reported (Yamanashi et al., 2022). All studies were conducted under slightly different conditions and most importantly in various phases of plant ontogeny. This may explain the contradictory results of some studies, and it is possible that AtKUP9 is involved in K^+ partitioning under certain conditions or at certain developmental stages.

This summary clearly shows that characterization of AtKUP9 is still incomplete and in some cases, studies are contradictory. During my experiments, I addressed possible roles of AtKUP9 in plant response to K^+ shortage. My results support the involvement of AtKUP9 in physiological processes outside of RAM (Šustr et al., 2023) I showed that cessation of growth is more prominent in lateral roots compared to primary root in *atkup9*. Moreover, I observed an altered carbohydrate allocation in *atkup9* plants cultivated *ex vitro* in a hydroponic culture.

2.4.4. Transport of Cs^+ as chemical analogue of K^+ via KT/HAK/KUP transporters

The possibility that other monovalent cations are transported through the high-affinity K^+ uptake system has been known since pioneering studies in mineral nutrition. Epstein and Hagen (1952) found that transport of both Rb^+ and K^+ at low concentrations is mediated by transporters. Later, Rb^+ was used as K^+ analogue to analyse K^+ uptake kinetics (Epstein et al., 1963). Another perspective has been applied when uptake kinetics of Cs^+, as an environmental pollutant, has been measured. It came out that Cs^+ uptake declines 30-fold between 50 and 250 μM of external K^+ in wheat plants (Smolders et al., 1996). Since then, it has been generally accepted that high-affinity K^+ uptake system is much less selective for K^+ than low-affinity system. Indeed, when *AtHAK5* was cloned, the high-affinity transport of Cs^+ was confirmed in yeast (Rubio et al., 2000). This hypothesis was later evaluated in *A. thaliana* knock-out mutants. *Athak5* mutants took up less Cs^+ than wild type (Qi et al., 2008), while Cs^+ uptake into *atakt1* mutants was even higher than in wild type (Broadley et al., 2001), probably because *atakt1* mutants had enhanced expression of *AtHAK5* (Rubio et al., 2008). Similar data were published for Rb^+. It was shown that AtHAK5 does not discriminate between K^+ and Rb^+ when *athak5*, *atakt1* and respective double mutants were examined (Rubio et al., 2008).

In the work of Adams et al. (2019), knock-out mutants of 10 KT/HAK/KUP transporters were compared in their ability to take up Cs^+. Most of the mutants took up Cs^+ in the same manner as wild type, while *atkup9* accumulated larger amounts. Details reviewing putative involvement of AtKUP9 in Cs^+ and K^+ uptake and distribution can be found in previous chapter 2.4.3. Also, I tested possible involvement of AtKUP7 in Cs^+ uptake and allocation and I found that *atkup7* took up less Cs^+ into both root and shoot in 24 h exposure (Šustr et al., 2020). For these Cs^+ uptake measurements, I established a cooperation with National Radiation Protection Institute (SÚRO v.v.i.), with Ing. Tereza Ježková. Obtained results were in agreement with the role of AtKUP7 in K^+ uptake and xylem loading (Han et al., 2016), however observed transport phenotype did not translate into improved Cs^+ tolerance in long-term experiments (Šustr et al., 2020).

2.5. Adaptation to serpentine soils and the possible role of KUP9

During my studies, I established a collaboration with laboratory of RNDr. Filip Kolář Ph.D. (Department of Botany, Faculty of Science, Charles University), based on their discovery of AaKUP9 transporter as a potential major contributor to adaptation of *Arabidopsis arenosa* to serpentine soils (Konečná et al., 2021). In this chapter, I briefly explain what are the specifics of serpentine soils and how AaKUP9 was discovered.

Serpentine soils are ultrabasic naturally toxic soils, which occur as islands in the landscape and provide no intermediate habitats between them and surrounding soils that support plant growth much better than serpentines. This makes serpentine habitats very good models for studying the colonization of toxic substrates and plant adaptations to them.

Serpentine soils are characterized by very low Ca/Mg ratio and high concentration of toxic metals such as Co, Cr, Ni and Zn. These hostile characteristics are more or less often accompanied by relative scarcity of macronutrients such as N, P and K and propensity to drought (Konečná et al., 2020).

Plants colonizing this adverse environment developed a number of adaptations called serpentinomorphoses and these unique plants were studied from the first half of 20th century (reviewed in Whittaker, 1954). Only recently, two studies have dealt with the genetic background of serpentine adaptations. Several serpentine and non-serpentine populations of *Arabidopsis arenosa* and *Arabidopsis lyrata* were sequenced to uncover possible shared alleles that are recruited during serpentine adaptation. Both studies overlapped in two candidate genes, one of which was K^+ transporter KUP9 and the other was Ca^{2+} channel TPC1 (Two-pore channel 1) (Turner et al., 2010, Arnold et al., 2016). Recently, Konečná et al. (2021) studied three independently adapted serpentine populations of *A. arenosa* and their closest non-serpentine counterparts and found that all of them recruited *AaKUP9* alleles to some extent.

In our recent publication, we developed a medium which allowed us to examine growth traits of serpentine and non-serpentine populations exposed solely to low Ca/Mg ratio *in vitro*. These cultivations showed that serpentine populations are adapted to low Ca/Mg availability even when this factor is singled out under artificial conditions. Also, serpentine populations exhibited various degree of adaptation further confirming parallel evolution of these adaptations (Konečná et al., 2022).

Currently, our collaboration continues in the effort to characterize specific serpentine alleles of *AaKUP9* by heterologous expression in *kup9* mutants of *A. thaliana*.

3. Aims

My doctoral project followed three interconnected objectives focused on KT/HAK/KUP transporters and plant potassium management.

The main objective of my research was to characterize selected potassium transporters from KT/HAK/KUP family by using T-DNA insertion mutants of *Arabidopsis thaliana* and other experimental approaches. I wanted to establish novel functions for less studied KT/HAK/KUP transporters AtKUP9 and AtKUP7 with focus on root system growth.

The second objective was more theoretical. I summarized how root growth and development is affected by K^+ scarcity in various plant species, in a review paper. Deep understanding of this topic helped me to interpret data gained in my research.

Third objective was based on the collaboration with the team of RNDr. Filip Kolář Ph.D. (Department of Botany, Faculty of Science, Charles University) and focused root growth of serpentine and non-serpentine populations of *Arabidopsis arenosa* cultivated *in vitro.* This objective was based on the previous identification of *AaKUP9* gene as a candidate involved in the serpentine tolerance.

Detailed aims were as follows:

1. To analyse growth characteristics of *atkup9* and *atkup7* under various nutritional stress conditions with focus on K^+ deficiency and Cs^+ toxicity.

2. To evaluate how *AtKUP9* and *AtKUP7* mutants accumulate alkali ions with focus on K^+ and $^{134}Cs^+$ and to further analyse allocation of these ions within a plant under low-K conditions.

3. To investigate possible functions of AtKUP9 outside of root apical meristem.

4. To summarize effects of K^+ starvation on various aspects of root growth and development in a review paper.

5. To test how low Ca/Mg ratio typical for serpentine soils affects serpentine and non-serpentine populations of *Arabidopsis arenosa in vitro*.

4. Summary of publications

Potassium transporter KUP9 regulates plant response to K^+ deficiency and affects carbohydrate allocation in *A. thaliana*

Šustr, M., Konrádová, H., Martinčová, M., Soukup, A., Tylová, E.

Journal of Plant Physiology (2024) 292 IF_{2024}: 4,3

Cited by: N/A (Web of science) 1 (Google scholar)

In this publication, we focused on the role of KT/HAK/KUP transporter in plant response to K^+ starvation and root system growth. We broaden the knowledge regarding functions of AtKUP9 transporter in *A. thaliana*. By characterization of *atkup9* knock-out mutants cultivated *in vitro* and under hydroponic conditions, and by localization of *AtKUP9* expression we clearly showed, that AtKUP9 participated in number of responses to low-K environment and its function exceeded the processes in RAM maintenance and ion homeostasis described so far (Zhang et al., 2020).

We focused on root system architecture of 10-day-old plants cultivated *in vitro* and we showed that root system of *atkup9* in K^+ shortage distributed unevenly its growth. Growth of lateral roots was impeded in comparison to wild type, while primary root was not significantly affected. Parameters of RAM proliferation were analysed by multiple approaches in primary and lateral roots and it further confirmed that lateral roots of *atkup9* were affected by K^+ scarcity to a greater extent than primary root.

In subsequent experiments, we localized *AtKUP9* expression by engineering plants carrying *pKUP9:GUS* reporter constructs. This approach demonstrated *AtKUP9* expression along vascular tissues in shoot and in various root tissues including rhizodermis and stele. We also showed that expression pattern in roots shifted significantly in response to K^+ starvation, and we confirmed by qRT-PCR that *AtKUP9* expression was enhanced in root and shoot in K^+ scarcity.

Next, we studied whether AtKUP9 function in RAM was responsible for observed phenotype. While auxin maxima in *atkup9* lateral roots were disturbed in low-K, exogenous auxin did not reverse the phenotype and we concluded that other factors had to contribute to the short lateral root phenotype on the whole-plant level. We therefore focused on long-distance transport in plants and studied the possibility that AtKUP9 affects phloem transport towards the roots. We found that *atkup9* transported increased amounts of K^+ and non-structural carbohydrates under control conditions. These data drew our attention to carbohydrates and their allocation. We found that *atkup9* plants accumulated significantly more soluble carbohydrates in shoots under low-K conditions. On the other hand, K^+ contents in tissues was only slightly altered in *atkup9*. Based on these results we concluded that *AtKUP9* mutation affected carbohydrate allocation, although its exact role in the response to low-K is still not fully clear.

Contribution to presented manuscript: I contributed to the conceptualization of the research topic and experimental design. I also conducted all cultivations and most of the other experimental procedures with the exception of qRT-PCR (Quantitative real-time polymerase chain reaction) and the measurements of carbohydrate content. Moreover, I analysed the data and I created all the figures presented in the manuscript. I wrote draft of the manuscript and finalized it for submission.

^{134}Cs Uptake and Growth at Various Cs^+ and K^+ Levels in *Arabidopsis* AtKUP7 Mutants

Šustr, M., Doksanská, T., Doležalová, B., Soukup, A., Tylová, E.

Plants (2020), 9, 11 IF_{2020}: 3,9

Cited by: 1 (Web of science) 4 (Google scholar)

In this publication, we focused on the role of KT/HAK/KUP transporters in radiocesium translocation in plants, which is the question of high ecological importance regarding the spread of radionuclides in the environment and their entrance into the food chain. The involvement of AtHAK5 in caesium uptake in roots has already been shown (Qi et al., 2008) but other KT/HAK/KUP transporters may translocate it into the shoot, which have not been analysed so far. AtKUP7, participating in K^+ uptake and loading into xylem (Han et al., 2016), was the obvious candidate. We focused on AtKUP7 and tested two *atkup7* mutant lines regarding their growth in K^+ deficiency and in toxic concentrations of Cs^+. In addition, their uptake and allocation of K^+ and $^{134}Cs^+$ was analysed in the cooperation with National Radiation Protection Institute v.v.i.

We found that the accumulation of toxic $^{134}Cs^+$ in *atkup7* roots and, to a greater extent, in shoots was reduced after a 24-hour incubation in a solution containing $^{134}Cs^+$. We therefore hypothesized that *atkup7* plants might also be more resilient to toxic concentrations of Cs^+ and we cultivated these plants over a range of Cs^+ and K^+ concentrations *in vitro*. It turned out that *atkup7* is not more tolerant to Cs^+ than wild type and, contrary to published data (Han et al., 2016), it is not strongly susceptible to growth retardation in low K^+ availability.

In the subsequent experiments, we grew two mutant lines *atkup7-1* and *atkup7-2* on several types of media with both high-K and low-K treatments. We confirmed that neither of these mutants show strong growth retardation under low-K conditions in comparison to wild type under our cultivation conditions. Moreover, growth of these lines was slightly but significantly different from each other. To assess the differences between these mutant lines further, we analysed whether these two mutant lines differ in K^+ content. Surprisingly *atkup7-1*, the line with better growth, contained less K^+ than *atkup7-2* and wild type. We also tested if this phenotype of *atkup7-1* is manifested in hydroponics as well. In hydroponics K^+ content in *atkup7-1* did not differ significantly from wild type, which contradicts previous findings and suggest that effect of *AtKUP7* mutation on the tolerance to K^+ shortage is relatively weak.

Contribution to presented paper: I contributed to the conceptualization of the research topic and experimental design. I also conducted some of the cultivations and other experimental procedures. I cooperated with Ing. Tereza Doksanská from National Radiation Protection Institute v.v.i. on experiments regarding uptake and allocation of $^{134}Cs^+$. I coordinated master student Mgr. Barbora Doležalová, who conducted some of the cultivations and measurements of K^+ content. Moreover, I analysed the data and I created all the figures presented in the manuscript. I wrote a draft of the manuscript and finalized it for submission.

Potassium in Root Growth and Development

Šustr, M., Soukup, A., Tylová, E.

Plants (2019), 8, 10 IF_{2019}: 2,8

Cited by: 82 (Web of science) 142 (Google scholar)

In this review, we summarized how K^+ availability affects root system growth and development. As K^+ shortage can inhibit root cell volume growth, the role of K^+ transport proteins in this process was summarized. For the most part, this review focused on root growth and root system architecture, however nutrient allocation and stress tolerance in K^+ scarcity was also discussed.

In the first part, we summarized the molecular mechanisms that play a role in root cell volume growth. We speculated on the role of AtKUP2/6/8 transporters and AtKAT1/2 channels in complex with the SNARE (Synaptosomal-associated protein receptor) protein SYP121 in roots. The role of AtAKT1 and AtTRH1 in root hair development was also highlighted.

In the next part of the review, the responses of the root system architecture to K^+ deficiency were analysed. Under K^+ deficiency, the root-to-shoot ratio is generally lower than under K^+ sufficiency. This is in stark contrast to deficiencies of other macronutrients such as N and P, where plants enlarge their root system to enhance the foraging ability. The most commonly observed change in root system architecture under K^+ scarcity is the increased root branching into higher orders. Interestingly, different ecotypes of the key plant model species *Arabidopsis thaliana* have significantly different root system architecture under K^+ deficiency. Some ecotypes (including Columbia 0) strongly prefer the growth of the primary root, while others supress the growth of the primary root in favour of lateral roots. The signalling involved in the growth response to low-K is not fully understood. It is apparent that AtPIN1-mediated auxin transport is inhibited, which retards RAM activity and root growth. The role of ethylene and nitric oxide (NO) in this process was also documented, but the complete signalling pathways are not yet known.

We also evaluated the role of phloem transport in response to K^+ deficiency. K^+ transported via xylem is largely recirculated in the phloem and contributes to root growth under low-K conditions. More importantly, carbohydrate transport to roots is reduced under K^+ deficiency. Carbohydrate loading is partly facilitated by the K^+ gradient through the action of the AtAKT2 channel. It is not clear whether the reduced carbohydrate loading is due to K^+ deficiency in shoot or whether it is driven by reduced demand for carbohydrates in roots.

In the last section, we reviewed how the availability of K^+ can alleviate the toxicity of other monovalent ions such as Na^+, Cs^+ and NH_4^+. In addition, a number of documented effects of K^+ status on resistance to biotic stress were summarized.

Contribution to presented paper: I authored draft chapters reviewing root system architecture, phloem transport and stress resistance. I participated in proofreading and finalizing the manuscript for submission.

Genomic basis and phenotypic manifestation of (non-)parallel serpentine adaptation in *Arabidopsis arenosa*

Konečná, V., **Šustr, M.,** Požárová, D., Čertner, M., Krejčová, A., Tylová, E., Kolář, F.

Evolution (2022), 76, 10 IF_{2022}: 3,3

Cited by: 2 (Web of science) 4 (Google scholar)

In this work I collaborated with the team of RNDr. Filip Kolář Ph.D. (Department of Botany, Faculty of Science, Charles University). In this publication I tested the fitness and analysed root system growth of *A. arenosa* plants naturally inhabiting serpentine or non-serpentine habitats *in vitro* on medium that mimics low Ca/Mg ratio typical for serpentine soils. This experiment enabled comparison of the populations in their ability to respond to unfavourable ratio of bivalent cations. It also confirmed different levels of adaptation between the populations.

Throughout the study, three pairs of *A. arenosa* populations were compared for their ability to thrive on serpentine soils. Each pair consists of one population colonizing serpentine soil and one neighbouring population growing on common soil. Apparently, all serpentine populations had higher fitness in comparison to their non-serpentine counterparts when grown on serpentine soils. This adaptation manifested in different magnitudes among the population pairs. Moreover, these adaptations come with a trade-off of impeded growth on common soil only for S1 and S2 populations while S3 has superior fitness to its N3 counterpart on both soils. These results confirmed a hypothesis of parallel adaptation in these populations.

In the second part, the accumulation of heavy metals (Co, Cr, Ni), the Ca/Mg ratio in plant tissue and several fitness traits were evaluated to determine the parallelism among all population pairs. Most of the traits appeared to be parallel among populations with the somewhat surprising exception of the Ca/Mg ratio. Plants from the S1 population showed a lower Ca/Mg ratio in their tissue than N1 when grown on serpentine soil, whereas S2 and S3 plants are apparently able to ameliorate the unfavourable Ca/Mg ratio in the substrate by selective uptake of these cations.

We further studied this phenomenon by exposing the plants to a single stress factor, low Ca/Mg ratio, in cultivation on agar-solidified medium *in vitro*, which was the experimental part I was responsible for. Under these conditions, all serpentine populations grew better on the low Ca/Mg, suggesting that the S1 population might have partially different mechanisms to cope with unfavourable Ca/Mg ratio.

In the subsequent part, the putative parallelism of the three adaptations was studied using genomic tools. Significant parallelism was found for a relatively small number of genes. When gene function was considered, the level of significant parallelism increased. The most shared biological processes between the pairs were those specific to serpentine adaptation, such as: transport, protein localization, stress response and developmental processes. Finally, functional networks were constructed from all genes that are recruited in serpentine adaptation in any population pair. It was shown that in each population, different genes from the same pathway are often targeted for serpentine adaptation. Among these shared pathways, processes relevant to serpentine adaptation were found to include: iron homeostasis, inorganic ion transport including K^+ transport proteins, steroid hormone synthesis, and hormone signal transduction.

Contribution to presented paper: I took part in the planning of the *in vitro* cultivation design. Subsequently, I tested the designed media, performed the cultivations and measured the root traits. I wrote the methodology of this cultivation for the paper.

5. Discussion

5.1. Effect of KT/HAK/KUP transporters on root growth in K^+ deficiency

When analysing plants with modulated gene expression (knock-out, knock-down or overexpressing lines), identifying the growth phenotype is usually the initial step in the characterisation process. During my studies, I discovered K^+ specific root growth retardation in *atkup9* plants, which I subsequently studied in more detail. I also summarised the current knowledge on the relationship between root growth and K^+ supply in a review article. In this chapter, findings of these two papers are synthesised and further discussed.

As summarised in my review (Šustr et al., 2019), plants usually compromise root growth in K^+ deficiency, while deficiencies of other macronutrients trigger preferential allocation of biomass into roots (an increase of root/shoot ratio). For example, a moderate N deficiency induces lateral root elongation, leading to an enlarged root system, while only a very severe N deficiency causes a decline in root growth compared to control conditions (Gruber et al., 2013). Preferential biomass allocation to roots enable plants to forage for nutrients in the heterogeneous soil environment. The elongation of the primary root facilitates penetration into deeper soil layers, while the growth and branching of lateral roots enables radial exploration and efficient foraging in the topsoil (Giehl and von Wirén, 2014).

The apparent lack of preferential allocation of biomass to roots in K^+ deficiency is an interesting phenomenon from the evolutionary perspective. It is either not worth for plants to forage for K^+ horizontally and radially, or K^+ deficiency is such a burden that plants cannot maintain sufficient root growth. The reported variability of the available K^+ concentration within a habitat is usually in an order of magnitude (Jackson and Caldwell, 1993, Skálová et al., 2023), which arguably rules out the unworthiness of root foraging. Alternatively, inhibition of root growth might be a mean to retain sustainable K^+ concentration in the tissue. K^+ is a crucial ion maintaining homeostasis in plant cells. It is a key factor for enzyme activity (Walker et al., 1998) and for photosynthetic apparatus, where it is involved in osmoregulation and maintenance of membrane integrity in plastids (Kunz et al., 2014), so that K^+ homeostasis in photosynthetically active shoot is important for plant survival upon K^+ shortage. Based on these findings, we can assume that root foraging for K^+ is useful, but plants are only able to perform it to a limited extent, because internal reserves of K^+ most likely do not allow for significant growth without external K^+ supply. It is also worth mentioning that the ability to maintain root growth to the maximum extent under K^+ deficiency, as well as increased K^+ uptake, is an important trait of low-K tolerant cultivars, which has been described in tomato (Chen and Gabelman, 1995, Chen and Gabelman, 2000). In conclusion, root proliferation is beneficial in K^+ shortage, but only some plants can sustain it, probably due to sufficient K^+ uptake.

Root growth defects triggered by K^+ starvation were reported in a number of mutants in KT/HAK/KUP transporters as well as in other K^+ transporting proteins. AtHAK5 is the main contributor to high-affinity K^+ uptake, and knock-out mutants in this gene had growth phenotype only at very low K^+ concentrations (Qi et al., 2008, Pyo et al., 2010), while knock-outs in its rice orthologue *OsHAK5* had impeded growth in wider range of K^+ concentrations as OsHAK5 participates in K^+ uptake in these concentrations (Yang et al., 2014). Knock-out mutants in closely related *OsHAK1* showed more general phenotype, when root growth retardation among other phenotypes was prominent even under control conditions (Chen et al., 2018). A severe growth phenotype was reported for *atkup7* plants (Han et al., 2016), although AtKUP7 is redundant to previously described AtSKOR, AtHAK5 and AtAKT1, respectively, in its putative role in xylem loading and K^+ uptake. In my work, I cultivated *atkup7* plants but failed to reproduce the low-K root growth phenotype under various K^+ deficient experimental

conditions using two independent T-DNA insertion lines of *A. thaliana* (Šustr et al., 2020). I also discovered significant differences in the growth of these two *atkup7* mutant lines, which leads to caution for future experiments based on these mutants. In *atkup9* seedlings, elongation of primary root is hampered by unusual mechanism, where auxin crucial to RAM maintenance is not transported from storage in ER in sufficient manner (Zhang et al., 2020). Low-K triggered growth defect is known only in one channel knock-out *atakt1* (Hirsch et al., 1998, Dennison et al., 2001). This phenotype is somewhat expected as AtAKT1 channel procures large amounts of K^+ uptake across most conditions (Gierth and Mäser, 2007).

The signalling underlying the plant growth response has not been thoroughly studied so far, but the AtAKT1 channel was suggested as a sensor controlling the root growth response to low-K (Li et al., 2017). When *atakt1* seedlings were replanted from control conditions to low-K, or split root cultivation was used, it was shown that roots of these mutants grow in the same manner in low-K as under control conditions, while growth of wild type was diminished (Li et al., 2017). Although these results seemingly contradict the previously reported growth retardation in *atakt1* (Dennison et al., 2001), it was well defended including a documented pathway involving auxin signalling and degradation of AtPIN1 (Li et al., 2017). We can assume that in long term, the effect of disrupted K^+ uptake in *atakt1* causing inhibited growth prevails.

While a reduced allocation of biomass towards the roots was discussed previously, changes and trade-offs in root system architecture could perhaps play a role in response to K^+ shortage. Enhanced branching of lateral roots was reported in K^+ deficiency (Jia et al., 2008, Song et al., 2018). This growth pattern would enable plants to infiltrate K^+ rich topsoil more efficiently (Giehl and von Wirén, 2014), assuming that the elongation of newly emerged roots is restored when they are provided with the nutrient in question, as was demonstrated for P deficient roots (Sánchez-Calderón et al., 2005). Interestingly, selective growth of lateral roots in K^+ rich patches was not observed in classical studies in barley (Drew, 1975), however recently it was described in *Arabidopsis thaliana* (Li et al., 2017).

Further insight into changes in RSA (root system architecture) was brought by studying the diversity in *Arabidopsis thaliana* ecotypes. In this study, ecotypes were divided into 5 clades based on their RSA properties under low-K and control conditions. The most apparent difference among the ecotypes was the trade-off between elongation of primary root and lateral roots in K^+ scarcity (Kellermeier et al., 2013). This data suggest that various ecotypes of *A. thaliana* may have different strategies how to cope with K^+ deficiency based on the soil characteristics in their natural environment, however this hypothesis has not been tested yet. Interestingly, Columbia-0, genetic background to most of the T-DNA insertion lines used today, is strongly preferring elongation of primary root over lateral roots under K^+ shortage (Kellermeier et al., 2013). This phenotype is very similar to the phenotype of *atkup9* plants that I observed in my experiments (Šustr et al., 2023). It may suggest that AtKUP9 is somehow involved in root growth allocation between primary root and lateral roots in K^+ deficiency.

So far, AtKUP9 has been characterized in the quiescent centre, where its function was tied to auxin transport (Zhang et al., 2020), but there is growing evidence that AtKUP9 plays a role in processes outside the RAM, such as distribution of cations and carbohydrates or growth partitioning within the root system (Tenorio-Berrio et al., 2018, Genies et al., 2021, Yamanashi et al., 2022, Šustr et al., 2023). Also, AtKUP9 function might not be solely related to auxin homeostasis maintenance as auxin supplementation failed to fully rescue low-K phenotype of *atkup9* (Zhang et al., 2020, Šustr et al., 2023). Based on similarities between *atkup9* root growth phenotype and Col-0 response to K^+ limitation (Kellermeier et al., 2014, Šustr et al., 2023), my current hypothesis is that AtKUP9 is a negative regulator of root response to K^+ limitation and upon its mutation the low-K root growth phenotype is more severe. In this role, AtKUP9 may take part in various pathways influencing root growth apart from auxin as discussed further in this chapter.

The involvement of AtKUP9 in growth allocation between primary root and lateral roots and determination of relevant pathways can be investigated in the future experiments. It was shown in tobacco (Song et al., 2015a) and in *A. thaliana* (Šustr et al., 2023) that lateral roots with arrested growth in K^+ scarcity have impaired auxin maxima in comparison to primary root and control conditions. Disruption of auxin maxima might be caused by an altered AtPIN1 localization, which was described in primary roots of very young seedlings during K^+ starvation (Li et al., 2017). Analysis of AtPIN1 localization in the *atkup9* background may contribute significantly to the understanding of root growth allocation in K^+ scarcity, but there are also other elements possibly involved in the control of growth allocation. Gibberellin and NO signalling were given a role in growth response of lateral roots in K^+ scarcity (Song et al., 2018, Hetherington et al., 2021). As external application of gibberellic acid reversed the cessation of growth in lateral roots under K^+ limiting conditions (Hetherington et al., 2021) it might be worth trying to grow *atkup9* in low-K media enriched with gibberellic acid or NO precursors as well as with substances blocking these signalling pathways. These cultivations together with determination of expression and interaction partners of AtKUP9 may provide further insight into the role of AtKUP9 in growth allocation between primary root and lateral roots.

5.2. Carbohydrate allocation in K^+ deficiency and its regulation by KT/HAK/KUP transporters

As I discussed in my review article (Šustr et al., 2019), export of carbohydrates from photosynthetically active tissue is diminished under low K^+ availability, which was documented in a number of species including bean and maize (Cakmak et al., 1994, Amtmann et al., 2008, Martineau et al., 2017, Berg et al., 2018, Cui et al., 2020). In my experiments, I showed that hydroponically grown *Arabidopsis thaliana* followed this trend, moreover retention of carbohydrates in shoot was exacerbated in *atkup9* mutants (Šustr et al., 2023). Metabolic response to K^+ deficiency was analysed also in *A. thaliana* cultivated *in vitro* with carbohydrates provided in the medium (Armengaud et al., 2009), which might significantly skew the carbohydrate allocation compared to natural growth conditions. In spite of that an elevated concentration of soluble carbohydrates in the shoot was found.

Increased carbohydrate retention in *atkup9* is interesting phenomenon in relationship to its root growth phenotype. The causal relationship between K^+ deficiency and cessation of root growth is still not fully resolved as well as potential causes and effects of decreased carbohydrate transport in K^+ deficiency. The causes of decreased carbohydrate transport from leaves might be three-fold: 1) Lack of carbohydrate demand in K^+ starved roots, 2) Potassium shortage in phloem of leaves, which makes energisation of carbohydrate loading difficult (Dreyer et al., 2017) or 3) Cessation of root growth enables plants to maintain K^+ homeostasis. As outlined in the previous chapter, it is probable that K^+ starved roots are unable to grow under these conditions, therefore the decreased demand for carbohydrates probably contributes significantly to carbohydrate retention in shoot. However, causal relationship between K^+ scarcity and altered carbohydrate allocation has not been experimentally resolved yet.

The nature of the response to K^+ deficiency was studied by transcriptomic approach in various species. Comparison of gene expression changes in timepoints after the onset of K^+ deficiency may provide useful insight into the causal relationship between various responses. While the expression of various genes is altered in K^+ deficiency, genes coding carbohydrate transporters were not found in transcriptomic studies conducted on various plant species exposed to K^+ scarcity (Armengaud et al., 2004, Ma et al., 2012, Shankar et al., 2013, Zeng et al., 2015). In potato, two carbohydrate transporters StSUT1 and StSUT4 were upregulated after prolonged K^+ starvation (Koch et al., 2019). Besides that, I was not able to find any data suggesting transcriptional regulation of carbohydrate transporters in K^+ deficiency. These

transporters might be regulated post-translationally by a variety of kinases and phosphatases, which are responsive to K^+ deficiency according to the transcriptomic studies. So far, no specific candidates for post-translational regulation of carbohydrate transporters have been suggested. This is partly due to the lack of characterized genes in studied species and partly due to limited research interest in this topic in well studied model organisms such as *A. thaliana* or *O. sativa*.

While the mechanism behind decreased carbohydrate transport in response to K^+ deprivation remains elusive, there is a clear shift in the metabolism of carbohydrates, organic acids and nitrogen rich compounds documented by transcriptomic and metabolomic studies (Armengaud et al., 2004, Armengaud et al., 2009, Shankar et al., 2013, Zeng et al., 2015). Decreased synthesis of N rich metabolites from hydrocarbon precursors and decreased demand for carbon rich compounds in roots might sufficiently explain increased carbohydrate concentration in shoot.

A role for a KT/HAK/KUP transporter in carbohydrate allocation in K^+ deficiency was found in rice. OsHAK1 regulates source-sink relationship resulting in smaller yield of *oshak1* mutant plants as well as decreased carbohydrate allocation into roots. Roots of *oshak1* plants also have decreased expression of several carbohydrate transporters MST (Monosaccharide transporter) and impeded activity of sucrose synthase and two invertases (Chen et al., 2018). This work showed, that KT/HAK/KUP transporters can affect carbohydrate allocation, but the exact mechanism is unknown. To verify the possibility that AtKUP9 works in a similar way I plan to test the carbohydrate and transcriptomic profile of *atkup9* and wild type short after the onset of K^+ deficiency. Moreover, the study of Tenorio-Berrio et al. (2018) found decreased seed size in *atkup9*, which supports the notion that AtKUP9 is somehow involved in phloem transport and/or source-sink relationship.

K^+ participates in phloem loading via the K^+ channel AtAKT2. *In silico* modelling showed that K^+ released through AtAKT2 is crucial for maintaining a negative membrane potential in the sieve element/companion cell complex under the conditions triggering ATP shortage (Gajdanowicz et al., 2011). Insufficient supply of ATP to power H^+ ATPases is relatively common in phloem tissue. Among others, the role of the AtAKT2 in phloem loading was experimentally supported by the fact that *atakt2* plants grew worse than wild type under energy restricting conditions such as short day, low light intensity and low oxygen concentration (Gajdanowicz et al., 2011). I grew *atkup9* under energy limiting conditions such as short day (8/16 h day/night cycle) and *in vitro* without added saccharides. Neither of these conditions further diminished the growth of *atkup9* (Šustr et al., 2023), suggesting that AtKUP9 does not contribute to the phloem loading in a manner similar to AtAKT2.

Based on the current knowledge, AtKUP9 seems involved in the regulation of source-sink relationship in K^+ deficiency, which is manifested as reduced lateral root growth *in vitro* and by altered carbohydrate allocation *ex vitro*. The exact mechanism of this regulation is yet not known as well as the cause-effect relationship between diminished root growth, auxin homeostasis and saccharide transport.

5.3. Cellular localization of KT/HAK/KUP transporters

Cellular localisation is crucial for functioning of all transporters and recruitment of transporters through endomembrane system to the membrane of destination may serve as a regulatory pathway. Nowadays, the process of gene characterisation in *A. thaliana* usually comprise of analysis of knock-out mutant lines and cellular localisation of the protein using fluorescent markers.

Canonical functions of K^+ transport proteins in K^+ uptake, allocation or movement within a tissue assume localisation of the transporter to plasma membrane. Several KT/HAK/KUP

transporters from *Arabidopsis thaliana* and *Oryza sativa* suit this profile very well. AtHAK5 (Qi et al., 2008), OsHAK5 (Yang et al., 2014b), OsHAK1 (Chen et al., 2018) and OsHAK21 (Shen et al., 2015) are all members of clade I in KT/HAK/KUP family as described by Nieves-Cordones et al. (2016b) and Santa-María et al. (2018) and were localised to plasma membrane by fusion with florescent markers. So far, all characterised transporters belonging to Clade I mediate canonical functions and are localised to plasma membrane (Santa-María et al., 2018). Members of other clades such as ATKUP4/TRH1 (Rigas et al., 2013), AtKUP7 (Han et al., 2016) and AtKUP6 (Osakabe et al., 2013) may be targeted to plasma membrane as well.

While Clade I transporters are primarily active on plasma membrane, they might be recruited there only under specific conditions. For example, AtHAK5 is detectable only on endomembranes in sufficient K^+ (Qi et al., 2008), while it is recruited to plasma membrane by CIPK/CBL complex (Ragel et al., 2015) and AtILK1 (Brauer et al., 2016) under K^+ deficient conditions. These mechanisms are crucial in regulating high-affinity K^+ uptake in *A. thaliana* (Ragel et al., 2019) and it is possible that other KT/HAK/KUP transporters are regulated in a similar manner.

KT/HAK/KUP transporters may reside in other membrane compartments as well and several of them have been localized to various cell components. AtKUP12 was found in chloroplasts (Kleffmann et al., 2004), while OsHAK10 was localized to tonoplast by fusion with fluorescent protein (Bañuelos et al., 2002). By proteomic techniques AtKUP5, AtKUP7 and AtKUP12 were found on tonoplast as well (Jaquinod et al., 2007, Whiteman et al., 2008), although their main function might be in other compartments (Kleffmann et al., 2004, Han et al., 2016).

Two transporters from *Physcomitrella patens* PpHAK2 and PpHAK3 were functionally characterized on endoplasmic reticulum (Haro et al., 2013). Recently, AtKUP9 was localized by C-terminal GFP (green fluorescent protein) fusion to endoplasmic reticulum in primary root quiescent centre (Zhang et al., 2020). In other report, efforts to localize AtKUP9 by N-terminal GFP fusion resulted in expression of truncated protein and a lack of fluorescent signal (Genies et al., 2021). Interestingly, different splicing variants of AtKUP9 were used in mentioned studies, which might impact observed localisation in major ways as discussed further in this chapter.

As a part of my research project, I also tried to localize AtKUP9 protein into particular cell compartment by fusion with GFP. I used two genetic constructs in which expression of AtKUP9 coding sequence fused with GFP on C-terminus was driven by either native promoter (*pKUP9:KUP9::GFP*) in the genetic background of *atkup9* mutant or by strong 35S promoter from tobacco mosaic virus (p*35S:KUP9::GFP*) in the background of *rdr6* (RNA dependent RNA polymerase 6; this mutant has impaired RNA interference and therefore overexpression of transgenic constructs should not be disturbed) and in *Arabidopsis thaliana* ecotype Columbia-0. In the transgenic plants expressing *pKUP9:KUP9::GFP* I was not able to detect any fluorescent signal by confocal microscopy, moreover these plants retained the mutant phenotype under low-K conditions (unpublished data) suggesting a lack of the functional protein. The presence of the construct was repeatedly proved by PCR genotyping. Plants overexpressing *AtKUP9::GFP* under 35S promoter in *rdr6* background revealed a pattern typical for cytoplasmic localisation (Fig. 3). However, these plants showed diminished growth under K^+ limiting conditions (unpublished data), suggesting that overexpressed tagged protein hampered the function of the native *AtKUP9* gene.

Fluorescent marker proteins fused with cytoplasmic termini of transporters can impede their biological function. For example, transgenes of plant Pin-formed proteins remain active only when they are tagged with fluorescent protein at a specific position on the hydrophilic loop between trans-membrane domains (Mravec et al., 2009, Simon et al., 2016). On the other hand, functional capacity of AtKUP9 with C-terminal GFP was tested in earlier study by successful

mutant complementation (Zhang et al., 2020). From available data we can say that N-terminal fusion of AtKUP9 might cause problems as a production of incomplete protein was reported (Genies et al., 2021) while it is not clear which factor determines the success of C-terminal fusion.

It is also worth noting that AtKUP9 has four splicing variants according to the public database (TAIR, 2023). These splicing variants might pose different functions or localization. Differential presence of splicing variants in response to environmental and developmental cues have been shown for many plant genes (Tognacca et al., 2022). In recent studies, functional analyses of splicing variants under various stress conditions started to appear. Splicing variants of the ligase AtSRAS1 (Salt-responsive alternatively spliced 1) have antagonistic effects on salt stress response based on the presence or lack of ubiquitin ligase domain in *A. thaliana* (Zhou et al., 2021). In sugarcane, four splicing variants of the transcription factor SoMYBAS1 regulate drought tolerance differently based on the number of MYB (Myeloblastosis) domains (Fávero Peixoto-Junior et al., 2018). Interestingly, two published attempts to fuse AtKUP9 with fluorescent protein used different splicing variants. C-terminal GFP fusion with At4g19960.3 variant led to ER localised fluorescence (Zhang et al., 2020), while N-terminal fusion with variant at4g19960.2 produced only truncated transcript with no fluorescence signal (Genies et al., 2021). The splicing variant at4g19960.2 might lack a biological function, although there are no available data comparing the splicing variants of AtKUP9. In my genetic constructs full-length genomic DNA was used so these constructs should be processed into all available splicing variants.

As a part of my project to characterise AtKUP9, I put significant effort to localise AtKUP9 at cellular level and into a specific cell compartment. Impaired growth of resulting transgenic lines on low-K media cast a serious doubt on the reliability of observed localisation on ER in the plants overexpressing *AtKUP9::GFP* under 35S promoter. Due to this doubt the results were not published even if the localisation of AtKUP9 was not known by that time. In the meantime, AtKUP9 was localised to ER in quiescent centre of the root apical meristem (Zhang et al., 2020). However, there is growing evidence for AtKUP9 function outside of the

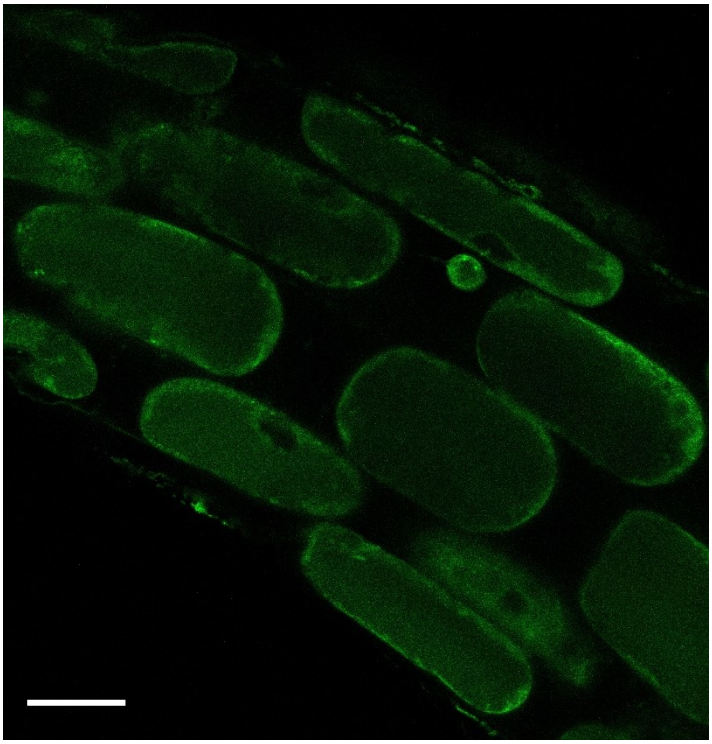

Fig. 3: Fluorescence in plasmolysed epidermal cells of *rdr6* plants transformed with *pKUP9:KUP9::GFP* captured by confocal microscopy. Scale bar = 25 µm. Unpublished results.

QC, as discussed later. Therefore, it is important to study its cellular localisation in other plant organs as well.

5.4. Involvement of KT/HAK/KUP transporters in K^+ accumulation

Accumulation of K^+ into root or shoot is controlled by K^+ uptake and its loading into xylem where key transporting proteins have been characterised. Significant amount of K^+ is also re-circulated into roots via phloem (Jeschke and Pate, 1991), but this process is significantly less understood and its role in K^+ shortage and involved transporters are not yet fully characterised.

In *Arabidopsis thaliana*, only small number of transport proteins were found by analysis of knock-out mutant lines to affect long-term K^+ accumulation. A mutation in *AtHAK5*, encoding the crucial transporter for high-affinity K^+ uptake, leads to lower K^+ contents in plants subjected to severe K^+ shortage (Gierth et al., 2005). Moreover, K^+ specific channel SKOR affect shoot K^+ concentration by procuring the bulk of xylem loading (Gaymard et al., 1998), and on top of that, loss of *AtKUP7* which is redundant to other mechanisms in both K^+ uptake and xylem loading leads to decreased K^+ content in shoot only under K^+ depleted conditions (Han et al., 2016).

In *Oryza sativa* several KT/HAK/KUP transporters affect K^+ accumulation. OsHAK5 is involved mainly in K^+ transport to shoot and its overexpression driven by 35S promoter lead to increased K^+ content in shoot at the expense of roots (Yang et al., 2014). OsHAK1 has major impact on K^+ content as both root and shoot suffer from decreased K^+ concentration upon its loss (Chen et al., 2018), while OsHAK21 is involved in response to saline conditions when it ensures sufficient K^+ supply to the whole plant (Feng et al., 2019).

While characterising KT/HAK/KUP transporters I studied K^+ accumulation in *atkup7* and *atkup9* mutant lines. For AtKUP7, the role in K^+ transport into shoot was already established, however in my *in vitro* experiments two mutant lines *atkup7-1* and *atkup7-2* differ in shoot K^+ content in K^+ deprivation, when only *atkup7-1* exhibited reduced K^+ content in comparison to wild type. I further tested *atkup7-1* in hydroponics where this line showed no phenotype regarding K^+ content. These results show that *in vitro* characterisation might not apply to plants grown in hydroponics or in soil and furthermore that future research in AtKUP7 by mutant analysis has to take in account different phenotypes of the mutant lines. There are striking differences in the topology of insertion in the mutant lines *atkup7-1* and *atkup7-2*. While in *atkup7-2* the T-DNA insertion is located in the first exon and mutant plants lack *AtKUP7* transcript (Han et al., 2016), in *atkup7-1* T-DNA insertion is located in the last exon and I was able to detect partial *AtKUP7* transcript in *atkup7-1* plants (Šustr et al., 2020). This transcript may encode partial protein with functional AMP cyclase domain in its N terminus (Al-Younis et al., 2015). However, it is not clear why *atkup7-1*, whose transcriptome is more reminiscent of the wild type, due to the partial *AtKUP7* transcript, is the line showing phenotype. It can be speculated that truncated protein in *atkup7-1* leads to a gain of function mutation, however this aspect has not been studied yet.

I also studied K^+ accumulation in *atkup9* knock-out plants. When plants were grown hydroponically under short-day conditions there was no difference in K^+ content between mutant and wild type when subjected to K^+ deficiency (Šustr et al., 2023). This is in agreement with most of the published data (Zhang et al., 2020, Genies et al., 2021) and as a whole these data suggest that AtKUP9 is not involved in K^+ uptake and/or its transport to the shoot. However, I registered diminished growth (lower biomass production) of *atkup9* in hydroponics which suggest that observed K^+ content ($mg.g^{-1}$ dry weight) is a result of a balance between production of biomass and K^+ uptake in *atkup9*. I also showed that growth cessation of *atkup9* is less pronounced in low illumination. These data and some of my unpublished results from

cultivations conducted under long-day conditions suggest that growth of *atkup9* plants is influenced by light duration and intensity in a different manner than in wild type. Under the conditions when growth of *atkup9* is equalised to wild type, such as altered illumination (as shown in Šustr et al. (2023)), K^+ content may differ as well. Detailed study of *atkup9* phenotype in various light regimes might contribute to the understanding of AtKUP9 function in an ongoing research.

5.5. Role of KT/HAK/KUP transporters in Cs^+ uptake and allocation

Caesium can be taken up into plant cells through a K^+ transport system due to its chemical similarity to K^+. While K^+ specific channels are very selective for K^+ among other alkali ions, KT/HAK/KUP transporters facilitate transport of K^+ chemical analogues to a larger extent (Rai et al., 2017, Ródenas et al., 2018). As capacity of different transport mechanisms to relocate Cs^+ is not precisely known even in *Arabidopsis thaliana*, Cs^+ is rarely used as a K^+ tracer in physiological experiments. However, Cs^+ transport and accumulation in plants is studied because its radionuclides are environmental pollutants with potential to accumulate in plant edible parts and enter the food chain (Wai et al., 2020).

I studied $^{134}Cs^+$ uptake and short-term allocation in several KT/HAK/KUP mutants in hydroponics conducted in National Radiation Protection Institute. In Šustr et al. (2020) I presented diminished uptake and shoot-ward transport of $^{134}Cs^+$ in *atkup7* plants. Shoot $^{134}Cs^+$ levels were reduced to a greater degree than root levels in *atkup7* compared to wild type. I also included *athak5* plants in my $^{134}Cs^+$ incubation experiments as a reference, since AtHAK5 was showed to mediate Cs^+ uptake (Qi et al., 2008). In the case of *athak5* plants, $^{134}Cs^+$ uptake was almost abolished, while shoot allocation was not further reduced. These data are well in agreement with the previously discovered AtKUP7 function in K^+ xylem loading with effect on K^+ uptake (Han et al., 2016). Under K^+ deficient conditions, which were used in our experiments, AtHAK5 is the major contributor to alkali ions uptake (Rubio et al., 2008) and it is a major contributor to Cs^+ uptake (Qi et al., 2008). This is also supported by my data (Šustr et al., 2020). Subsequently, *atkup7* resistance to toxic concentrations of Cs^+ was analysed, but none of the two *atkup7* mutant lines showed enhanced tolerance in comparison to wild type when subjected to Cs^+. Although a decreased uptake of Cs^+ contributed to plant Cs^+ resistance of *athak5* (Qi et al., 2008), this was not the case for *atkup7* (Šustr et al., 2020).

AtKUP9, another member of KT/HAK/KUP family, may also have a role in Cs^+ accumulation. Mutants in *AtKUP9* were shown to have increased long and short-term accumulation of Cs^+ (Adams et al., 2019, Genies et al., 2021). I tested *atkup9* ability to accumulate Cs^+ in 24- h experiments with similar results (Fig. 4, unpublished results). When the dynamics of Cs^+ uptake were studied in details, *atkup9* depleted Cs^+ from solution significantly faster than the wild type (Genies et al., 2021). On the other hand, Cs^+ release from roots into medium was hampered in *atkup9* (Genies et al., 2021). Role for AtKUP9 in Cs^+ release was suggested, although Cs^+ is not a biogenic element and there is no other evidence that AtKUP9 participates in the release of ions from the roots.

When multiple KT/HAK/KUP mutants were tested, no other transporter showed effect on Cs^+ accumulation (Adams et al., 2019). These studies are based on the notion that Cs^+ is taken up by the plants predominantly under K^+ scarce conditions (Hampton et al., 2005), where the KT/HAK/KUP transporters contribute essentially to K^+ uptake. Finding transporters that contribute significantly to Cs^+ uptake and its allocation into edible parts may enable the selection of crop varieties for cultivation on polluted soils in the future.

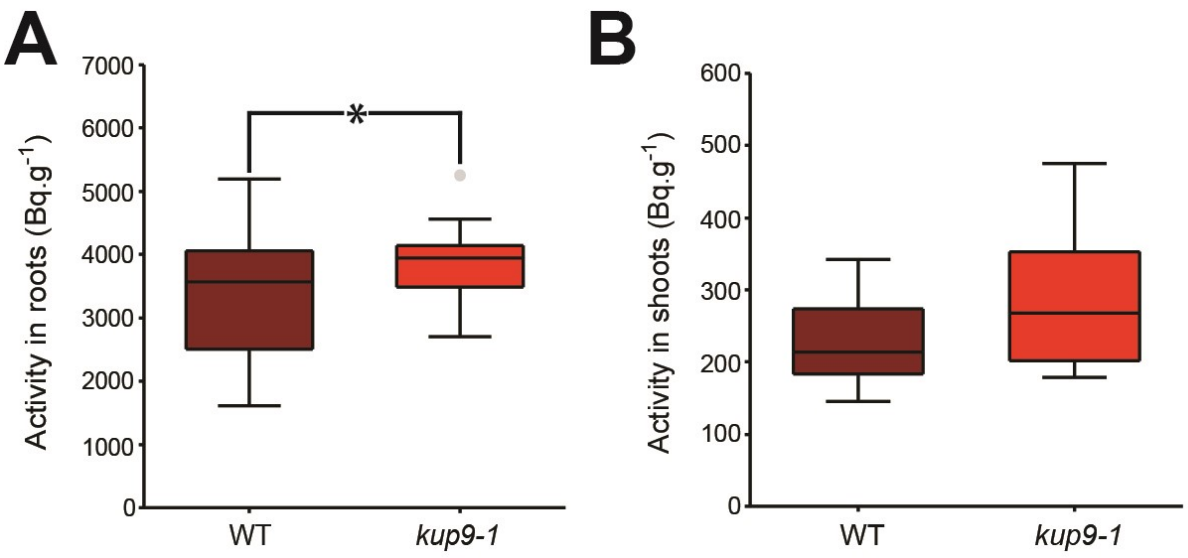

Fig. 4: Activity of $^{134}Cs^{+}$ in (A) roots and (B) shoots of wt and *kup9-1* plants exposed to K^{+} scarcity for one week and then incubated for 24 h in a medium containing $^{134}Cs^{+}$. * indicates significant difference between genotypes (Two sample T-test, $p \leq 0,05$, $n = 16$). Unpublished results.

5.6. KT/HAK/KUP transporters and plant adaptation to hostile environments

While sufficient K^{+} is crucial for plant response to stress in general (Wang et al., 2013), there is a growing notion that KT/HAK/KUP transporters might be directly involved in stress responses and adaptations, which do not directly involve K^{+} nutrition. Multiple studies found *KUP9* to be one of the most selected genes during adaptation to serpentine soils in *Arabidopsis arenosa* and *Arabidopsis lyrata* (Turner et al., 2010, Arnold et al., 2016, Konečná et al., 2021). Its function in serpentine-adapted plants is unknown. K^{+} does not seem to be a major limiting factor in serpentine soils, although these soils are generally considered infertile (Brady et al., 2005). Moreover, K^{+} accumulation was not altered between serpentine and non-serpentine populations of *A. arenosa* (Konečná et al., 2021). On the other hand, some serpentine relevant phenotypes were discovered in *Arabidopsis thaliana* mutants of KT/HAK/KUP genes. Old leaves of *atkup9* contained less Ca^{2+} and Mg^{2+} than wild type when subjected to low-K (Yamanashi et al., 2022), which might suggest a role in bivalent cation allocation for AtKUP9. Also, possibility that KT/HAK/KUP transporters may contribute to the resistance to heavy metals was brought to light by investigation of *atkup8.* While *atkup8* plants took up heavy metals in the same manner as wild type, they suffered less oxidative damage when exposed to heavy metals and their growth was more resistant to them (Sanz-Fernández et al., 2021).

I took part in the effort of RNDr. Filip Kolář Ph.D. and his team to characterise the factors contributing to adaptation of *Arabidopsis arenosa* to serpentine soils including involvement of AaKUP9. In the first project, which led to the publication included in this thesis (Konečná et al., 2022), I grew serpentine and non-serpentine *A. arenosa* plants *in vitro* on medium simulating serpentine soils. In the ongoing project we want to investigate properties of serpentine alleles from *Arabidopsis arenosa* by heterologous expression in *kup9* loos-of-function mutants of *Arabidopsis thaliana*. Comparison of plants expressing some of the serpentine alleles with *atkup9* and wild type in cultivations simulating serpentine conditions may help us understand the function of AaKUP9 as well as the function of KUP9 transporter in general.

5.6.1. Assessing the fitness of serpentine populations of *A. arenosa*

Serpentine soils are hostile environments and affect plant fitness in several ways. They occur in nature as relatively small islands divided by large areas of other soil types. Although there is a significant variation in soil properties among these islands, low ratio of available Ca^{2+} to Mg^{2+} and toxic concentrations of heavy metals Co, Cr and Ni are accepted as common characteristics of serpentine soils. On top of these, there are several properties common to serpentines that vary more between sites, such as low availability of macronutrients N, P and K, propensity to drought, high compactness, tendency to excessive warming and others (Brady et al., 2005, Rajakaruna, 2011, Konečná et al., 2020).

In the first part of presented paper transplantation pot experiment was conducted. Three pairs of neighbouring populations, which inhabit contrasting soils (serpentine (marked S)) or (non-serpentine (marked N)), were used. Plants of each population were grown on the soil of their origin and on the soil from neighbouring site. By this approach, the number of permutations between populations and soils remain manageable at the cost that universality of serpentine adaptation could not be fully addressed as serpentine populations were not grown on more than one serpentine substrate. The magnitude of adaptation to serpentine soils in several fitness metrics varied between the three serpentine populations suggesting parallel evolution of these adaptations. In addition, majority of analysed traits showed parallelism between soil types not between populations. Surprisingly, the Ca/Mg ratio in plant tissue was not one of them. In this case, serpentine S1 population had lower Ca/Mg ratio than its counterpart N1, while the entire opposite was true in the other population pairs. This phenomenon prompted us to study adaptation to low Ca/Mg ratio as a single factor *in vitro*.

Two media, based on 0,2 MS (Murashige-Skoog) medium, were designed. In one of them, Ca^{2+} and Mg^{2+} content was extrapolated from the element analysis of the serpentine soils used in the transplantation experiment in the previous study of Konečná et al. (2021). This medium contains more Ca^{2+} than usual 0,2 MS (2,15 $mmol.dm^{-3}$), while large quantities of Mg^{2+} is added into the media (6,97 $mmol.dm^{-3}$) resulting in Ca/Mg ratio of 0,27. The second medium was adapted from Bradshaw (2005). This medium contains 0,2 $mmol.dm^{-3}$ Ca^{2+}, which is lower than in 0,2 MS (0,59 $mmol.dm^{-3}$), and it is supplied with 4,5 $mmol.dm^{-3}$ Mg^{2+}, which largely exceeds common 0,3 $mmol.dm^{-3}$ in 0,2 MS. The resulting Ca/Mg ratio of 0,04 causes a severe stress to plants and differs significantly from the Ca/Mg ratio of 1,97 in 0,2 MS medium. High availability of other nutrients was a concern, however preliminary cultivations showed that both media have negative impact on non-serpentine *A. arenosa* populations.

Results of the cultivation clearly showed that low Ca/Mg ratio causes diminished growth of non-serpentine populations, while serpentine populations thrived on the medium with the Ca/Mg ratio of 0,04 (Konečná et al., 2022). S1 population even grow better on 0,04 than on control medium. The varying degree of tolerance to 0,04 Ca/Mg ratio among serpentine populations supported again the hypothesis of parallel adaptation. S1 population is very interesting when all the data are put together. It has very low Ca/Mg ratio in its tissue, yet it is thriving on 0,04 medium. It is probable that S1 population evolved powerful adaptations to suboptimal Ca/Mg tissue content while S2 and S3 populations are rather avoiding this scenario by optimising the uptake of Ca^{2+} and Mg^{2+}.

When these adaptations were investigated by genomic techniques it was apparent that only a relatively small fraction of adapted genes is shared among the populations including AaTPC1 and AaKUP9. Further analysis was able to assign some of the genes to shared pathways such as cation membrane transport or iron homeostasis. However, there is still plenty of room for unparallel adaptations, as observed for S1 in the case of Ca^{2+} and Mg^{2+} homeostasis.

In these *in vitro* cultivations, we completely omitted a possible effect of heavy metals present in the serpentine soils. The response to heavy metals requires different adaptations than nutritional stress. In the continuation of this collaboration, I co-supervised Bc. Adéla Brucknerová who investigated growth of serpentine and non-serpentine populations subjected to toxic concentrations of Ni^{2+} *in vitro*. In her results, 50 μmol.dm^{-3} Ni^{2+} reduced the growth of non-serpentine population N1 in roughly the same manner as the application of 0,04 Ca/Mg ratio. Combination of these two stress factors (low Ca/Mg ratio and Ni toxicity) further reduced the growth of N1 plants. S1 population was less susceptible to all applied stresses.

In preparation for a planned phenotypic analysis of *atkup9 A. thaliana* plants transformed with serpentine and non-serpentine alleles of *AaKUP9*, as mentioned earlier, I also grew *atkup9* and wild-type *A. thaliana* on low Ca/Mg media. To our surprise, *atkup9* seemed to be responding slightly differently to low Ca/Mg than the wild type. Currently, I co-supervise Bc. Lucie Škopová, who participates in the cloning of *AaKUP9* alleles and realises preliminary cultivations of *atkup9* on media with toxic concentrations of Ni^{2+}.

Conclusions

The main project of my doctoral studies contributed to the characterization of KT/HAK/KUP transporter AtKUP9. I discovered that root growth phenotype of *atkup9* is characterized by uneven distribution of growth in favour of primary root over lateral roots. I also showed that this phenotype cannot be reversed by auxin application. Outside of roots, *AtKUP9* mutation is manifested by altered carbohydrate allocation under low-K conditions.

My understanding of processes that might take place in *atkup9* in K^+ deficiency was significantly broaden by my study of literature for a review article. In this review, I summarised several phenomena that shape root system growth and development in K^+ scarcity, including carbohydrate allocation and signalling pathways.

In my other project I focused on the AtKUP7 transporter. I found that $^{134}Cs^+$ uptake and transport to shoot is negatively affected in *atkup7*, but this is not reflected in enhanced tolerance to toxic Cs concentrations in long-term cultivation. In subsequent experiments I discovered significant differences in biomass production and K^+ content between two mutant lines of *AtKUP7*. It is important to take these differences into account in future mutant analysis of *AtKUP7*.

In the last project I cooperated on characterization of multiple adaptations of *Arabidopsis arenosa* to serpentine soil. My cultivations helped to discern parallel and non-parallel mechanisms that drive this adaptation in multiple populations.

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