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Review article

KT-HAK-KUP transporters in major terrestrial photosynthetic organisms: A twenty years tale

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ABSTRACT

Since their discovery, twenty years ago, KT-HAK-KUP transporters have become a keystone to understand how alkali cation fluxes are controlled in major land-dwelling photosynthetic organisms. In this review we focus on their discovery, phylogeny, and functions, as well as the regulation of its canonical member, AtHAK5. We also address issues related to structure-function studies, and the technological possibilities opened up by recent findings. Available evidence suggests that this family of transporters underwent an early divergence into major groups following the conquest of land by embryophytes. KT-HAK-KUPs are necessary to accomplish several major developmental and growth processes, as well as to ensure plant responses to environmental injuries. Although the primary function of these transporters is to mediate potassium (K^+) fluxes, some of them can also mediate sodium (Na^+) and cesium (Cs^+) transport, and contribute to maintenance of K^+ (and Na^+) homeostasis in different plant tissues. In addition, there is evidence for a role of some members of this family in auxin movement and in adenylate cyclase activity. Recent research, focusing on the regulation of the canonical member of this family, AtHAK5, revealed the existence of a complex network that involves transcriptional and post-transcriptional phenomena which control the enhancement of AtHAK5-mediated K^+ uptake when Arabidopsis thaliana plants are faced with low K^+ supply. In spite of the formidable advances made since their discovery, important subjects remain to be elucidated to gain a more complete knowledge of the roles and regulation of KT-HAK-KUPs, as well as to improve their use for innovative procedures in crop breeding.

1. Introduction

Twenty years ago, four research teams discovered that plants possess a relatively large, and by that time, almost unknown family of transport proteins, named KT-HAK-KUP (Quintero and Blatt, 1997; Santa-María et al., 1997; Fu and Luan, 1998; Kim et al., 1998). Although the primary function of these proteins proved to be the transport of potassium (K^+), further research revealed that members of this family could play additional roles, some related to the transport of other alkali cations, others associated with plant development, and still others that are just starting to emerge. Following those early reports, a growing amount of work has revealed the pivotal role of these proteins in major physiological processes such as the uptake of K^+ by roots from dilute solutions, auxin movement and control of water status (Véry et al., 2014; Li et al., 2018). However, key aspects of the structure, cellular functions, regulation and roles of these transporters in major land-dwelling photosynthetic organisms (i.e. embryophytes), remain essentially under shadows, particularly when their diversity is taken into account. Nevertheless, considerable progress has been achieved. In this review, we attempt to provide an integrative view of these transporters. Particularly, we present a brief historical overview on the discovery of KT-HAK-KUPs in plants, consider new aspects of their phylogeny and roles, and discuss issues derived from recent structure-function studies, and from an increasing knowledge of the regulation of the canonical member of this family, AtHAK5. Finally, we consider some possibilities stemming from our current understanding of these transporters. Recently, Li et al. (2018) offered a review centered on the roles and regulation of KT-HAK-KUPs; the reader is referred to their work for a complementary point of view on these topics.

2. A brief historical overview of the discovery of KT-HAK-KUP transporters

Since the pioneering work of Emanuel Epstein and co-workers more than half a century ago (Epstein and Hagen, 1952; Epstein et al., 1963), it has been thought that the inward flux of K^+ into the roots of higher plants can be mediated by high- and low-affinity transport “mechanisms” located in the plasma membrane of outer root cell layers, and

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operating at low and high K^+ concentrations, respectively. For many years, the precise nature of the transporters involved in those "mechanisms" remained unknown, while the tools necessary to solve the issue waited to appear. A decisive step towards this goal was taken by Wolfgang Epstein and co-workers who, in the seventies, identified *Escherichia coli* mutants defective in K^+ transport. In a series of interesting works, they provided genetic evidence showing that several distinct systems, originally grouped under the general designation of TRK (for "Transport of K^+"), could be involved in K^+ transport in this Gram negative bacteria (Epstein and Kim, 1971; Dosch et al., 1991). Some years later, the use of molecular biology tools provided nucleotide and putative amino acidic sequences for each of those systems. One of them, TRKD, was later renamed KUP (for "K^+ Uptake Permease"), and its characterization indicated that it contributes to low-affinity K^+ transport in *E. coli* (Bossemeyer et al., 1989; Schleyer and Bakker, 1993). Meanwhile, other important advances were made with the unicellular eukaryotic organism *Saccharomyces cerevisiae* (baker's yeast) by Rodríguez-Navarro and collaborators (Ramos et al., 1985), which resulted in the identification of a yeast mutant unable to grow in a low-K^+ medium. Further studies led to the cloning of two fungal K^+-transport systems, TRK1 and TRK2 (Gaber et al., 1988; Ko and Gaber, 1991), which do not share homology with the KUP system of *E. coli*. The disruption of *TRK1*, or both *TRK1* and *TRK2,* led to a conditional (K^+-dependent) yeast phenotype which proved to be extremely important for the early cloning and characterization of plant K^+ transport systems through the use of complementation procedures (Anderson et al., 1992; Sentenac et al., 1992; Schachtman and Schroeder, 1994).

The possibility that the earliest identified plant K^+-transport proteins, which belong to the Shaker-channel (KAT1 and AKT1) and HKT-transporter (TaHKT2, formerly named as HKT1) families, contribute to the transport of K^+ into roots from very dilute solutions, was considered at that time to be unlikely. This doubt was based on apparent thermodynamic constraints imposed on K^+ channels (Maathuis and Sanders, 1994), and on analyses comparing the properties of high-affinity K^+ transport observed in roots (Epstein et al., 1963; Maathuis et al., 1996) with those determined for the TaHKT2 transporter (Rubio et al., 1995; Gassmann et al., 1996). The precise identity of the transporters involved in high-affinity K^+ transport by roots, while unsolved, led to an interesting debate (Walker et al., 1996). Among the systems previously identified in *E. coli*, potential plant homologues to the KUP transporter would not have been considered good candidates to account for the high-affinity transport of K^+ observed in roots because of the high K_M displayed by this bacterial transporter. However, in 1995 this view was modified when Bañuelos et al. found that expression of a KUP homologue from the soil yeast *Schwanniomyces occidentalis* into a *trk1 S. cerevisiae* mutant, conferred the capacity to mediate high-affinity K^+ transport. This fungal transport system was named HAK ("High Affinity K^+"; Bañuelos et al., 1995). HAK transport exhibited an enormous concentrative capacity as the intracellular concentration of K^+ displayed by yeast cells expressing this transporter was several orders of magnitude higher than the extremely low concentration in the external medium (Bañuelos et al., 1995). It was in this context that research teams, performing either "in silico" cloning (Quintero and Blatt, 1997; Kim et al., 1998), an RT-PCR based approach (Santa-María et al., 1997), or a direct complementation procedure (Fu and Luan, 1998), offered evidence indicating that higher plants also possess a large family of these proteins, and that some have the capacity to mediate high-affinity K^+ transport. Because the research teams used three different acronyms (KT, HAK and KUP) for this family, it is now generally referred to as KT-HAK-KUP or KUP-HAK-KT, or sometimes by just one of the three acronyms. Interestingly, soon after the identification of KT-HAK-KUPs in plants, it was found that the inward flux of K^+ into roots of *Arabidopsis thaliana* from dilute solutions ($> 10\,\mu M$) can be actually mediated by the Shaker-like channel, AKT1, due to the strong membrane hyperpolarization observed in this plant species upon K^+ starvation (Hirsch et al., 1998).

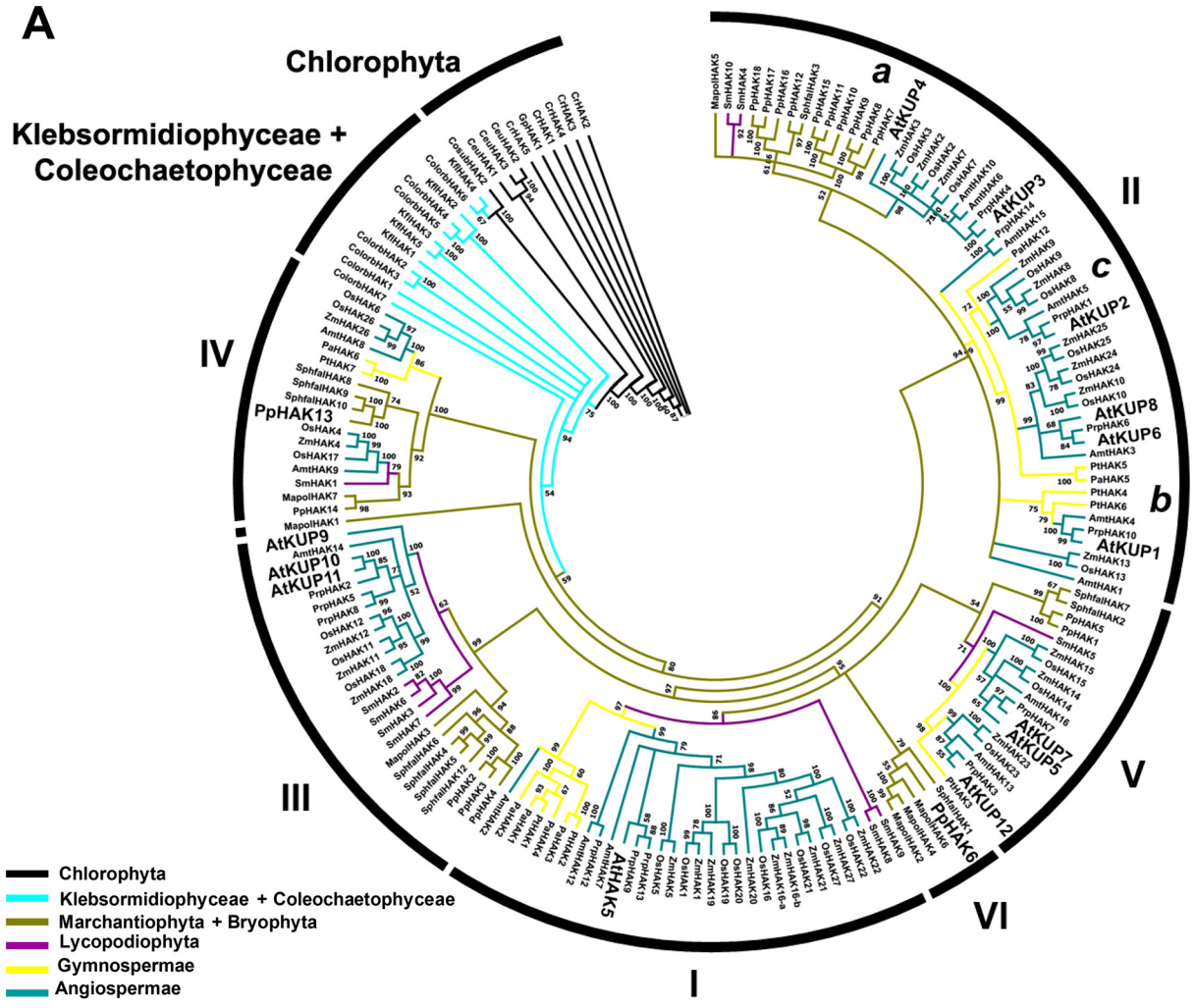
3. Diversity and phylogeny of KT-HAK-KUP proteins in photosynthetic organisms

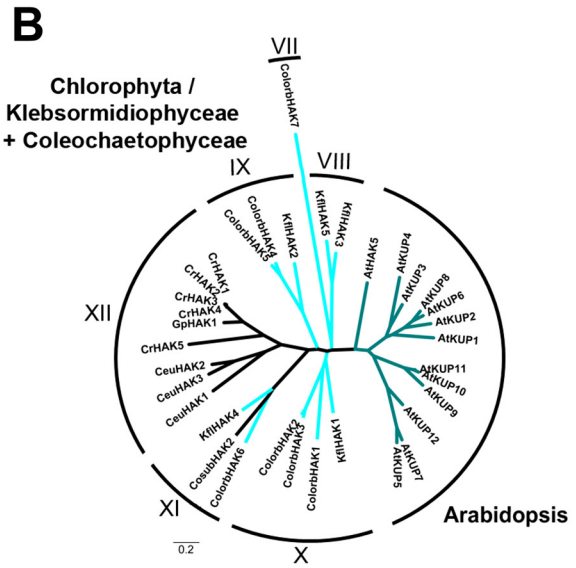
An emerging question following the discovery of plant KT-HAK-KUP transporters centered on their diversity and phylogeny. A first assessment of this issue was provided by Rubio et al. (2000), while later studies (Yang et al., 2009; Gomez-Porras et al., 2012; Nieves-Cordones et al., 2016) took advantage of the increasing amount of data coming from photosynthetic-organism genome projects, to perform a more comprehensive phylogenetic analysis. In addition, several works have provided assessments of KT-HAK-KUPs diversity at the species level, including rice (Bañuelos et al., 2002; Gupta et al., 2008; Yang et al., 2009), poplar (He et al., 2012), maize (Zhang et al., 2012), tomato (Hyun et al., 2014) and peach (Song et al., 2015b).

It should be mentioned that HAK-KUP transporters can be identified in Gram-negative and Gram-positive bacteria, some archaea, fungi, and other taxa such as amoebozoa. In these taxa, KUP-HAK transporters appear to be typically encoded by just one or two genes; however, there are exceptions to this, as exemplified in the Gram-negative bacterium *Legionella pneumophila*, which possess three *KUP* genes (Hori et al., 2013), and the fungus *Allomyces macrogynus*, which has four HAK genes (Benito et al., 2011). Equally important, members of some of those taxa do not contain KUP-HAK transporters, as was first found with *Saccharomyces* and later with many other fungi (Benito et al., 2011). This suggests that, in those species, other transporters could entirely replace their function, or alternatively that the function originally played by them is not crucial to complete the life cycle, and has been lost. In this regard, it is worth mentioning that the known functions of KUP-HAKs in these organisms have been mainly associated with high-affinity alkali cation transport, as in the fungi *S. occidentialis* and *Neurospora crassa* (Bañuelos et al., 1995; Haro et al., 1999), or with low-affinity K^+ transport in bacteria under hyperosmotic and moderately acidic media (Trchounian and Kobayashi, 1999).

KT-HAK-KUPs are also present in members of the red algae group and probably in at least one dinoflagellate. A search for the presence of KT-HAK-KUPs in green algae of Phytozome 12 performed in the present work, suggests that these transporters are absent in species like *Dunaliella salina, Micromonas pusilla or Ostreococcus lucimarinus*, but present in others like *Coccomyxa subellipsoidea* (Fig. 1, Supplementary Table 1) and *Chlamydomonas reinhardtii* (He et al., 2012, Fig. 1). On the other hand, they are present in all embryophytes so far studied (Véry et al., 2014; Nieves-Cordones et al., 2016). It is in these photosynthetic organisms where the diversity of KT-HAK-KUP transporters appears to reach its maximum, with the peak, as of now, being in the grass *Panicum virgatum*, for which more than 50 transporters are present (Nieves-Cordones et al., 2016). It remains unknown how many of the *KT-HAK-KUP* genes present in this and other polyploid species code for functional proteins, particularly when considering that most of the angiosperm genomes harbor around 14–20 KT-HAK-KUP transporters, as indicated in the detailed work of Nieves-Cordones et al. (2016).

KT-HAK-KUP transporters found in angiosperms have been recently re-grouped into five major clades, I to V, with clades I and II subdivided into two (a and b) and three (a, b and c) subclades, respectively (Nieves-Cordones et al., 2016). Whether the divergence of KT-HAK-KUPs into those groups took place before or after the conquest of land by green organisms has not been formerly assessed, due to the scarce information about this family in green algae. In Fig. 1, however, we provide such an assessment, on the basis of *Chlamydomonas reinhardtii* sequences, formerly available (He et al., 2012), and those obtained by us from databases (Supplementary Table 1), that correspond to the basal chlorophytes *Chlamydomonas eustigma, Gonium pectorale* and *Coccomyxa subellipsoidea*, the Klebsormidiophyceae member *Klebsormidium flaccidum*, and the Coleochaetophyceae member *Coleochaete orbicularis,* which is thought to be closely related to the lineage from which land plants evolved. In addition, data on basal land plants formerly available only for *Physcomitrella patients* (Gomez-Porras et al., 2012; Nieves-





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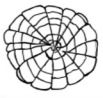
	Group	Organism	Number of KT-HAK-KUPs
	Chlorophyta	*Chlamydomonas reinhardtii*	5 (5)
		Chlamydomonas eustigma	3 (3)
		Gonium pectorale	2 (1)
		Coccomyxa subellipsoidea	5 (1)
	Klebsormidiophyceae + Coleochaetophyceae	*Klebsormidium flaccidum*	5 (5)
		Coleochaete orbicularis	7 (7)
	Basal Embryophyta	*Physcomitrella patients*	18 (18)
		Sphagnum fallax	13 (11)
		Marchantia polymorpha	8 (7)
	Higher Embryophyta	*Selaginella moellendorffii*	11 (10)
		Pinus taeda	7 (7)
		Picea abies	13 (7)
		Amborella trichopoda	16 (15)
		Arabidopsis thaliana	13 (13)
		Prunus persica	15 (13)
		Oryza sativa	27 (27)
		Zea mays	27 (26)

(caption on next page)

Cordones et al., 2016) can be accompanied with the information now available in databases for *Sphagnum fallax* and *Marchantia polymorpha* (Supplementary Table 1). This information, together with data available (Nieves-Cordones et al., 2016, Supplementary Table 1) for the lycopod *Selaginella moellendorffii*, two gymnosperms (*Pinus taeda* and *Picea abies*), the basal angiosperm *Amborella trichopoda*, two dicots

Fig. 1. A Phylogenetic tree of KT-HAK-KUPs transporters corresponding to green photosynthetic organisms based on 176 sequences from Chlorophyta, Charophyta (Klebsormidiophyceae + Coleochaetophyceae), basal land plants (Bryophyta + Marchantiophyta), Lycopodiophyta, Gimnospermae and Angiospermae. Sequences of the chlorophytes *Chlamydomonas reinhardtii* (He et al., 2012) and *Coccomyxa subellipsoidea*, denoted as "Cr" and "Cosub" respectively, were retrieved from Phytozome 12. It was also the source for sequences corresponding to the bryophyte *Sphagnum fallax* and to the marchantophyte *Marchantia polymorpha* ("Sphfal" and "Mapol", respectively). Sequences from the gymnosperm *Pinus taeda* "Pt" were retrieved from Congenie.org. Sequences from *Coleochaete orbicularis* "Colorb" and *Klebsormidium flaccidum* "Kfl", were retrieved by performing Blast on a transcriptome shotgun assembly database. In turn, sequences from *Chlamydomonas eustigma* "Ceu" and *Gonium pectorale* "Gp" were retrieved from NCBI. The remaining sequences corresponding to the moss *Physcomitrella patients* "Pp", the lycopodiophyte *Selaginella moellendorffii* "Sm", the gymnosperm *Picea abies* "Pa", the basal angiosperm *Amborella trichopoda* "Amt", the dicots *Arabidopsis thaliana* "At" and *Prunus persica* "Prp" as well as the monocots *Oryza sativa "Os"* and *Zea mays* "Zm" were obtained from Nieves-Cordones et al. (2016). Only full length sequences were used once confirmed that they contain a region with potential homology to the putative first transmembrane domain as well as the highly conserved GGT(A/L/I/P/S)F(L/A)A(S)L(V/I/M/A)YS(T/A) motif. Sequences were aligned using MAFFT (Katoh et al., 2017) and the alignment was curated through G-Blocks (Castresana, 2000). The sequences used to construct the tree are provided in Supplementary Table 6. The maximum likelihood tree was inferred through the use of the PhyML 3.0 program (Guindon and Gascuel, 2003). Only branches supported by more than 50% bootstrap values are shown (999 iterations): black: chlorophyte algae, cyan: charophyte algae, ocre: basal land plants, violet: lycopodiophytes; yellow: gimnosperms and blue: angiosperms. B. Radiation tree for green algae sequences and Arabidopsis showing the green algae clades. The tree was constructed through the procedure above described. C. Table showing the number of sequences corresponding to KT-HAK-KUP transporters in the organisms subjected to the above analysis. Between brackets the number of sequences that meet the criteria above outlined is also given.

(*Arabidopsis thaliana* and *Prunus persica*), and two monocots (*Oryza sativa* and *Zea mays*), were used to generate the phylogenetic tree in Fig. 1. This analysis shows that all KT-HAK-KUPs from chlorophyte and charophyte algae diverged from land plant clades. This suggests that the diversification of KT-HAK-KUPs into currently recognized clades occurred after land conquest. A next question refers to the subsequent evolutionary steps taken by KT-HAK-KUP transporters. Gomez-Porras et al. (2012), on the basis of a comparative study that included *Physcomitrella patients* as the unique basal green lineage, suggested the possibility that two additional clades of KT-HAK-KUPs may be present in basal land plants. However, according to our analysis, the group called "V" by Gomez-Porras et al. (2012) probably corresponds to an early branch of Clade III, while that called "VI" seems to be a subgroup of Clade II; this is in agreement with the analysis performed by Benito et al. (2012). Interestingly, clades II, III, and IV, and also clade V (with a weak bootstrap support of 54%), all contain sequences from basal land taxa. The presence of sequences of basal embryophytes in clade I was not supported in this analysis, however, suggesting the presence of a possible additional clade (VI) that only includes members of basal land plants. This clade is related to I and III. In turn, a single sequence from *Marchantia polymorpha*, MapolHAK1, diverges from all those clades. Whether the position of MapolHAK1 corresponds to the presence of an additional clade in basal land plants remains unknown. The presence of basal embryophyte sequences in clades II, III, IV and V suggest that the conquest of land by plants, and/or the early evolutionary steps following it, constituted an opportunity for the divergence of KT-HAK-KUPs into major groups probably due to the selection pressures acting in terrestrial ecosystems, including the division of work between aerial and underground plant parts and the appearance of entirely new tissues with specialized functions. Nevertheless, the extent to which members of different clades actually evolved to play alternative functions remains essentially unknown (see next section). A detailed survey of the subsequent, and highly dynamic, evolution within these clades in angiosperms has been recently provided (Nieves-Cordones et al., 2016).

3.1. Divergence among algal KT-HAK-KUPs

The analysis above also suggests the possibility that algal KT-HAK-KUPs diverged into several major groups. A radiation tree built from sequences from green algae and Arabidopsis indicates that green-algal KT-HAK-KUPs can be tentatively assembled into several clusters, all of them distinct from KT-HAK-KUPs of Arabidopsis (Fig. 1B). The alignment of the corresponding sequences (Supplementary Fig. 1) and further pair comparisons among representatives of all clusters (Supplementary Table 2, 3) indicate that algal clades share low similarity scores relative to those shared by land plant clades (the highest similarity score among algal representatives corresponded to the pair KflHAK3/KflHAK4, and was 48.6%, coincident with the lowest score among representatives of embryophyte clades, which corresponded to the pair PpHAK13/AtKUP4). The apparent distance among algal clusters, as well as the fact that some of them (IX, X, XI and XII, respectively) contain sequences from at least two distantly related organisms, seems to be consistent with an early divergence scenario. Among these groups, Cluster XI seems to display a unique position as it contains sequences from a chlorophyte (*Coccomyxa*) and the charophyte algae *Klebsormidium* and *Coleochaete*. On the other hand, Cluster XII contains, with the exception of CosubHAK2, all the sequences from chlorophyte, while clusters IX and X seem to be specific of charophyte algae. In turn, the positions of the two remaining clusters (VII and VIII) are more uncertain as they contain sequences from a single species, ColorbHAK7 being particularly interesting as it seems to be highly divergent from other KT-HAK-KUPs. Whether or not this pattern corresponds to a taxon-specific or to clade-divergent evolution is currently unknown. Clearly further studies, with a broader set of species, will be necessary to fully describe the phylogenetic relationships of KT-HAK-KUPs in green algae.

4. *In planta* functions of KT-HAK-KUP transporters

The putative function of the first identified *KT-HAK-KUP* genes was inferred from heterologous expression studies in yeast and bacterial systems. One of those studies indicated that *HvHAK1a*, from *Hordeum vulgare,* conferred to Saccharomyces *trk1trk2* cells the capacity for high-affinity transport of K^+ and of the K^+ analogue, Rubidium, Rb^+ (Santa-María et al., 1997). The low K_M estimated for each of these transport processes, the inhibitory effect exerted by external sodium (Na^+) and ammonium (NH_4^+) on Rb^+ transport, as well as the observation that *HvHAK1* expression was stimulated by K^+ deprivation, provided consistent evidence that this, and/or closely related, transport systems, could participate in the high-affinity transport of K^+ long ago observed by Epstein in barley roots (Epstein et al., 1963). Pharmacological approaches provided further support to this notion and, together with the disruption of the inward rectifying K^+ channel AKT1 (Hirsch et al., 1998), opened up the possibility that K^+ transport from dilute solutions into plant roots could involve two separate components whose contributions depend on the ionic environment encountered by plants during growth: a KT-HAK-KUP transporter component, and an AKT1 channel component (Spalding et al., 1999; Santa-María et al., 2000; Rubio et al., 2008; Coskun et al., 2013). However, conclusive evidence supporting this possibility had to wait until the characterization of the phenotype displayed by *athak5* plants, which lack AtHAK5, the Arabidopsis homologue of HvHAK1 (both from clade I; Gierth et al., 2005). Since then (see Table 1), studies with other plant species have confirmed that a primary function of the KT-HAK-KUPs clustered in this clade is to mediate the inward transport of K^+ from dilute K^+ solutions, as shown *in planta* for the rice OsHAK1 and OsHAK5 transporters (Bañuelos et al., 2002; Yang et al., 2014; Chen et al., 2015b), and also in heterologous studies in yeast systems expressing LeHAK5 from tomato

Table 1
Functions of selected Arabidopsis and rice KT-HAK-KUP transporters, including tissular and sub-cellular localization.

Transporter	Clade	Evidence for K^+ transport in Saccharomyces and other hallmarks	Tissue expression	Cellular localization	Function (s) in plant (based on "*in planta*" studies)	References
AtHAK5	I	Yes (a version carrying the L776H substitution in WΔ3 strain)	Root cortex and stele	Endomembrane/Plasma membrane	K^+-uptake under K^+-deficiency condition; Innate immunity	Rubio et al. (2000), Gierth et al. (2005), Qi et al. (2008), Brauer et al. (2016)
AtKUP2	II	Complements the growth of the *E. coli* TK2463 strain	Growing tissues	–	Cell enlargement; control of cell turgor	Elumalai et al. (2002); Osakabe et al. (2013)
AtKUP4	II	Yes (M398 strain)	Peripheral root cells, central cylinder	Tonoplast, endomembrane/ Plasma membrane	Auxin movement; root hair growth; gravitropic response	Rigas et al. (2001), Desbrosses et al. (2004), Vicente-Agullo et al. (2004), Whiteman et al. (2008), Rigas et al. (2013), Daras et al. (2015)
AtKUP6	II	Complements the growth of the *E. coli* TK2420 strain	Root tip, vascular tissue, guard cells	Plasma membrane	Cell enlargement, response to osmotic stress, ABA-dependent stomatal closure, lateral root formation	Osakabe et al. (2013), Ahn et al. (2004)
AtKUP7	V	Yes (5421 strain). It rescues the growth of the *E. coli* strain defective in AC activity (recombinant AtKUP7 protein)	Epidermis, endodermis, procambium	Endomembrane/Plasma membrane	K^+-uptake, K^+-translocation. *In vitro* adenylate cyclase (AC) activity	Whiteman et al. (2008), Al-Younis et al. (2015); Han et al. (2016)
OsHAK1	I	Yes (PLY246 strain)	Root (epidermis, vascular cells); Shoot (apical cells, vascular bundles)	Plasma membrane	K^+-uptake and long-distance transport; K^+:Na^+ homeostasis under salt stress; main pathway for Cs^+ transport in rice. Drought tolerance.	Bañuelos et al. (2002), Chen et al. (2015b), Nieves-Cordones et al. (2017), Rai et al. (2017), Chen et al. (2017)
OsHAK5	I	Yes (CY162 strain)	Root epidermis, vascular tissue, mesophyll cells	Plasma membrane	K^+-uptake and long distance transport; response to salt stress	Horie et al. (2011), Yang et al. (2014)
OsHAK21	I	Yes (CY162 strain)	Xylem parenchyma cells, endodermis, anthers	Plasma membrane	Acclimation to salt stress; K^+-uptake and probably long distance transport	Shen et al. (2015)

Global expression studies have been performed for Arabidopsis KT-HAK-KUP transporters by Ahn et al. (2004) and for rice by Gupta et al. (2008).

(Nieves-Cordones et al., 2008), ThHAK5 from the halophyte *Thellungiella halophila* (Alemán et al., 2009), and transporters from other plant species. In the case of OsHAK1, current information indicates that it contributes, *in planta*, to K^+ transport over a wide range of external K^+ concentrations (Chen et al., 2015b). In turn, AtKUP1 (also named as AtKT1, Clade II), as determined early on in heterologous systems, may be involved in biphasic transport of K^+ (Fu and Luan, 1998; Kim et al., 1998), while other KT-HAK-KUPs could be involved in K^+ efflux, as suggested for OsHAK7 and OsHAK10 (Clade II; Bañuelos et al., 2002). It can be speculated that, at least in the case of OsHAK10, this efflux could play a role in the control of K^+ movement between the vacuole and the cytoplasm. However, its actual relevance needs to be further evaluated by *in planta* studies. The possibility that some KT-HAK-KUP transporters could be involved in reversible K^+ transport has been suggested by studies in *E. coli* with the CnHAK1 transporter (Clade II) from the sea grass *Cymodocea nodosa* (Garciadeblas et al., 2002).

4.1. Members of different clades contribute to in planta potassium transport

It is interesting to note that the capacity to contribute to *in planta* K^+ transport from dilute K^+ solutions is not confined to clade I (Table 1). Recent findings, based on the use of a disruption mutant, showed that AtKUP7 (group V), which is ubiquitously expressed in Arabidopsis (including root hairs), could be involved in the depletion of K^+ from a 250 μM solution, suggesting that it could participate in net K^+ uptake from low to moderate external K^+ concentrations in K^+-starved plants (Han et al., 2016). In the same way, disruption of *AtKUP4/TRH1* (group IIa), which is expressed in multiple tissues including the root epidermis, results in reduced Rb^+ uptake from a low-μM K^+ (^{86}Rb) solution (Rigas et al., 2001). On the other hand, the multiple disruption of *AtKT2(AtKUP2)*, *AtKUP6* and *AtKUP8* genes (group IIc) enhanced the root capacity to transport Rb^+ from a solution of low K^+ ($^{86}Rb^+$) concentration (Osakabe et al., 2013). These results suggest that the transporters can mediate or influence the influx of K^+ from dilute K^+ media. Noticeably, in Arabidopsis it has been shown that the ability to transport K^+ from very low, or even moderate, external K^+ concentrations is essentially abolished in *akt1/athak5* plants, which lack the activity of both AKT1 and AtHAK5 (Pyo et al., 2010; Rubio et al., 2010). Therefore, the effects derived from the disruption of KT-HAK-KUP transporters distinct from AtHAK5, at those external K^+ concentrations, could be attributed to their possible role in K^+-efflux (as proposed for AtKT2, AtKUP6, AtKUP8, Osakabe et al., 2013) and/or to the possibility that they modulate the activity of AtHAK5 and via side effects on root architecture. This last possibility could be important for the particular case of the *atkup4(trh1)* mutant, which exhibits tiny root hairs, a condition that could negatively impact Rb^+ uptake, as shown in studies with another root-hair-defective mutant (Ahn et al., 2004). In turn, modulation of KT-HAK-KUPs could involve physical interactions among different subunits, thus modifying the transport capacity of AtHAK5. Although evidence for this kind of interaction has been documented for plant Shaker-like K^+ channels (Véry et al., 2014), scarce information has been available on this subject for KT-HAK-KUP transporters. Interestingly, a recent work, based on the use of bimolecular fluorescence complementation, indicates that for AtKUP4(TRH1) there is a physical self-interaction among subunits (Daras et al., 2015). The evidence obtained by the authors of this work supports the notion that this transporter could tend to form homomers over heteromers. The extent to which this notion can be extended to other KT-HAK-KUP transporters deserves further studies, particularly for closely related transporters, which may share extensive homology. An alternative hypothesis to explain the effect of the disruption of KT-HAK-KUP transporters that do not belong to clade I on Arabidopsis root K^+ uptake include a possible role of some of them in low-K^+ signaling, which could regulate AtHAK5 uptake capacity, as outlined below. Clearly, studies with multiple mutants for KT-HAK-KUP transporters, including AtHAK5, will be necessary to fully understand their roles in ensuring

K^+ acquisition under low K^+, as well as in other ionic environments. In this context, the refinements recently introduced in protocols for measuring K^+-fluxes at different external K^+ concentrations (Coskun et al., 2016) could be valuable to unequivocally resolve the unidirectional fluxes specifically mediated by each transporter.

Notably, some KT-HAK-KUP transporters are likely involved in long-distance transport of K^+ to the shoot including OsHAK5, OsHAK1, AtKUP7 and probably OsHAK21 (Yang et al., 2014; Chen et al., 2015b; Shen et al., 2015; Han et al., 2016; Table 1). In addition, certain KT-HAK-KUP transporters are probably involved in local alkali cation movements among tissues. In this regard, recent work performed in Arabidopsis suggests a possible role of AtKT2 and AtKUP11 in determining the local fluxes of K^+ between bundle and mesophyll cells (Wigoda et al., 2017). Other specialized, more subtle, roles can be also played by KT-HAK-KUP transporters, as reported for DmHAK5 from the carnivorous plant *Dionaea muscipula*, which mediates the flux of K^+ derived from digested prey in the trap (Scherzer et al., 2015) and belongs to clade I (Nieves-Cordones et al., 2016).

4.2. KT-HAK-KUPs are allocated to both outer and inner cell membranes

All the transport phenomena above mentioned, including uptake, root to shoot movement, and movements among tissues, likely require the presence of transporters in the plasma membrane. In fact, members of clade I already characterized (Table 1) seem to be mainly targeted to this membrane, as suggested for AtHAK5 (Qi et al., 2008), OsHAK5 (Yang et al., 2014); OsHAK1 (Chen et al., 2015b; Liu et al., 2016); OsHAK21 (Shen et al., 2015) and OsHAK19 (Liu et al., 2016). It is noteworthy that the targeting of the canonical member of this group, AtHAK5, to the outer membrane, seems to depend on growth conditions: at sufficient K^+ levels, AtHAK5 is preferentially found in the endoplasmic reticulum, while under K^+ scarcity, a fraction is allocated to the plasma membrane (Qi et al., 2008). Members of other groups such as AtKUP7 (Han et al., 2016), AtKUP4/TRH1 (Rigas et al., 2013) and AtKUP6 (Osakabe et al., 2013) can also be found in the plasma membrane. However, some KT-HAK-KUP transporters can be primarily allocated to inner organelles. The first evidence supporting this statement was obtained for OsHAK10 (group II), which was found to be associated with the tonoplast by Bañuelos and co-workers, who used an approach based on localization of a green fluorescent fusion protein (Bañuelos et al., 2002), as well as for AtKUP12 (group V), which was found in chloroplasts (Kleffmann et al., 2004). There is strong evidence that members of group III, specifically PpHAK2 and PpHAK3 from *Physcomitrella*, are localized in endomembranes, where it has been proposed that they mediate the efflux of K^+ from the lumen of the endoplasmic reticulum to the cytosol (Haro et al., 2013); the role of PpHAK2 is probably critical in maintaining reticulum functionality at low K^+ and in weakly acidic media. It seems likely that AtKUP4(TRH1) could also be localized in the endomembrane system (Rigas et al., 2013). Current evidence indicates that, in Arabidopsis, AtKUP4(TRH1) and AtKUP8 from group II, as well as AtKUP5, AtKUP7 and AtKUP12 from group V, can be found in tonoplast-enriched fractions (Jaquinod et al., 2007; Whiteman et al., 2008), but some could also be targeted to other membranes. Localization of KT-HAK-KUP transporters in the tonoplast fraction suggests their possible role in alkali-cation movement within the cell, thereby contributing to the regulation of cellular water homeostasis.

4.3. KT-HAK-KUP contribute to major physiological processes

Evidence that KT-HAK-KUP transporters participate in the control of water movement at the plant level has been supported by studies with the *atkt2/atkup6/atkup8* triple mutant (Osakabe et al., 2013). More recently, a role for OsHAK1 in plant responses to drought has been advanced (Chen et al., 2017). Additionally, it is worth considering that some KT-HAK-KUPs likely play an important role in determining Na^+ uptake from low concentrations, which could be beneficial when plants are exposed to low K^+ conditions. This has been shown for the moss PpHAK13 (group IV) and also for the yeast transporter YlHAK1 from *Yarrowia lypolytica* (Benito et al., 2012), which may play roles in ensuring water accumulation and charge balance. In addition, some KT-HAK-KUP transporters likely play a major role in determining the magnitude of Na^+ fluxes when plants are exposed to salt stress, as indicated by several expression studies (e.g. Su et al., 2002; Fulgenzi et al., 2008), and by the analysis of the protective effect conferred by their expression in yeast (Mangano et al., 2008) and plants (Shen et al., 2015). Thus, the primary functions of KT-HAK-KUPs in plants seem to be the transport of K^+ and, directly or indirectly, the control of Na^+ transport. In addition there is compelling evidence that KT-HAK-KUP transporters such as AtHAK5, AtKUP9, OsHAK1, and SlHAK5 could also provide a pathway for the transport of the toxic element Cs^+ into the roots (Rubio et al., 2000; Qi et al., 2008; Kobayashi et al., 2010; Nieves-Cordones et al., 2017; Rai et al., 2017; Ródenas et al., 2017). Additional studies performed by Qi et al. (2008) with the *akt1* mutant lend support for this notion.

Early evidence suggested a major role of members of this family of transporters in key developmental and growth processes, as shown by studies with mutants displaying tiny root hairs (trh1) and short hypocotyls (shy3), accompanied by small leaves and short flowering stems, which were shown to correspond to mutant versions of AtKUP4(AtKT3) and AtKUP2(AtKT2) by Rigas et al. (2001) and Elumalai et al. (2002), respectively (Table 1). A role of KT-HAK-KUPs has been proposed in nodulation (Desbrosses et al., 2004; Rehman et al., 2017), gravitropism and root hair growth (Rigas et al., 2001; Desbrosses et al., 2004; Vicente-Agullo et al., 2004), lateral root formation in response to exogenous auxin (Osakabe et al., 2013), fiber elongation (Ruan et al., 2001), pollen tube growth (Liu et al., 2016) as well as whole shoot (Elumalai et al., 2002; Chen et al., 2015b) and root growth (Chen et al., 2015b). These transporters have also been proposed to play a role in innate immunity (Brauer et al., 2016), as well as in ABA-mediated stomatal closing (Osakabe et al., 2013). It is worth mentioning that the precise role of a KT-HAK-KUP transporter in K^+ efflux during stomatal closing needs to be further evaluated, as K^+-outward channels provide a major conductance which could make unnecessary, from a quantitative point of view, the contribution of a low conductance transport system. It is not clear whether the contribution of KT-HAK-KUPs in all these processes primarily involves alkali cation movement, as KT-HAK-KUPs can also transport other substances and play additional roles. An interesting example of this possibility has been offered for AtKUP4(TRH1), which was firstly shown to be necessary for tip growth in Arabidopsis root hairs (Rigas et al., 2001), and also shown to rescue the growth of *trk1* yeast cells, while *trh1* plants displayed a reduced capacity to mediate Rb^+ transport from a solution with low Rb^+ concentration. However, the phenotype of *trh1* plants was not rescued by enhancing K^+ supply, thus stablishing an important difference with *athak5* plants, which exhibit short root hairs only under low K^+-supply conditions (Zhao et al., 2016). In turn, Desbrosses et al. (2004) have shown that the *akt1* mutant displays a reduction of root hair length compared with WT plants at high external K^+-concentrations, while root hairs of *trh1* plants are short over a wide range of external K^+ concentrations. Thus, both AKT1 and TRH1 are required for the control of root hair growth, but they have different functions in this process. Furthermore, a study showed that the *trh1* mutant is more sensitive to exogenous auxin addition, and that, when expressed in yeast cells, AtKUP4(TRH1) confers the capacity to mediate auxin efflux (Vicente-Agullo et al., 2004). Moreover, it was shown that root segments of *trh1* plants retain more auxin than those of WT plants. All these data suggest that AtKUP4(TRH1) mediates auxin efflux, and it has been proposed that it provides an autonomous pathway acting independently downstream of root hair initiation (Rigas et al., 2013; Daras et al., 2015).

The suggestion of an entirely new function for KT-HAK-KUP transporters comes from the recent demonstration that AtKUP7 can mediate,

at least *in vitro*, the conversion of ATP to cAMP (Al-Younis et al., 2015; Table 1). In other words, it displays adenylate cyclase activity, which suggests a possible role in signaling. As AtKUP7 is also able to mediate K^+ transport, the authors advanced the interesting suggestion that cAMP production could be linked to the transport of K^+, thus opening the possibility that AtKUP7, and probably other related transporters, could act as K^+-sensors.

5. A brief note on transport properties and structure-function studies with KT-HAK-KUP proteins

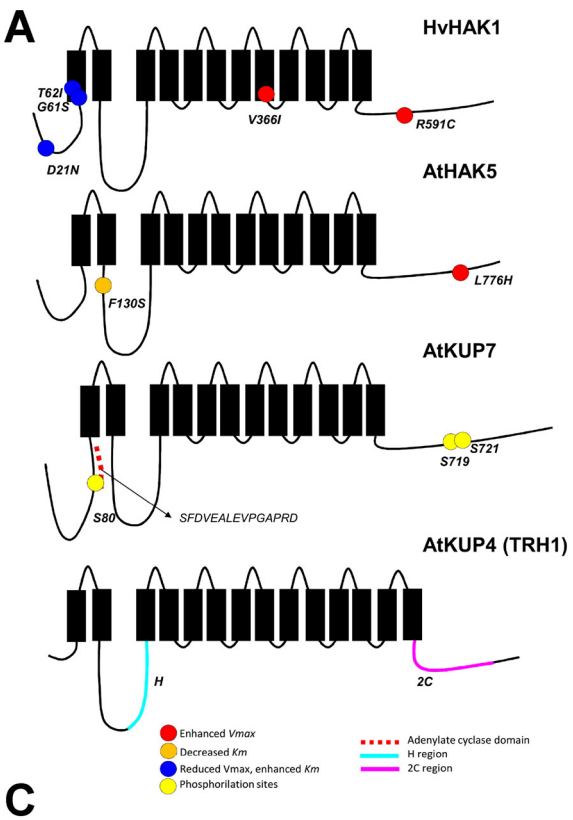
While important advances have been made in understanding the structure of K^+ channels, few studies have dealt with this subject for KT-HAK-KUP transporters. A recent study (Sato et al., 2014) provided the first experimental evidence, through the use of protein-reporter fusion constructs, that the KUP protein from *E. coli* contains 12 transmembrane domains (TMs), in agreement with several algorithms for the prediction of membrane topology (Supplementary Fig. 2). That work also determined that both the N and the C termini of this protein are located in the cytoplasm, and that endogenous cysteine residues are not necessary to ensure K^+ transport. An immediate consequence of these findings, as stated by the authors, is that the structure of KUP is dissimilar to that of other known bacterial and plant K^+ transport proteins. In turn, computational 3D structural models have been recently offered for AtKUP7, AtKUP4(TRH1) and OsHAK1 (Al-Younis et al., 2015; Daras et al., 2015; Rai et al., 2017). In Fig. 2, both topological and 3D models for the structure of AtKUP7, AtKUP1(TRH1), HvHAK1a and AtHAK5, are shown (see also Supplementary Table 5). A common attribute of the 3D models is the presence of a hydrophobic core containing the TMs. Outside this core, three components can be recognized. One corresponds to the N terminus, another to the C terminus, and a third one to a region containing approximately 70 residues situated between TM II and TM III. These three components are predicted to be oriented towards the same side. It should be emphasized that the extent to which these models actually mimic the structure of KT-HAK-KUP transporters requires further assessment.

Topology information obtained by Sato et al. (2014) did not describe the structure of the pore region, or how transport is controlled at the protein level. However, some works have addressed these questions, by examining the effect of mutations on transporter function. The use of truncated C-terminus versions of the *E.coli* KUP protein has yielded equivocal results, and it is unclear to what extent this domain is actually essential for the transport of K^+ by this transporter (Schleyer and Bakker, 1993; Sato et al., 2014). Nevertheless, a role for this region in determining the maximum rate of transport (Vmax) for members of group I of plant KT-HAK-KUP transporters seems to be apparent, because in both the Arabidopsis AtHAK5 and the barley HvHAK1a transporters (Fig. 2), amino acid mutations in this region strongly influence the rate of transport, with only a minor effect (if any) on the K_M for the transport of Rb^+ (Rubio et al., 2000; Mangano et al., 2008; Alemán et al., 2014). This notion is reinforced by the fact that the C-terminus (T759) of AtKUP6 (Osakabe et al., 2013), as well as residues S719 and S721 in the C-terminus of AtKUP7 (Han et al., 2016), are likely involved in phosphorylation, thus potentially affecting *in vivo* protein activity. In turn, the substitution of an amino acid residue (V366I), which is conserved in 39% of the KT-HAK-KUPs analyzed (Fig. 2), at the putative eighth TM of the HvHAK1a transporter, also conferred an increased Vmax (Mangano et al., 2008). On the other hand, it has been observed that the F130S substitution in AtHAK5 (at a site also highly conserved among KT-HAK-KUPs), results in a reduction of K_M for Rb^+ transport of almost two orders of magnitude, indicating a major effect on the apparent affinity of the transporter (Alemán et al., 2014). This substitution occurred in the region between the second and the third TMs. The construction of chimeric versions of HvHAK1a that contain different parts of HvHAK2 (group IIc) suppress the capacity of HvHAK1a to restore the growth of a *trk1trk2* yeast mutant (Senn et al., 2001). Interestingly, in that work it was found that the substitutions D21N, G61S and T62I (all found near the N-terminus, with the last two probably within the first TM) resulted in a several-fold increase of K_M, and, to a lesser extent, reduced the Vmax for Rb^+ transport (Fig. 2). The emerging picture from these studies indicates that while several parts of the protein are likely to contribute in determining the Vmax, only the N terminus and the region proximal to it, including the region between the II and III TM domains, appear to be involved in determining the affinity of the transporter. An unexplored, but likely, possibility is that the II–III loop region is a critical component of the pore region of the transporter, but further research is necessary to fully clarify this point. In addition, it seems possible that the hydrophilic N-extreme could play additional roles. One of them may be to participate in phosphorylation processes as proposed for AtHAK5 (Ragel et al., 2015), and suggested by the effect of the substitution S80A in AtKUP7 in yeast and *in planta* (Han et al., 2016). Regarding this transporter, it should be mentioned that its adenylate cyclase activity, demonstrated in *in vitro* assays with a recombinant protein, seems to be associated with a 14-amino-acid motif also found in the N terminus of the protein, which is likely localized in the cytosol. Interestingly, other transporters of the same clade from both dicot and monocot species, also contain this motif (Al-Younis et al., 2015), suggesting that this function has an ancient origin. Another interesting, but yet not fully explored, structural observation refers to AtKT2(AtKUP2/SHY3), in which it found early on that the substitution G419R determines a dwarf phenotype (Elumalai et al., 2002). This indicates that the substitution, which does not seem to alter the capacity of AtKT2 to complement an *E.coli* K^+-defective strain, confers an *in planta* gain of function.

The above findings refer to the structure of KT-HAK-KUP transporter subunits (Fig. 2). However, as mentioned, the recent study by Daras et al. (2015) suggests that the AtKUP4(TRH1) transporter, at least, can likely form homodimers. Evidence obtained by these authors suggests that sub-region 2C of the C-terminus, and sub-region H in the loop between TM domains II and III, could be involved in the interaction between different AtKUP4(TRH1) subunits. While the interaction was found to be weak in the loop zone, it was high at the C-terminus; suggesting that the 2C zone, and to lesser extent the H one, are major determinants in the conformation of the homomer.

5.1. Features of KT-HAK-KUPs in green algae

In 2011 Greiner et al. made the important discovery that some *Chlorella* viruses contain a KT-HAK-KUP transporter encoded in their genomes. Moreover, heterologous expression of one of these genes, *n110r*, rescued the growth of a *trk1trk2* yeast strain and bestowed upon it the capacity to mediate Rb^+ influx from a 500-μM Rb^+ solution (Greiner et al., 2011). Interestingly, the authors found that the genome of the host, *Chlorella variabilis*, encodes a short protein with high similarity to KT-HAK-KUP transporters. An analysis of this protein with the TMHMM 2.0 program reveals that it only contains the first five TM homologous domains. It could be speculated that this short protein, which is the only known member of the KT-HAK-KUP family in this green alga, could be involved in K^+ transport. If so, it could constitute the minimal KT-HAK-KUP functional system. Unfortunately, no studies to date have been carried out to test this possibility. On the other hand, an examination of sequences from other green algae indicates that, while representatives of most recognized clades tend to exhibit features similar to those found in land plants (with some degree of variation; Supplementary Table 4), members of cluster XII differ from the common pattern in several ways. Firstly, all members of this clade are very large proteins, ranging from 921 residues in GpHAK1, and up to 1752 in CrHAK5. Secondly, the length of the loop between TMs II and III in the members of this clade almost double that found for AtHAK5 and related transporters (Supplementary Fig. 1 and Table 4). Given the potential functions played by this loop, such a difference could exert major effects on the functional properties of these proteins. As with the



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HvHAK1	All	Clade I	Algae
D21	nd	100	nd
G61	64.7	100	94.4
T62	83.8	100	94.4
V366	39.3	60.1	16.7
R591	nd	21.2	nd

AtHAK5	All	Clade I	Algae
F130	98.3	100	100
L776	66.5	72.7	55.6

AtKUP7	All	Clade V	Algae
S80	nd	73.7	nd
S719	nd	10.50	nd
S721	nd	52.6	nd

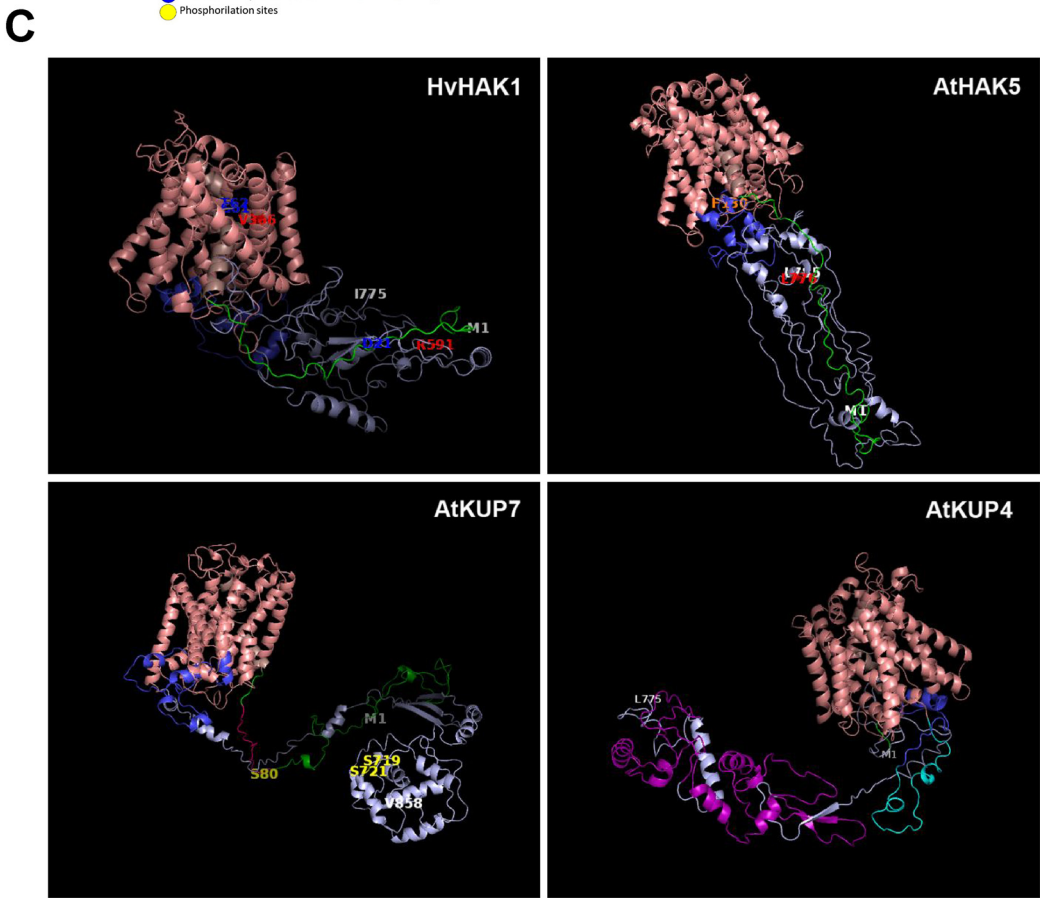

Fig. 2. A. Topological models of HvHAK1a, AtHAK5, AtKUP7 and AtKUP4(TRH1), as inferred from the analysis shown in Supplementary Table 5. Information obtained for the plant KT-HAK-KUP transporters in structure:function studies is also shown. Aminoacid substitutions labelled in blue correspond to those that confer reduced Vmax and enhanced Km, red: enhanced Vmax; orange: decreased Km. Residues labelled in yellow correspond to phosphorylation sites. The dashed red line corresponds to the putative adenylate cyclase (AC) domain. Cyan and violet corresponds to the H and 2C regions proposed to be involved in the formation of multimers. Note that regions are schematically represented and their extension do not necessarily reflect the actual one (in this regard see Supplementary Table 5). B. Frequency of conservation of the above mentioned residues (as determined through an alignment made with the 176 sequences plus HvHAK1a) for all KT-HAK-KUP transporters analyzed, for the specific clade involved as well as for green algae. Frequencies corresponding to residues situated in regions that may not be well conserved among clades were no determined (nd). C. Predicted 3D structures of HvHAK1a, AtHAK5, AtKUP7 and AtKUP4(TRH1). Models were constructed through the use of the Phyre 2 application, by the intensive mode. In all cases c3giaA, corresponding to the apct transporter, was automatically selected as the template with the highest confidence, between 41 and 52% of the residues being modelled by *ab initio*. Salmon: transmembrane helices region, green: N-terminus, grey: C-terminus, blue: region between transmembrane domains II and III. Residues and regions involved in specific functions are shown with the same colors used in A. The first and final residues are labelled in white. The region corresponding to the last two TMs predicted for AtKUP4 by TOPCONS, TMHMM 2.0 and TMPred, was placed within the C-terminus according to this 3D model.

HAK from Chlorella, this subject remains to be tackled experimentally.

5.2. Are KT-HAK-KUPs H^+/K^+ symporters?

A next question refers to the transport mechanism involved. Evidence obtained both in fungi and plant roots has suggested that high-affinity transport of K^+ from the external medium is coupled to H^+ currents (Rodriguez-Navarro et al., 1986; Blatt et al., 1987; Maathuis and Sanders, 1996). For *Neurospora*, several lines of evidence strongly support K^+/H^+ symport as the mechanism involved in determining K^+-fluxes mediated by the NcHAK1 transporter (Haro et al., 1999; Rivetta et al., 2013). First, NcHAK1 can mediate the inward transport of K^+ from low K^+ solutions (Haro et al., 1999). Second, under conditions of low K^+ and carbon supply NcHAK1 is the main contributor to K^+ uptake (Rivetta et al., 2013). Finally, under the above-mentioned conditions membrane currents attributable to K^+/H^+ cotransport were described (Rodriguez-Navarro et al., 1986; Rivetta et al., 2013). Indirect evidence also support K^+/H^+ symport as a mechanism for the *E. coli* KUP transporter (Trchounian and Kobayashi, 1999). In this regard, it has been shown that K^+ influx may be enhanced at low pH under hyper-osmotic shock, a condition under which K^+-transport is essentially mediated by KUP. Furthermore, under these growth conditions K^+ flux is inhibited by the presence of the ionophore CCCP, which could affect the H^+-gradient (Trchounian and Kobayashi, 1999). However, the recent study of Sato et al. (2014) indicated that growth of *E. coli* was not stimulated at acidic pH values, either in the *WT* version of KUP or in three variants in which histidine residues, which likely contribute to the sensing of external H^+, were substituted by alanine. In this context, it should be noted that major technical limitations prevented the resolution of the transport mechanism in heterologous studies using either *S. cerevisiae* or *E. coli* cells. In addition, early attempts to measure KT-HAK-KUP currents in oocytes proved to be unsuccessful (Kim et al., 1998; Rubio, personal communication). However, recent work performed with the DmHAK5 transporter showed that co-expression of the corresponding cRNA with that of CBL9/CIPK23 (but not DmHAK5 alone), generates inward K^+ and Rb^+ currents in *Xenopus* oocytes (Scherzer et al., 2015). Interestingly, those currents were enhanced as external pH decreased, thus suggesting that, at least for this transporter, K^+ currents are coupled with H^+.

6. Regulation of AtHAK5

The way in which plants modulate the contribution of KT-HAK-KUP transporters to the above-mentioned processes has recently become a very active area of research. Early on, several studies indicated that the environment encountered by plants during their ontogeny could strongly influence expression patterns of the genes coding for these proteins (see Véry et al., 2014, and references therein). Later, evidence for post-transcriptional modulation of KT-HAK-KUP transporters was suggested by heterologous studies in yeast with *HvHAK1a* (Fulgenzi et al., 2008), as well as by studies on phosphorylation sites in the tonoplast-enriched fraction in Arabidopsis (Whiteman et al., 2008). The existence of this sort of regulation received conclusive support by recent studies suggesting that phosphorylation processes could play a major role in determining KT-HAK-KUP activities *in planta* (Osakabe et al., 2013; Ragel et al., 2015; Scherzer et al., 2015; Brauer et al., 2016; Han et al., 2016; Liu et al., 2016). It should be noted that most of the cutting-edge research on the regulation of KT-HAK-KUPs has focused on the way in which low K^+ signaling enhances the accumulation of transcripts and activity of AtHAK5. Therefore, we will specifically focus on the findings obtained for this transporter under this particular stress condition (Fig. 3).

6.1. Transcriptional control of AtHAK5

Early works by Schachtman, Shin and co-workers indicated that,

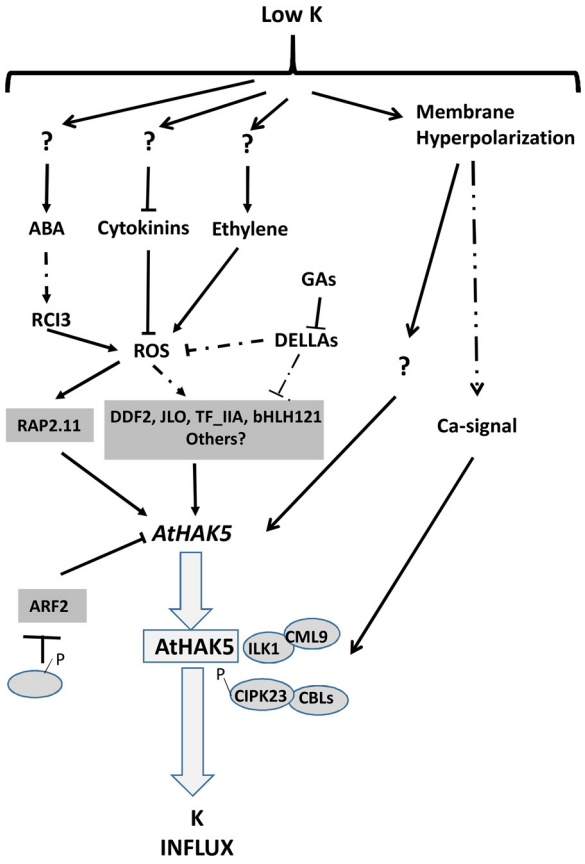

Fig. 3. Scheme showing the signaling network linking low K^+ supply with the induction of AtHAK5 mediated K^+ transport in Arabidopsis. Both potential transcriptional and post-transcriptional regulatory elements are shown. Dashed lines indicated possible links that require to be fully established. The need of further research for particular links is denoted by including an interrogative symbol (?). Further explanations can be found in the main text.

under low K^+ supply, both ROS (Shin and Schachtman, 2004; Shin et al., 2005) and ethylene (Jung et al., 2009) become increased; genetic and pharmacological evidence has supported a role for them in the control of *AtHAK5* expression. A role of ethylene in the regulation of AtHAK5 is also supported by the observation that under salt stress, loss of ETO1 function results in increased accumulation of both ethylene and *AtHAK5* transcripts (Jiang et al., 2013), suggesting the presence of common elements in the pathways controlling *AtHAK5* accumulation by salt and low-K^+ stresses. A relevant question is how ROS, ethylene, and other hormonal regulators are linked with *AtHAK5* transcript accumulation. In this regard, the use of activation tagging lines showed that the transcription factor RAP2.11 had the capacity to bind, both *in vitro* and *in vivo*, the *AtHAK5* promoter, leading to enhanced *AtHAK5* transcription (Kim et al., 2012). Soon after, another set of transcription factors (TFs) with the capacity to enhance *AtHAK5* transcription was identified by using that approach, combined with the use of a TF FOX library (Hong et al., 2013). Among the TFs identified, four received particular attention (DDF2, JLO, TF_IIA, and bHLH121), and were found to be able to bind the *AtHAK5* promoter. Apart from these TFs, other candidates identified by the authors could be also relevant, but further research is required to unveil their precise roles on *AtHAK5* expression in roots, if any, during low K^+ signaling. On the other hand, the TF ARF2 (Auxin Response Factor 2) has been recently proposed to be involved in the modulation of *AtHAK5* transcription (Zhao et al., 2016). The *arf2* mutant displays a phenotype similar to that conferred by overexpression of *AtHAK5*, which results in increased high-affinity K^+ uptake. Complementary experiments showed that ARF2 could be phosphorylated following K^+-deprivation, and that phosphorylation

suppresses its capacity to bind *AtHAK5*, leading to relieve the inhibitory effect exerted by ARF2 on *AtHAK5* transcription. It is important to note that the *arf2* phenotype does not seem to be rescued by exogenous auxin addition; thus suggesting that its effects do not involve auxin signaling. Having in mind the opposite effects exerted by RAP2.11, DDF2, and other TFs compared with those of ARF2, it seems likely that regulation of the activity of TFs acting positively and negatively on *AtHAK5* transcription is necessary to determine cooperatively the accumulation of the corresponding transcripts.

Interestingly, RAP2.11 expression was shown to be positively influenced by low K^+ supply as well as by the addition of ethephon or H_2O_2; thus suggesting the possibility that RAP2.11 forms part of a signaling pathway linking the detection of a low K^+ signal with enhanced *AtHAK5* transcription through ethylene and ROS. Providing further support for a pivotal role of ROS, it was also shown that RCI3, a member of the type III Peroxidase family, when overexpressed, determines a higher accumulation of both ROS and *AtHAK5* transcripts; with the opposite pattern found for *rci3* plants (Kim et al., 2010). In this case, the available evidence indicates that ethylene is probably not involved in the control of RCI3. It has been suggested that RCI3 is under the control of ABA levels (Kim et al., 2010); which increase following K^+-deprivation in both roots and leaf (Kim et al., 2009). In turn, the control of ROS levels and *AtHAK5* under K^+-deficiency likely involves other hormones, as suggested by the observation that cytokinins became reduced during K^+ deprivation, and that a cytokinin-deficient mutant displays, under this condition, enhanced accumulation of both ROS and *AtHAK5* transcripts (Nam et al., 2012). On the other hand, the possibility that DELLA proteins, which participate in the Gibberellin (GA) – GID receptor – DELLA regulatory module, contribute to determine ROS levels should also be taken into consideration, as it has been shown that DELLAs influence the antioxidant response of Arabidopsis plants under salt stress (Achard et al., 2008). For example, wheat plants with altered-function versions of the DELLA-coding genes *Rht–1B* and *Rht-1D* show enhanced antioxidant responses in leaves under K^+ deficiency (Moriconi et al., 2012). More recently, it was shown that Arabidopsis plants with an altered-function version of the DELLA protein GAI (*gai-1*, which is poorly recruited by GAs) showed a decreased induction of *AtHAK5* transcript accumulation under K^+ deprivation, accompanied by low Rb^+ uptake from dilute Rb^+ solutions (Oliferuk et al., 2017b). Given that the primary function of DELLAs is to modulate the activity of TFs, the possibility that the observed effect is due to the modulation of the TFs mentioned above (or others) has been advanced as an speculative model for the action of DELLAs (Oliferuk et al., 2017a). The model assigns a possible role to ethylene in the accumulation of the TFs that modulate positively *AtHAK5*, as well as in the movement of GAs among different tissues in the root differentiation zone; DELLAs could act negatively over those TFs. GA-induced DELLA degradation in epidermal cells could thus relieve the restriction imposed by these proteins in that zone, while, in the apical zone where DELLAs are accumulated, they could influence the growth restriction that takes place under low K^+ conditions. Following this model, the precise spatial location of the events leading to enhanced *AtHAK5* expression is likely to be extremely relevant as additionally suggested by the observation that the differential accumulation of ROS following K^+ deprivation takes place preferentially in the epidermal cells of the root differentiation zone (Shin et al., 2005). The possibility that other hormones, particularly jasmonic acid and auxins, are also involved in the control of *AtHAK5* expression should be not excluded, as these two hormones are thought to play important roles in plants suffering from K^+ deficiency (Armengaud et al., 2004; Song et al., 2015a).

6.2. Post-transcriptional regulation of AtHAK5

Evidence obtained by Rubio and co-workers indicate that, while accumulation of *AtHAK5* transcripts is associated with phosphorus, nitrogen and K^+ deficiencies, conditions that lead to plasma membrane hyperpolarization (Rubio et al., 2014), the induction of high-affinity K^+ transport mediated by AtHAK5 is a specific process that requires a low-K^+ signal; this indirectly suggests a role of post-transcriptional regulation *in planta*. A further step towards understanding the way in which AtHAK5 activity is modulated at this level was provided by Scherzer et al. (2015), who, as mentioned above, provided evidence that the DmHAK5 transporter is modulated by CBL9/CIPK23. CBL9 is a Ca^{2+}-responsive, calcineurin B-like protein with the capacity to recruit CIPK23 kinase. Ragel et al. (2015) indicated that enhanced AtHAK5 activity, both in yeast and Arabidopsis, also requires the action of CBL9 and CIPK23, as previously shown for the AKT1 channel (Li et al., 2006; Xu et al., 2006), thus providing evidence that AtHAK5 shares part of the signaling network with the other main contributor to K^+ uptake from dilute K^+ solutions. Furthermore, these findings support a role of Ca^{2+} signaling in the induction of these transporters, which is in line with a recent study showing that K^+ deficiency induces two spatially and temporally different Ca^{2+} signals (Behera et al., 2017); in yeast, a modification of intracellular Ca^{2+} was also reported (Lauff and Santa-María, 2010). An interesting work by Brauer et al. (2016) provided evidence for a second Ca^{2+}-dependent pathway acting on AtHAK5 during low-K^+ signaling. These authors showed that the Calmodulin-like protein 9 (CML9) interacted with INTEGRIN LINKED KINASE1 (ILK1), leading to the increase of AtHAK5 activity in a way that probably involves an interaction between ILK1 and AtHAK5. It is worth mentioning that in rice, recent evidence indicates that the receptor-like kinase RUPO, in its phosphorylated form, specifically interacts with the C-terminus of OsHAK1, OsHAK19 and OsHAK20, but not with other members of clade I (Liu et al., 2016); an interaction likely to be critical for pollen tube growth. Thus, it seems possible that kinases other than CIPK23 and ILK1 also participate in the tissue-specific and transporter-dependent control of the activities of certain transporters of this clade.

These results indicate that perception of low K^+ supply induces a complex signaling network that controls the accumulation of transcripts as well as the activity of AtHAK5, a statement which likely can be partially extended to other KT-HAK-KUP transporters that became induced by K^+ starvation. It should be considered that the first steps leading to the subsequent cascade of signals, particularly those controlling phytohormone levels, have received scarce attention; an additional effort will be needed to explore this issue. In addition, the hypothesis that the sensing of low K^+ could involve the coupling of K^+ transport with fast signaling processes through one or more KT-HAK-KUP transporters is an attractive one, particularly when the possible role of K^+ in plant signaling (Shabala, 2017) is taken in consideration.

7. Possible uses of *KT-HAK-KUP* genes

An emerging and interesting research issue is the possibility of using *KT-HAK-KUP* genes for different practical purposes. In this regard, efforts have been directed towards the possibility of over-expressing KT-HAK-KUP proteins as a way to resist particular stress conditions. The rationale behind those efforts is that, as maintenance of K^+ nutrition is critical for the survival and productivity of plants exposed to several stress conditions (Cakmak, 2005), the over-expression of specific KT-HAK-KUP transporters could help to maintain or improve K^+ homeostasis. This can be particularly relevant for plants grown under high external NH_4^+ concentrations, drought, and salinity. Regarding the last of these, which is thus far the most well studied, it has been shown that expression of the rice *OsHAK5* in BY2 tobacco cells enhances their capacity to resist moderate salt stress levels (Horie et al., 2011). Later work indicated that overexpression of *OsHAK5*, under the control of the *CaMV35S* promoter, enhanced dry matter accumulation at the seedling stage while improving the K^+/Na^+ ratio in shoots (Yang et al., 2014). The suitability of this approach has been reinforced by a study showing that overexpression of *ApKUP4* from *Alternanthera philoxeroides*, under the control of *35S*, conferred to Arabidopsis plants some degree of resistance to 100 mM NaCl (Song et al., 2014). Similarly, expression of

Aeluropus littoralis AlHAK1, under the control of the same promoter, conferred to cotton plants resistance to 150 mM NaCl (Liu et al., 2015). These results are auspicious and will promote the further refinement of strategies to use KT-HAK-KUPs to improve stress resistance. In this regard, the possibility of using engineered versions of KT-HAK-KUP proteins to improve plant performance has, to our knowledge, not been explored at the plant level. However, it was previously shown that mutant versions of the HvHAK1a transporter carrying single amino-acid substitutions confer to *S. cerevisiae* cells a dramatic enhancement in resistance to both salt and high NH_4^+ stresses (Mangano et al., 2008). Moreover, a chimera between two of these mutants confers an even more enhanced tolerance than that found with single mutations. A further examination of such mutants suggested the possibility that an increased K^+ influx can support plasma-membrane depolarization, and thus reduce the flux of Na^+ and/or NH_4^+ (Mangano et al., 2008), thereby reducing the incidence of toxic effects. Results obtained in rice plants with a disrupted OsHAK21 transporter are consistent with this view (Shen et al., 2015). While all these results are potentially promising, it remains unknown to what extent findings obtained in individual cells, or even in plants grown under controlled conditions, can actually be extrapolated to the field. In addition, to our knowledge, no studies have attempted to drive the expression of *WT* or altered-function alleles of *KT-HAK-KUP* genes under the control of promoters that specifically act in the window of response appropriate to salinity.

A second interesting approach takes advantage of the tissue specificity and the dependence of *KT-HAK-KUP* expression on growth conditions. Isolation of the corresponding promoters can be used to drive tissue-specific expression of proteins that could enhance the performance of plants under K^+ deprivation, a condition that may enhance the expression of certain *KT-HAK-KUP* genes, particularly those belonging to group I. An interesting example in this regard can be seen in the expression of the *WOX11* homeobox gene, under the control of the promoter of the root-expressed rice *OsHAK16* gene (Chen et al., 2015a). The resulting transgenic plants display a large root system under K^+-deficiency, which in turn exerts a positive effect on K^+ acquisition (i.e. the amount of K^+ taken up by plants) that is not significantly associated with enhanced K^+-net-uptake rate. This suggests that the primary effect of WOX11 on K^+ accumulation is mainly due to the increase of root size. These results indicate a possible way to improve the efficiency of K^+ acquisition by roots, which is important for plants growing in low-K^+ soils. Prospects to improve the efficiency of K^+ use by crops rests also on the capacity to improve so-called utilization efficiency, which relates biomass generation with the accumulation of nutrients in plant tissues (Santa-María et al., 2015), an aspect that can be strongly influenced by the pattern of K^+ distribution among plant organs and tissues (Yang et al., 2004). As KT-HAK-KUP transporters participate in long-distance and local movement of K^+, it seems likely that they are involved in determining K^+ utilization. The existence of theoretical tools to estimate K^+ utilization in hydroponic culture, without the masking effect of differences in K^+-capture (Moriconi and Santa-Maria, 2013), will help to evaluate the influence of ectopic-tissue-specific expression or editing of *KT-HAK-KUPs* mutants on K^+-utilization efficiency; this subject remains essentially unexplored.

Finally, an additional, and very important, recent possibility is that of generating crop plants with a reduced accumulation of toxic elements or radioactive isotopes, such the accumulation of radioactive Cs^+ in grains of crops growing near sites of nuclear accidents. In a recent paper, Nieves-Cordones et al. (2017) provided evidence in rice that the accumulation of Cs^+ from external Cs^+ concentrations potentially found in contaminated soils is mediated by the OsHAK1 transporter. Consequently, inactivation of the *OsHAK1* gene by use of a CRISPRCas system led to reduced Cs^+ accumulation in rice plants grown in hydroponics. Importantly, when tested in contaminated soils collected near the Fukushima Daiichi reactor, the plants displayed reduced levels of $^{137}Cs^+$ in shoots and roots. Notably, a parallel screening for rice mutants with reduced Cs^+ accumulation was performed by Rai et al. (2017). The three mutants isolated by this team correspond to the *OsHAK1* locus, providing confirmatory evidence that the encoded transporter is the main one contributing to Cs^+ influx. Moreover, when cultured in $^{137}Cs^+$-contaminated soil, the accumulation of radioactive Cs^+ in the grain was 10 times lower than in the WT rice. A search for transporters with similar function in barley, wheat and maize, where orthologues of OsHAK1 have been already identified (Santa-María et al., 1997; Zhang et al., 2012), could eventually extend this strategy to other relevant cereals. Nevertheless, given the dynamic history of the specific subgroup of OsHAK1 homologues, which in the particular case of Triticeae likely experienced recent duplications (Santa-María et al., 1997; Rubio et al., 2000), this may not necessarily be a straightforward procedure.

8. Conclusions

Since the first cloning of KT-HAK-KUP transporters in barley and Arabidopsis twenty years ago, our knowledge of these transport proteins has increased impressively. It is now clear that the primary function of these transporters, which diverged into most major clades following land colonization, is to mediate the transport of K^+ and other alkali cations in different plant organs and tissues. Specific transporters are well suited to mediate the transport of Cs^+, and it is possible to minimize the accumulation of this toxic element by use of lack-of-function mutants, or by modifying K^+/Cs^+ selectivity. In performing their physiological roles, these transporters, as exemplified by AtHAK5 from clade I, are subjected to a complex regulatory network that involves both transcriptional and post-transcriptional phenomena. In addition, certain KT-HAK-KUPs that belong to clade II are involved in auxin movement, while a subgroup of those that belong to clade V likely has adenylate cyclase activity, thus providing a potential link between K^+ transport and signaling. In spite of the important advances made thus far, some grey areas can be identified; here, we mention just three of them. i) Much remains to be discovered about the relevance of different protein domains on the transport capacity and overall activity of KT-HAK-KUPs, about the potential capacity of KT-HAK-KUP subunits to interact with others, and about the precise molecular mechanism involved in their functions. ii) More studies are needed which use multiple mutants, particularly those potentially participating in K^+ capture, but also in other processes, to disclose the way in which different KT-HAK-KUP transporters contribute to K^+ (and Na^+) transport, as well as to other not-yet-identified phenomena. iii) Finally, it is also necessary to integrate the different factors involved in the regulatory networks into a spatially and temporally well resolved picture. We are witnessing just the beginning of this tale, which will likely have positive impacts on biotechnology programs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jplph.2018.04.008.

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