Title

Genomic and Transcriptomic Analyses of Nutrient Transporters in Plants

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Publication Date

2021

Peer reviewed|Thesis/dissertation

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By

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A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Plant Biology

in the

Graduate Division

of the

University of California, Berkeley

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Spring 2021

ABSTRACT

Genomic and Transcriptomic Analyses of Nutrient Transporters in Plants

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Plants require Nitrogen (N), phosphate (P), potassium (K) to support numerous physiological processes. These nutrients are acquired and transported by large families of nutrient transporters found in various tissues and cell types. Decades of research on nutrient channels and transporters in the model plant, *Arabidopsis thaliana*, have expanded our understanding of how these nutrients are transported, stored, remobilized, utilized and recycled within plants. However, our knowledge of the nutrient transport systems in crops are lacking. Whole-genome sequencing and transcriptomic analyses of diverse plant species are increasing exponentially each year, which can be utilized for comparative genomic analyses to understand the evolution of specific family of nutrient channels and transporters, their functional conservation and divergence at the sequence and tissue- and cell-type-specific levels.

Camelina sativa is an emerging oilseed crop that contributes to food, feeds and fuels. It is considered to be tolerant to various harsh environments including low-nutrient stresses. However, it is not known how this polyploid might have evolved these adaptive traits at a gene level. To initiate functional genomic studies in this crop in response to low-nutrient stresses, we identified all the major nutrient channels/transporters of P and K present in the *Camelina* genome, and performed comprehensive and comparative genomic and transcriptomic analyses. We found that a whole-genome triplication event was the major driving force for the gene expansion, with three homoeologs for each Arabidopsis ortholog. In addition, tandem gene duplications have further expanded a specific nutrient transporter family in the *Camelina* genome. We also examined the phylogenetic relationship of nutrient transporters between *Camelina* and Arabidopsis, and analyzed their gene structures and protein domains to determine potential functional conservation and divergence at the sequence level. *In silico* RNA-seq analysis revealed potential candidate nutrient transporter genes that might function in specific tissue or organ for nutrient uptake, translocation and/or distribution. These studies represent the first effort in characterizing nutrient transporters in *Camelina*, and provide opportunities for future functional studies.

The expression of nutrient transporter genes in specific cell, tissue and in response to various nutrient stresses are under the control of transcriptional factors (TFs). In Arabidopsis, AtSTOP1, a zinc finger TF is known to be involved in different abiotic stress

response by modulating the transcript accumulation of different transporters. However, its involvement in low-K response have not been evaluated. Our study in Arabidopsis have revealed it is indeed involved, but the mechanism of its regulation is not understood. We isolated T-DNA insertion mutants of AtSTOP1 homolog in rice, named "*Sensitive to K starvation 1*" (*sks1*) based on its phenotype. RNA-seq analysis was performed in root and shoot tissues of wild type (WT) and *sks1* mutants under low-K and the control conditions to understand its transcriptional program in low-K response. We identified a large number of genes to be mis-regulated in the mutants that are potentially involved in signal transduction, ROS production, immune response, cell wall synthesis, ion transport and homeostasis. Several low-K-inducible genes in WT such as TFs and signaling genes were controlled by SKS1 in roots, whereas many metabolic enzymes in shoots. We additionally found that SKS1 might be involved in signal integrations between nutrients, and between roots and shoots in response to low-K stress.

While all these studies focus on gene expression of nutrient transporters and regulators at a tissue level, our understanding of the diverse transcriptional programs present in different cell types of a specific tissue is sparse. Recent advances in single-cell transcriptomics of Arabidopsis roots provided us with the opportunity to dissect the distribution of large sets of nutrient channels and transporters at a single cell resolution that work together to transfer nutrients from the soil to different cell-types of root cells and eventually reach vasculature for a massive flow. For this, we profiled the transcriptional patterns of putative nutrient transporters in different cell types of roots using the single-cell transcriptomics datasets from Arabidopsis root. Such analyses identified a number of NPK transporters expressed in the root epidermis to mediate NPK uptake and distribution to the adjacent cells. Some transport genes showed cortex- and endodermis-specific expression to direct the nutrient flow towards the vasculature. For long distance transport, a variety of transporters were shown to express and potentially function in the xylem and phloem. In the context of subcellular distribution of mineral nutrients, the NPK transporters at subcellular compartments were often found to show ubiquitous expression pattern, which suggests function in house-keeping processes. Overall, these single cell transcriptomic analyses provide a map of nutrient transport route from the epidermis across the cortex to the vasculature, which can be further tested experimentally in the future.

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Chapter 1: Introduction

Nutrients are essential for all living organisms for growth and survival. Nitrogen (N), phosphorus (P) and potassium (K) are the three major macronutrients that are needed in a large abundance, but their presence in natural soils are limited (Cordell et al., 2009; Maathuis, 2009; Luan et al., 2017). Fertilizer application has become a common practice to support the plant nutritional demands. While this has a positive impact on crop production, it also led to the rapid decline in the fertilizer-based natural resources and eutrophication. To support sustainable agriculture, researchers have been trying to decode the complex nutrient transport systems to engineer crops that can grow under minimal nutrient supplies. However, our current understanding of how nutrients are acquired from soil, transported and distributed between plant cells and tissues are mainly from the diploid model plant, *Arabidopsis thaliana*. A previous study using diverse Arabidopsis accessions indicated that polyploid Arabidopsis are better able to acquire nutrients and tolerate abiotic stresses compared to its diploid and haploid counterparts (Chao et al., 2013). However, studies of nutrient transport systems in polyploids are sparse.

Camelina sativa, a polyploid oilseed crop, is a close relative of Arabidopsis and has many attributes that contribute to the sustainable agriculture, including high tolerance to low-nutrient status (Kagale et al., 2014, 2016; Vollmann and Eynck, 2015; Malik et al., 2018). However, the mechanisms of its low-nutrient adaptability have not been elucidated at a genome level. For such understanding, identification and characterization of nutrient channels and transporters are the primary steps to dissect the intricate systems that contribute to low-nutrient adaptation. To initiate such studies, we leverage the publicly-available genome and developmental transcriptomic atlas of *Camelina (Kagale et al., 2014, 2016)*, which allowed us to examine the functional conservation and divergence of nutrient transporters at a sequence level and expression level in various tissues. Chapter 2 of this dissertation focuses on the comparative genomic and transcriptomic studies of P transporter families in *Camelina* and Arabidopsis, whereas Chapter 3 provides a comprehensive review on K channel and transporter families in angiosperms and expanded the study to *Camelina*.

The transcription of nutrient transporters at tissue- and cell-specific levels are controlled by transcriptional factors (TFs) in response to the changing nutrient status. However, not many TFs involved in low-K adaptation have been identified, even in Arabidopsis (reviewed in Chapter 2). AtSTOP1 (Sensitivity to proton rhizotoxicity) TF in Arabidopsis is shown to regulate signaling and transporter genes to combat various abiotic stresses (Sawaki et al., 2009; Sadhukhan et al., 2019; Ojeda-Rivera et al., 2020). It also modulates the expression of AtCIPK23 (CBL-Interacting Protein Kinase 23), one of the key regulators of K channel and transporter involved in K uptake under low-K stress. Through mutant analysis, our lab identified that indeed AtSTOP1 is also important for low-K adaptation. To understand its transcriptional program in response to low-K, we transitioned our study to diploid rice crop, and isolated the T-DNA insertional mutants of SKS1 (Sensitive to K Starvation 1), the homolog of AtSTOP1. In Chapter 4, RNA-sequencing was performed to unravel the candidate genes involved in low-K response in rice roots and shoots that are transcriptionally regulated by SKS1.

While different tissues have distinct transcriptional programs, the same is true for different cell types of a specific tissue. This is clearly indicated in recent breakthrough studies of Arabidopsis root single-cell transcriptomics (Jean-Baptiste et al.; Denyer et al., 2019; Ryu et al., 2019; Shulse et al., 2019; Zhang et al., 2019; Shahan et al., 2020). We took advantage of these studies to understand how NPK channels and transporters are transcriptionally distributed in different cell-types of the Arabidopsis root system. Chapter 5 maps the NPK transport routes from root epidermis to vasculature, initiated by diverse families of NPK channels and transporters, and Chapter 6 contains concluding remarks.

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Chapter 2: Genome-Wide Analysis of the Five Phosphate Transporter Families in *Camelina sativa* and Their Expressions in Response to Low-P

The following chapter (excluding protions of the preface) has been accepted for publication as a peer-reviewed research article in the Special Issue, “Ion Transport and Homeostasis in Plants” of *International Journal of Molecular Sciences*, and is reproduced here with permission.

Lhamo, D., Shao, Q., Tang, R., and Luan, S. (2020). Genome-Wide Analysis of the Five Phosphate Transporter Families in *Camelina sativa* and Their Expressions in Response to Low-P. *Int. J. Mol. Sci.* 21. doi:10.3390/ijms21218365.

Contributions

D. Lhamo designed and performed the bioinformatic analysis and experiments, and wrote the manuscript. R. Tang assisted in the experimental design and Q. Shao in repeating the experiments. S. Luan edited the manuscript.

Preface

P is one of the major macronutrients, acquired by plants in the form of inorganic phosphate (Pi). Arabidopsis contains five major families of phosphate transporters (PHTs) that play pivotal roles in phosphate (Pi) acquisition from the soil and distribution throughout a plant. While the current knowledge of Pi transport systems in Arabidopsis are well-established, their studies in crops are at infancy stages. Polyploid crops have been shown to have better tolerance to adverse environmental conditions because of their large genome sizes. One such crop is the hexaploid oilseed crop, *Camelina sativa* that can grow well under low-Pi supply. Although the *Camelina* genome was published in 2014, the low-Pi-responsive Pi transporters have not been identified and characterized. To initiate such studies, this chapter of the dissertation presents a comprehensive genomic analysis to identify all the Pi transporters belonging to the five major PHT families in *Camelina*, their chromosomal distributions, evolutionary relationships to Arabidopsis, conservation and divergence in gene structures, protein domains and *cis*-regulatory elements. In addition, *in silico* transcriptomic analysis reveals the putative functions of these Pi transporters in various tissues that cover the whole life cycle of *Camelina*. Moreover, the following chapter identifies low-Pi sensitive and tolerant *Camelina* accessions and examines the expression of Pi transporters in these accessions under low-Pi stress to identify the candidate transporters that may contribute to low-Pi adaptation.

INTRODUCTION

Phosphorus (P) is an essential macronutrient in all organisms, which provides a backbone to nucleic acids, a polarity to cell membranes, and participates in energy transfer and signal transduction processes (Nussaume, 2011; Gu et al., 2016). Plants acquire P in the form of inorganic phosphate (Pi), which is present at a very low level (<10 μM) in the soil due to its uneven distribution and low solubility (Luan et al., 2017; Wang et al., 2017). Therefore, application of fertilizers has become a common practice in agriculture to ensure crop productivity (Gu et al., 2016; Luan et al., 2017). Unfortunately, this has led to a rapid decline of non-renewable P-rock reserves that may last only 50-100 years if mined at the current pace (Nussaume, 2011; Luan et al., 2017; Wang et al., 2017). Heavy use of fertilizers not only threatens sustainable food production but also pollutes water resources, raising environmental concerns. To support sustainable agriculture and the environment, a widely accepted strategy is to minimize fertilizer use by breeding crops with higher P use efficiency (PUE). To achieve this goal, it is necessary to understand the molecular mechanism underlying Pi uptake from soil by roots, translocation from roots to the above-ground organs, storage into and remobilization from various subcellular compartments, and recycling from source (senescing leaves) to sink (young leaves, reproductive tissues and roots). All these processes require a specific set of transport proteins located in the plasma membrane of various plant cells and membranes of their subcellular compartments.

In plants, PHOSPHATE TRANSPORTERs (PHTs) are classified into five major families, PHT1-5, based on their sequences and subcellular localizations (Gu et al., 2016; Wang et al., 2017). The PHT1 family consists of H^+/Pi symporters located in the plasma membrane of root cells for Pi uptake from the soil and translocation to the shoots (Muchhal et al., 1996; Nussaume, 2011). It consists of nine members (AtPHT1;1-1;9) in *Arabidopsis*, among which AtPHT1;1 and AtPHT1;4 are the key players in Pi uptake (Misson et al., 2004; Shin et al., 2004). AtPHT1;8 and AtPHT1;9 are also shown to function in Pi uptake and translocation from root to shoot under low-P (Remy et al., 2012; Lapis-Gaza et al., 2014). AtPHT1;5 is involved in the redistribution of Pi from source to sink organs (Nagarajan et al., 2011).

The other PHT family transporters are found in different subcellular compartments and play important roles in Pi storage and remobilization. AtPHT2;1 is the only PHT2 member in *Arabidopsis* that cotransports H^+/Pi into chloroplast (Daram et al., 1999; Versaw and Harrison, 2002). The PHT3 family encodes three members in *Arabidopsis*, AtPHT3;1-3;3 (also referred as AtMPT1-3) that exchange Pi between the mitochondrial matrix and cytosol for ATP synthesis (Takabatake et al., 1999; Jia et al., 2015). Overexpression of *AtPHT3;1/AtMPT3* in *Arabidopsis* resulted in pleiotropic effect, suggesting a vital role of this gene in plant growth and development (Jia et al., 2015). Among the six members in the AtPHT4 family, AtPHT4;1 is involved in Pi and pH homeostasis in chloroplasts, and plant defense against pathogens (Wang et al., 2011;

Karlsson et al., 2015). AtPHT4;2 mediates Na^+-dependent Pi export from plastids in cells of sink organs (Irigoyen et al., 2011). AtPHT4;6 exports Pi from the *trans*-Golgi compartment to cytosol, essential for Golgi functions (Guo et al., 2008b; Cubero et al., 2009; Hassler et al., 2012, 2016). AtPHT5;1/AtVPT1 is located in the tonoplast to sequester excess Pi into vacuoles, and found to be essential for maintaining systemic Pi allocation for reproductive development along with AtPHT5;3/AtVPT3 (Liu et al., 2015, 2016c; Luan et al., 2019).

With increasing effort in whole-genome sequencing, genome-wide identification of PHT families has been conducted in a number of plant species, including rice, maize, barley, potato, tomato, soybean, foxtail millet, sorghum, wheat, poplar, apple, and rapeseed (Rae et al., 2003; Liu et al., 2011, 2016a, 2017; Fan et al., 2013; Ceasar et al., 2014; Chen et al., 2014; Zhang et al., 2016; Sun et al., 2017; Teng et al., 2017; Li et al., 2019; Wang et al., 2019; Yang et al., 2020). Such genomic analysis of nutrient transporters is not performed in *Camelina sativa* (false flax or gold-of-pleasure), an emerging oilseed crop for biofuel production. *Camelina* is an annual plant adapted to temperate growing regions, with centers of origin in southeastern Europe and southwestern Asia (Kagale et al., 2014; Vollmann and Eynck, 2015; Berti et al., 2016; Malik et al., 2018). It belongs to the Crucifer family (Brassicaceae) that is closely related to *Arabidopsis thaliana*, and more distantly to *Brassica napus* (Kagale et al., 2014). *Camelina* has attracted considerable interest as an oil crop, primarily due to its numerous valuable agronomic traits that support sustainable agriculture. For example, a short life cycle of approximately 85-100 days, low input costs and ease of transformation using a simple floral dip procedure similar to that used for *Arabidopsis*, make *Camelina* an attractive host for genetic and metabolic engineering (Kagale et al., 2014, 2016; Vollmann and Eynck, 2015; Malik et al., 2018). In addition, *Camelina* has high tolerance to adverse environmental conditions (e.g., drought and low-nutrient status), resistance to pests and pathogens, and high oil content with a fatty acid profile suitable for fuel purposes (Kagale et al., 2014, 2016; Vollmann and Eynck, 2015; Abdullah et al., 2016; Malik et al., 2018). Although *Camelina* has been shown to grow fairly well under P deficiency in field conditions (Solis et al., 2013; Mohammed et al., 2017), nothing is known on the genetic basis and gene networks enabling this plant to cope with low-P status. By utilizing the published genome sequence (Kagale et al., 2014), we combine a comprehensive genomic and transcriptomic analyses of CsPHT transporters with low-P response of two *Camelina* accessions to initiate functional studies on the Pi transport system in this crop species.

RESULTS

Identification and Phylogeny of the Five Major PHT Families

We identified 73 *CsPHT* genes in *Camelina* by blasting the coding sequences of known *Arabidopsis PHTs* against the *Camelina* genome. These genes belonged to the five PHT families, which include 33 *CsPHT1s*, 3 *CsPHT2s*, 9 *CsPHT3s*, 19 *CsPHT4s*, and 9 *CsPHT5s* (**Table S1**). The proteins encoded by these *CsPHT* genes consisted of 309 to 704 amino acids, with the molecular weight (Mw) between 34.3 to 78.8 kDa, and isoelectric point (pI) value between 5.2 to 9.8. The number of transmembrane domains (TMDs) in CsPHT proteins varied between 4 to 13.

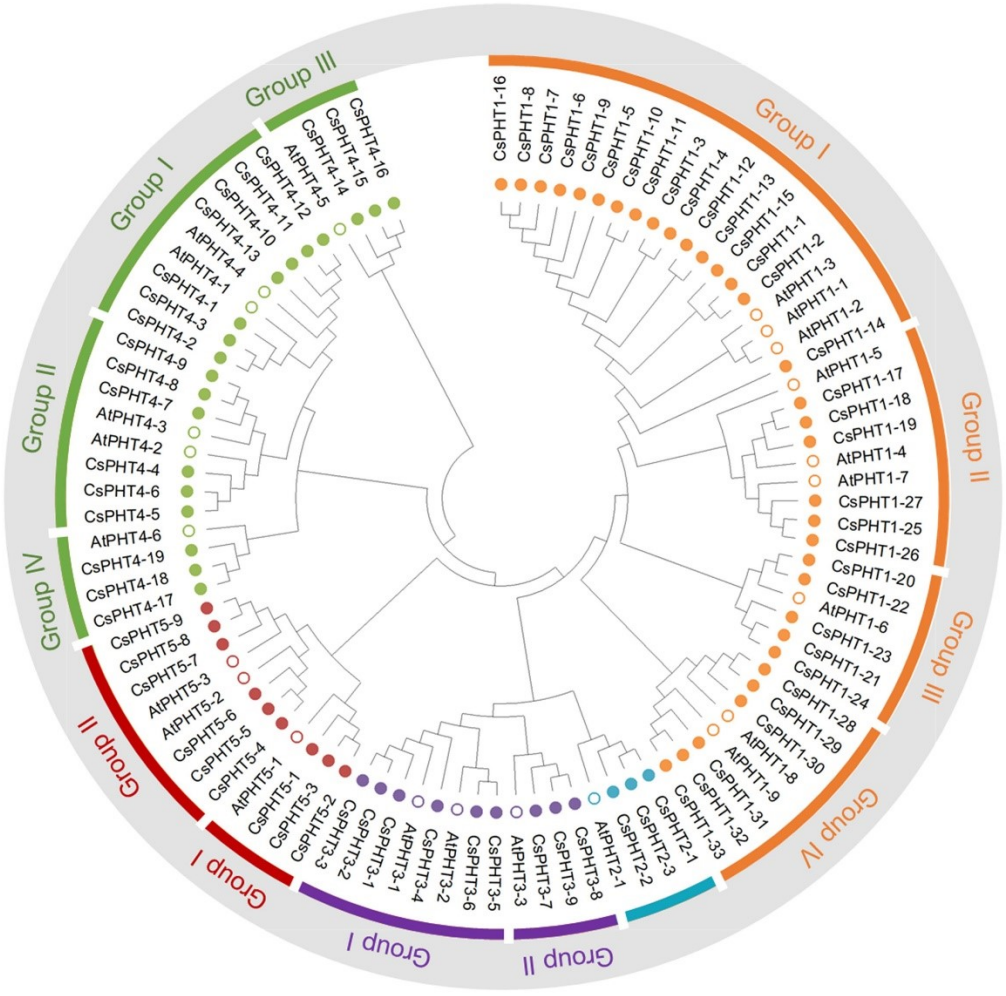

Figure 1. Evolutionary relationship between *Camelina* and *Arabidopsis* PHT transporters. The phylogenetic tree was constructed using neighbor-joining methods with the 1000 bootstraps in MEGA X program (Kumar et al., 2018). The PHTs of *Arabidopsis* are represented by open circles and *Camelina* by closed circles. Different PHT families are color-coded.

To investigate the evolutionary relationship between 73 *Camelina* and 22 *Arabidopsis* PHTs, their protein sequences were aligned to generate a phylogenetic tree using neighbor-joining method in MEGA X (Kumar et al., 2018) (**Figure 1**). Our results indicated that PHT1 family proteins were divided into four major groups, with AtPHT1;1/1;2/1;3 clustered in Group I along with 16 CsPHT1s. Group II and IV each contained 6 CsPHT1s corresponding to their close homologous pairs, AtPHT1;4/1;7 and AtPHT1;8/1;9, respectively. While AtPHT1;5 was categorized into Group II, the *Camelina* genome appeared to lack homoeologs in this group. AtPHT1;6 along with 5 CsPHT1s were classified into Group III. The PHT2 family contained one AtPHT2;1 and three CsPHT2 members. Two distinct groups were found for the CsPHT3 family, with AtPHT3;1/3;2 and 6 CsPHT3 members in Group I, and AtPHT3;3 and 3 CsPHT3s in Group II. CsPHT4 family proteins formed four separate groups, with 7 CsPHT4s corresponding to AtPHT4;1/4;4 homologous pairs in Group I, 6 CsPHT4s with AtPHT4;2/4;3 in Group II, and 3 CsPHT4s with AtPHT4;5 and AtPHT4;6 in Group III and IV, respectively. CsPHT5 family consisted of two distinct groups, AtPHT5;1 along with 3 CsPHT5s in Group I, and AtPHT5;2/5;3 with 6 CsPHT5s in Group II.

Chromosomal Distribution and Duplication of CsPHT Genes

CsPHT genes were mapped to *Camelina* chromosomes based on their genomic locations using the MapDraw program (Liu and Meng, 2003). Subgenome G1 and G3 each contained 25 *CsPHT* genes, and Subgenome G2 harbored 23 *CsPHT* genes (**Figure 2**). This difference resulted from fewer *CsPHT1* genes in Subgenome G2 as compared to Subgenome G1 and G3. Among the twenty chromosomes, *CsPHT1* genes were largely clustered on Chromosome 11, 18 and 20. *CsPHT2* and *CsPHT5* family genes were equally distributed across the three subgenomes. *CsPHT3* family genes were dispersed unevenly among the three subgenomes, with 4 *CsPHT3s* in Subgenome G2, 3 in Subgenome G1, and 2 in Subgenome G3. While *CsPHT4* family genes were equally distributed between Subgenome G1 and G2 with six genes each, one additional gene was found in Subgenome G3.

Camelina has undergone whole-genome triplication event that retained triplet copies (homoeologs) for each *Arabidopsis* ortholog, with the majority of CsPHT families containing three homoeologous genes for each *AtPHT* gene (Kagale et al., 2014) (**Figure 1** and **Table S1**). Among CsPHT families, the largest expansion beyond the ploidy norm was observed in CsPHT1 family. As a hexaploid, *Camelina* was expected to have 27 *CsPHT1* genes that correspond to 9 *AtPHT* genes. However, we identified 33 *CsPHT1* genes, although no homoeologous genes were found for *AtPHT1;5*. The further expansion in CsPHT1 family largely resulted from tandem duplication events (**Figure 2** and **Figure S1**). The presence of two or more homologous genes within the 200 kb region of chromosome is considered tandem duplicated genes (Liu et al., 2016b; Li et al., 2019).

Close examination of *Camelina* genome revealed five tandem duplicated regions on Chromosome 11, 18 and 20 (**Figure S1**).

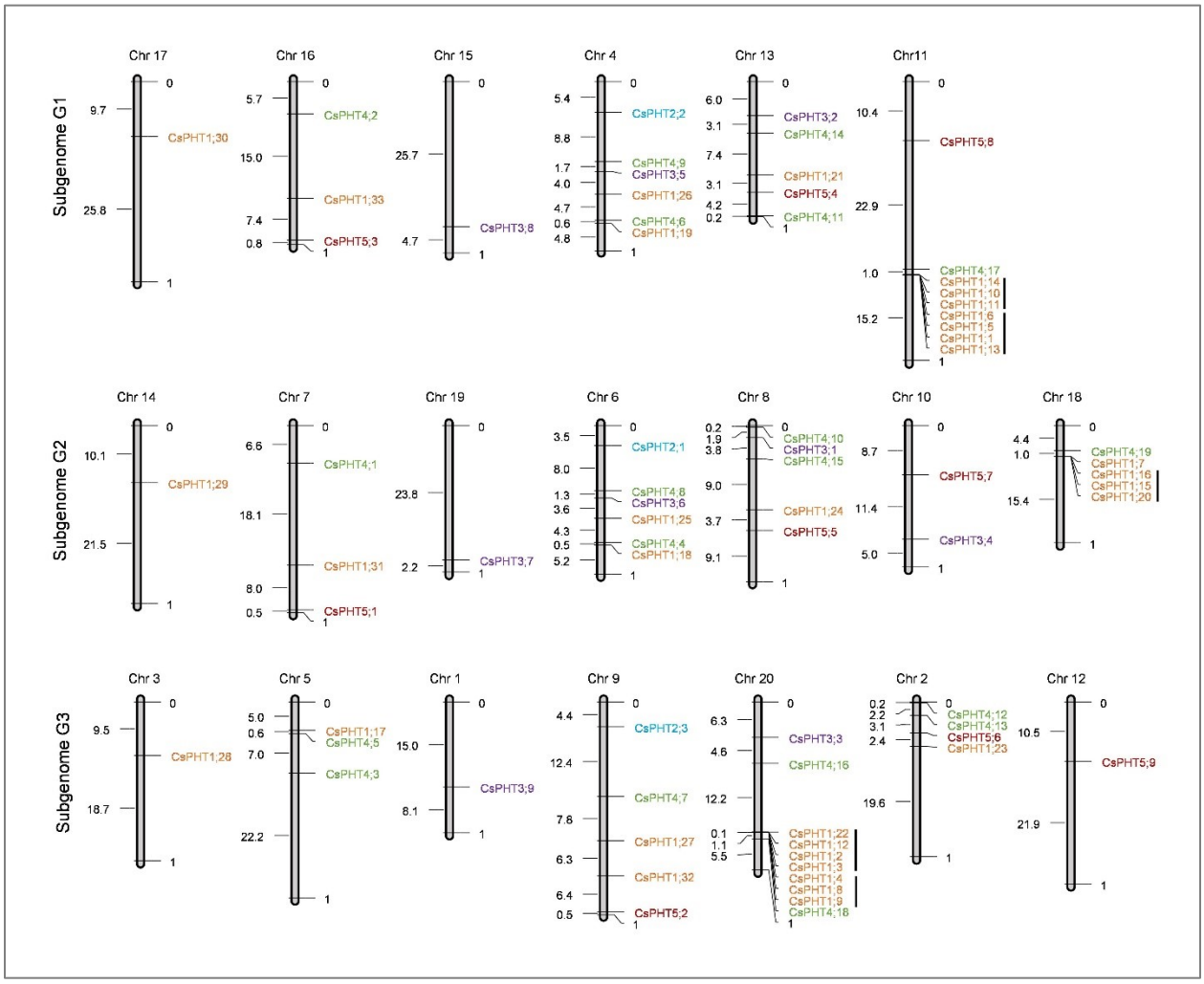

Figure 2. Chromosomal locations of *CsPHT* genes in the three sub-genomes. The genes were mapped using the MapDraw program (Liu and Meng, 2003). Black lines next to the clustered genes represent tandem duplication events.

Gene Structure and Protein Domain Analyses of CsPHT Members

To inspect the structural diversity among CsPHT families, gene structures were built using the *Camelina* genome annotation file in Tbtools (Chen et al., 2020). Noticeably, the exon number and organization were more conserved within the same family, with 1-2 exons in the majority of *CsPHT1* genes, 3 exons in *CsPHT2s* and 6 exons in *CsPHT3s* (**Figure 3A**). However, the intron lengths within *CsPHT1/3* family genes varied between different groups, which might alter their encoding protein sequences and functions (Choudhuri, 2014). *CsPHT4* family genes showed the most diverse gene structures, with exons ranging broadly between 1 to 15. Among these, *CsPHT4* genes in Group IV shared

highly conserved gene structures. *CsPHT5* family genes featured 9-16 exons, with Group II displaying a higher conservation in intron/exon organizations.

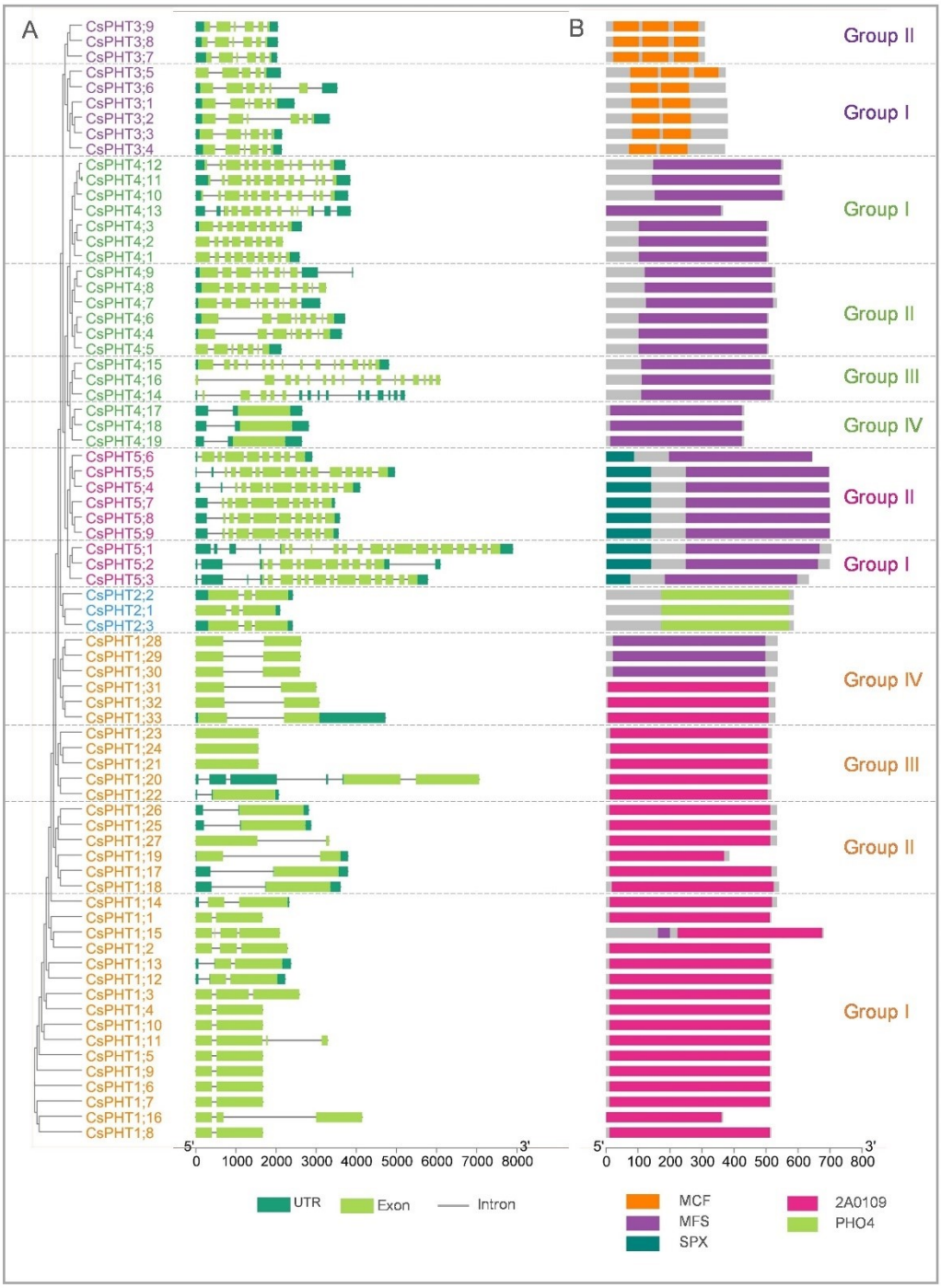

Figure 3. (A) Gene structures and (B) Conserved protein domains of CsPHT members. The intron/exon structures were built using *Camelina* genome annotation file (Kagale et al., 2014). The protein domains were searched in NCBI-CDD database (Marchler-Bauer et al., 2015). The graphics were generated using TBtools (Chen et al., 2020).

To find conserved domains within CsPHT family proteins, we scanned their protein sequences in NCBI's Conserved Domain Database (Marchler-Bauer et al., 2015). In general, some domains were specifically conserved within the same CsPHT family, while others were conserved across multiple CsPHT family proteins (**Figure 3B**). For instance, CsPHT1/4/5 family proteins all possessed conserved MFS or 2A0109 domain, which were found in Major Facilitator Superfamily proteins that transport Pi and other ions/substrates across plasma membrane and internal compartments including plastids, Golgi and vacuoles in *Arabidopsis* (Cubero et al., 2009; Karlsson et al., 2015; Liu et al., 2015, 2016c; Hassler et al., 2016; Niño-González et al., 2019; Xu et al., 2019). On the other, PHO4 (PHOSPHATE4) domain was specifically conserved in CsPHT2 family proteins, similar to their *Arabidopsis* ortholog, AtPHT2;1 that transports Pi into chloroplasts (Daram et al., 1999; Versaw and Harrison, 2002). CsPHT5 family proteins contained highly conserved SPX (SYG1/PHO81/XPR1)-MFS domains, which were specifically found on vacuolar Pi-transporters in *Arabidopsis* and rice to maintain Pi homeostasis (Wang et al., 2012, 2015; Liu et al., 2015, 2016c; Xu et al., 2019). MCF (Mitochondrial Carrier Family) domains were uniquely conserved in CsPHT3 family proteins, implying their roles as Pi-transporters in mitochondria (Takabatake et al., 1999; Rausch and Bucher, 2002; Jia et al., 2015). However, Group I of the CsPHT3 family contained two MCF domains while three were found in Group II, suggesting a possible functional divergence between these two groups in mitochondria.

Cis-Regulatory Elements of CsPHT Genes

Using 2-kb upstream regions of fully-sequenced *CsPHT* family genes (65/73) as queries, we searched the PLACE database (Higo et al., 1999) and found 231 putative *cis*-regulatory elements (**Figure S2**). These *cis*-elements are known to govern gene expression in specific tissues (e.g., pollen, seed, root, flower), or in response to specific conditions, such as defense, phosphate, nitrogen, sugar, dehydration, energy, light, and other abiotic stresses (Liu et al., 2011; Gu et al., 2016; Zhang et al., 2016). Particularly relevant are those Pi starvation-related *cis*-elements, including P1BS, W-box, PHO-like, helix-loop-helix (HLH) and TATA-box-like elements (**Figure 4**). Among these, P1BS element was found in 40 of the 65 *CsPHT* genes examined. Most of the *CsPHT1* family genes (28/31) contained P1BS, suggesting these genes can be transcriptionally induced by PHR1 (PHOSPHATE RESPONSE1) transcriptional factor (TF) in response to low-P for efficient Pi uptake (Rubio et al., 2001; Bustos et al., 2010; Castrillo et al., 2017). In addition, all *CsPHT1* genes contained high frequency of several W-box elements, suggesting their expressions might be regulated by multiple WRKY TFs to modulate Pi uptake in response to the changing P status (Devaiah et al., 2007; Wang et al., 2014a; Su et al., 2015). Similarly, the other *CsPHT* family genes might be transcriptionally regulated by WRKYs to mediate Pi distribution, as they also harbored high frequency of those W-box elements. Several PHO-like, TATA-box-like (TATABOX2) and HLH (CATATGGMSAUR) elements were present in the majority of *CsPHT* family genes except *CsPHT3s*. These *cis*-elements were implicated to mediate low-P responsive expression,

although not experimentally validated (Mukatira et al., 2001; Hammond et al., 2003; Gu et al., 2016).

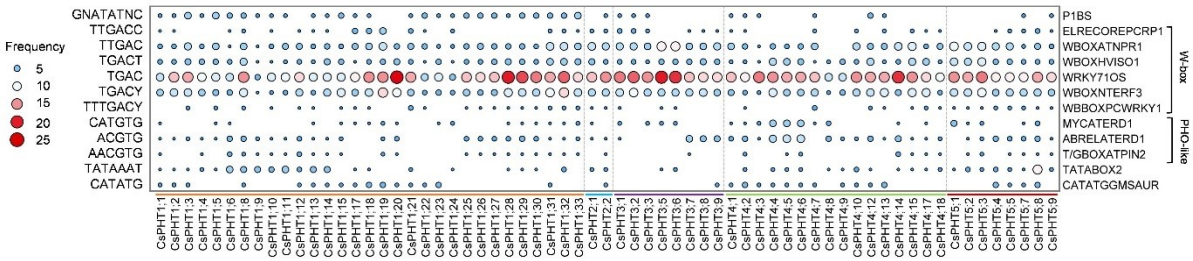

Figure 4. Frequency of Pi-starvation-related *cis*-elements in the 2-kb promoter region of *CsPHT* genes. The *cis*-elements were identified using New PLACE database (Higo et al., 1999) and the dotplot was generated using TBtools (Chen et al., 2020).

Expression Pattern of CsPHT Genes in Multiple Tissues

To investigate the expression pattern of *CsPHT* genes, we analyzed transcriptomic datasets generated for 12 different tissues covering four developmental stages (Kagale et al., 2016). The expression data for sixty out of seventy-three *CsPHT* family genes were analyzed (**Figure 5**). Because the datasets were acquired under normal growth conditions, the majority of *CsPHT1* family genes were not expressed, consistent with the presence of low-P responsive *cis*-elements in the promoter sequences of these genes. Nevertheless, we found both conservation and divergence in expression patterns among the three close homoeologous genes for each *Arabidopsis* ortholog. For instance, *CsPHT1:17-1;19*, the three homoeologs for *AtPHT1;4,* displayed similar expression patterns in germinating seed, senescing leaf, flower and seed development. In contrast, *CsPHT1;31 -1;33*, homoeologs for *AtPHT1;9,* were expressed similarly in senescing leaf, but differed sharply in stem, flower buds and seeds. In addition, *CsPHT1;27* corresponding to *AtPHT1;7* displayed a dominant expression during early and vegetative stages, while no expression was detected for the other two close homoeologs.

The three *CsPHT2* genes were exclusively expressed in leaves during early and vegetative stages. The closely-related *CsPHT3;1-3;3* genes of *AtPHT3;1* shared tissue-specific expression in germinating seed, whereas *CsPHT3;7-3;9*, homoeologs for *AtPHT3;3*, were more broadly expressed in cotyledons and reproductive organs. Most of the *CsPHT4* family genes including *CsPHT4;1-4;12* were expressed in leaves, but only *CsPHT4;1-4;3,* close homoeologs for *AtPHT4;1,* showed preferential expression in senescing leaves. *CsPHT4;1, -4;3, -4;4* and *-4;6* were additionally expressed at low level in flower, and *CsPHT4;4-4;6* and *4;9-4;12* in early seed development. Division in tissue expressions were observed in *CsPHT5* family genes. For example, only *CsPHT5;1-5;3*, close homoeologs for *AtPHT5;1/AtVPT1,* were expressed in root and stem during vegetative growth, with *CsPHT5;3* showing a ubiquitous and dominant expression. On the other, rest of the *CsPHT5* genes except *CsPHT5;5* and *-5;7*, were expressed during later stages of the seed development, with *CsPHT5;4* exhibiting a broader expression.

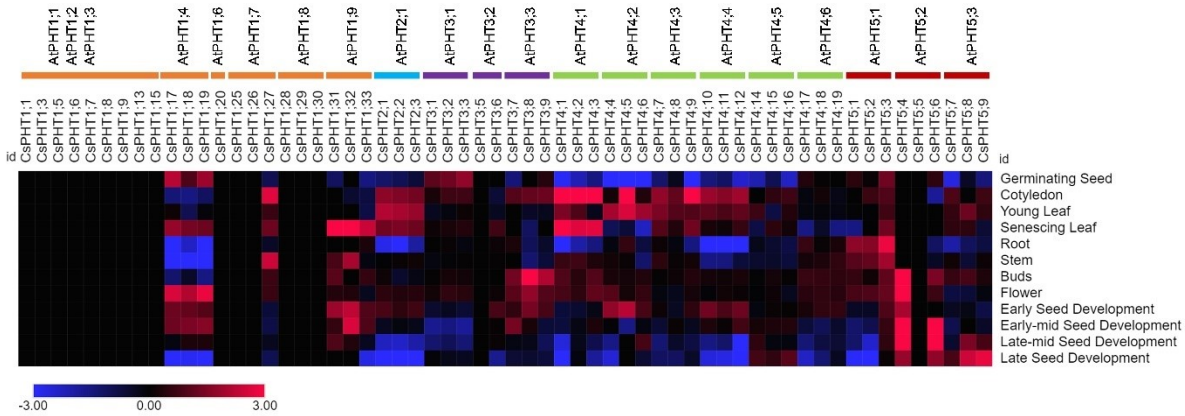

Figure 5. Expression pattern of *CsPHT* genes in 12 different tissues covering four developmental stages. The *Arabidopsis* PHT orthologs were shown on the top. The RNA-seq data (Log2 Ratio) were extracted from *Camelina* eFP browser (http://bar.utoronto.ca/efp_camelina/cgi-bin/efpWeb.cgi) (Kagale et al., 2016), and the heatmap was generated using MORPHEUS program (https://software.broadinstitute.org/morpheus/).

Camelina's Phenotype and Expression Analysis of CsPHT Genes in Response to Low-P

To examine low-P response in *Camelina*, we grew two different accessions under low-P (-Pi) and the control (CK) conditions (**Figure 6**). Phenotypically, Suneson displayed a high tolerance to low-P, whereas CS-CROO was highly sensitive (**Figure 6A**). Suneson retained ~70% of shoot biomass under low-P relative to the control condition, whereas CS-CROO only maintained ~30% (**Figure 6B**). In addition, Suneson accumulated a significantly higher root biomass under low-P compared to the control, whereas no difference was found for CS-CROO (**Figure 6C**).

To understand the genetic basis underlying low-P response of the two *Camelina* accessions, we analyzed the expression patterns of 15 genes from the five CsPHT families under low-P (-Pi) and the control (CK) conditions using quantitative Real-Time PCR (**Figure 7**). The high sequence homology between the closely-related genes and their homoeologous/paralogous genes posed a challenge in designing gene-specific primers, thus the expression patterns of only a subset of *CsPHT* genes were analyzed. In response to low-P, the majority of *CsPHT1* genes analyzed were highly upregulated in both shoots and roots of *Camelina* (**Figure 7A** and **7B**). Interestingly, a number of these genes were expressed at a higher level in the low-P-tolerant Suneson as compared to the low-P-sensitive CS-CROO, including *CsPHT1;1*, *-1;12* and *-1;31* in shoots, and *CsPHT1;12* and *-1;31* in roots. In addition, several *CsPHT4* family genes were upregulated at a slightly higher level in Suneson than CS-CROO under low-P, including *CsPHT4;1*, *-4;6* and *-4;15* in shoots, and *CsPHT4;1* and *-4;6* in roots. Suneson also exhibited slightly higher expression of *CsPHT3;7* and *-5;8* in shoots, and *CsPHT5;1* in

roots compared to CS-CROO under low-P. These genes might contribute to the better Pi uptake and distribution capabilities of Suneson in response to low-P.

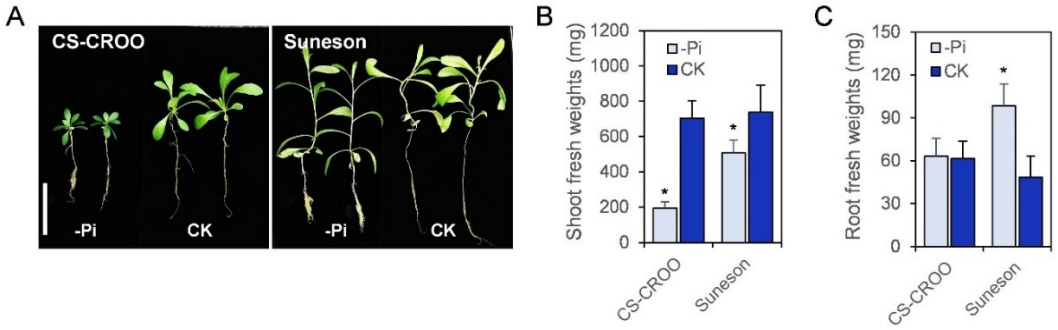

Figure 6. (A) Phenotype, (B) Shoot biomass, and (C) Root biomass of the low-P-sensitive CS-CROO and the low-P-tolerant Suneson grown under low-P (-Pi, 10 µM) and the control (CK, 312 µM) conditions for four weeks. White scale bar represents 5 cm. The biomass data represents the mean ± SD of one of the three experiments (n=10). Asterisk indicates a significant difference (*p < 0.0005) between -Pi and CK conditions.

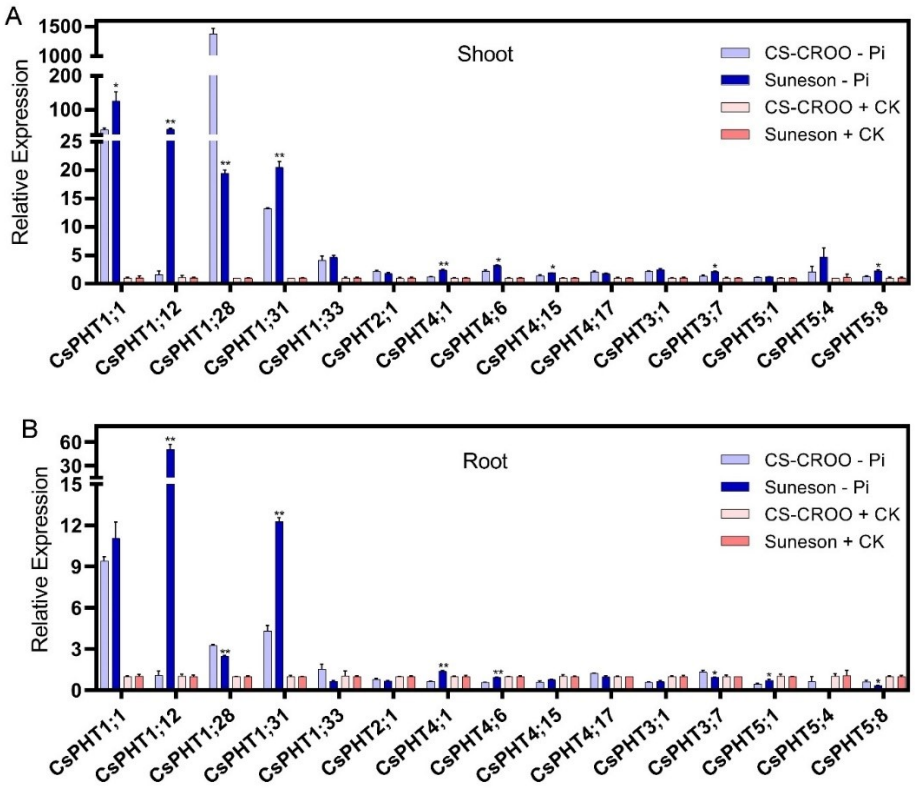

Figure 7. Expression analysis of 15 selected *CsPHT* genes in (A) Shoot and (B) Root of the low-P-sensitive CS-CROO and the low-P-tolerant Suneson treated under low-P (-Pi, 10 µM) and the control (CK, 312 µM) conditions for four weeks, as revealed by qRT-PCR. The mean ± SD of three biological replicates is presented. Asterisks indicate a significant difference (*p < 0.01 and ** p < 0.001) in gene expression between two *Camelina* accessions after Pi treatments.

DISCUSSION

Expansion and Structural Conservation in CsPHT Families

Camelina is an oilseed crop that is capable of growing on marginal lands with modest input of fertilizers (and other resources), thus serving as a strong candidate crop that may utilize non-agriculture lands for fuel purposes (Kagale et al., 2014, 2016; Abdullah et al., 2016; Berti et al., 2016). However, *Camelina's* response to low-nutrient status has not been studied at a genome level. In this study, we identified 73 genes encoding CsPHT transporters in the *Camelina* genome and analyzed their expression patterns in various tissues and in response to low-P, providing a road map to understand the transport and utilization of Pi in this promising oil crop.

The vast expansion of CsPHT families in *Camelina* mainly resulted from the whole-genome triplication event that occurred 17 million years ago (Kagale et al., 2014). In addition, gene duplications further expanded these CsPHT families. In particular, these events drove major evolutionary changes in the composition of CsPHT1 family. Although there is an overall expansion of genes beyond the ploidy norm (33 vs. 27), a specific loss of all homoeologs for *AtPHT1;5* was identified, presenting an interesting point for functional analysis in the future. Tandem gene duplication events were the major driving force for the expansion of these *CsPHT1* genes on Chromosome 11, 18 and 20. These tandem duplicated genes largely correspond to *AtPHT1;1/1;2/1;3* orthologs, which are functionally redundant genes that function as key players in Pi uptake (Shin et al., 2004; Ayadi et al., 2015). The significant expansion of this gene family in *Camelina* might contribute to the robust Pi acquisition and low-P tolerance in this polyploidy plant.

The three CsPHT2 members might share similar functions to their ortholog, AtPHT2;1 as H^+/Pi symporters since their gene structures, protein sizes and PHO4 domain were highly conserved (Daram et al., 1999; Versaw and Harrison, 2002). *Camelina* has nine CsPHT3 members with MCF domains, which are also found in AtPHT3 transporters that affect mitochondrial respiration processes (Takabatake et al., 1999; Rausch and Bucher, 2002; Jia et al., 2015). However, CsPHT3s in Group I differ in exon/intron organizations with two MCF domains, while Group II contain highly conserved gene structures with three MCF domains. Transport activity analysis of these two diverging groups might provide clues to the significance of this domain for Pi transport mechanism in mitochondria.

Camelina has 19 CsPHT4 members clustered largely in Group I and II with diverse gene structures, although all display conserved MFS domains. These suggest CsPHT4 members might have wide-range of functions as observed in *Arabidopsis* that transport Pi and other solutes such as ascorbate in different subcellular compartments, and are involved in plant defense and salt tolerance (Cubero et al., 2009; Wang et al., 2011; Hassler et al., 2012, 2016; Karlsson et al., 2015). A nine CsPHT5 members are found in *Camelina* with conserved SPX-MFS domains that are also found on vacuolar Pi-

transporters in *Arabidopsis* and rice that control vacuolar Pi fluxes (Wang et al., 2012, 2015; Liu et al., 2015, 2016c; Xu et al., 2019). For instance, Pi influx is mediated by *Arabidopsis* AtPHT5;1/AtVPT1, and rice OsSPX-MFS1 and OsSPX-MFS3, whereas Pi efflux from vacuoles is mediated by VPEs in rice and *Arabidopsis* (Wang et al., 2012; Liu et al., 2015, 2016c; Xu et al., 2019). In addition, these SPX-MFS proteins might have Pi-sensing abilities (Wild et al., 2016), which is worth further investigation.

CsPHT Gene Expressions in Tissues and in Response to Low-P

Understanding when and where a gene is expressed could provide valuable information about its role in plant growth and development. Therefore, we analyzed the expression pattern of *CsPHT* genes in various plant tissues of *Camelina* DH55 genotype (Kagale et al., 2016). In general, the *CsPHT1* genes displayed a broad expression in multiple tissues, implying their roles in Pi transport across various tissues throughout plant growth and development. However, these genes were not expressed in roots under the normal growth condition. This might be because a large number of CsPHT1 transporters are encoded in *Camelina*, therefore, a basal expression level in roots might be adequate to efficiently uptake Pi under sufficient-P condition. Nevertheless, these *CsPHT1* genes were transcriptionally induced under low-P, probably because these genes harbor P1BS (PHR1 binding site) and several W-box (WRKY binding site) elements on their promoter regions. AtPHR1 and AtWRKY45/75 in *Arabidopsis*, and OsPHR2 and OsWRKY74 in rice are shown to bind to those *cis*-elements to promote efficient Pi acquisition and translocation especially under low-P stress (Rubio et al., 2001; Devaiah et al., 2007; Zhou et al., 2008; Bustos et al., 2010; Liu et al., 2010; Wang et al., 2014a; Dai et al., 2016; Castrillo et al., 2017). These Pi transport processes are mediated by *AtPHT1;1, -1;4, -1;8* and *-1;9* in *Arabidopsis*, and *OsPHT1;2, -1;3, -1;9* and *-1;10* in rice (Misson et al., 2004; Shin et al., 2004; Ai et al., 2009; Remy et al., 2012; Lapis-Gaza et al., 2014; Wang et al., 2014b; Chang et al., 2019).

The *CsPHT2* genes displayed tissue-specific expression in leaves, similar to *Arabidopsis* and rice, suggesting their conserved roles in Pi-transport into chloroplasts (Daram et al., 1999; Ferro et al., 2002; Versaw and Harrison, 2002; Liu et al., 2020). *CsPHT3;7-3;9* and their ortholog, *AtPHT3;3* showed similar expression patterns in floral organs (Zhu et al., 2012), indicating their importance in reproductive development. However, *CsPHT3;1-3;3* genes exhibited tissue-specific expression in germinating seeds, in contrast to their orthologous gene, *AtPHT3;1* that showed ubiquitous expression (Zhu et al., 2012). This might infer a neofunctionalization of these *CsPHT3* genes in seed germination. The PHT3 family is the least studied among the five PHT families in *Arabidopsis* due to the lethality of homozygous mutants (Jia et al., 2015). Thus, it would be interesting to decipher their physiological roles in *Camelina* that contains multiple

copies, and knocking out one or more homoeologous genes might not pose such a challenge.

Many *CsPHT4* genes were highly expressed in leaves, similar to their orthologous genes in *Arabidopsis*, suggesting their putative roles in Pi transport into chloroplast as observed for AtPHT4;1 (Guo et al., 2008a, 2008b; Karlsson et al., 2015). However, the dominant expression of *CsPHT4;1-4;3* in senescing leaves confers their additional roles in source to sink Pi transport, as observed for AtPHT4;6 (Hassler et al., 2016). Intriguingly, the majority of *CsPHT5* genes were expressed in flowers and seeds, and the significance of these vacuolar transporters in maintaining Pi homeostasis during reproductive development has recently been confirmed in *Arabidopsis* (Luan et al., 2019). Among the *CsPHT5* family genes, *CsPHT5;3* showed a dominant expression from early to vegetative growth, whereas *CsPHT5;4* from flower to seed development. Thus, the functional studies of these two genes as key vacuolar Pi-transporters are worth further investigation.

To understand *Camelina's* response to low-P at a genetic level, we compared the growth and gene expression of two *Camelina* accessions under low-P and the control conditions. Our results indicated Suneson as the low-P-tolerant genotype, and CS-CROO as the low-P-sensitive genotype. Suneson might have a better Pi utilization compared to CS-CROO based on the gene expression analysis. For example, the high expression of *CsPHT1;12* and *-1;31* genes in roots of Suneson under low-P suggests a better Pi-uptake capability than CS-CROO, as demonstrated by the roles of these *PHT1* genes in *Arabidopsis* (Shin et al., 2004; Remy et al., 2012). The other *CsPHT* family genes are likely involved in Pi distribution involving Pi remobilization from subcellular compartments and Pi recycling from source to sink in order to maintain cellular Pi homeostasis. For example, the higher expression of *CsPHT4;6* in shoots and roots of Suneson implies a better Pi remobilization as illustrated by the function of its *Arabidopsis* ortholog, *AtPHT4;2* in exporting Pi from plastids (Irigoyen et al., 2011) In addition, *CsPHT4;1* was expressed at a higher level in shoots of Suneson than CS-CROO, and its tissue-specific expression in senescing leaves infers the potential role of this gene in recycling Pi from older to younger leaves under low-P. Similarly, although *CsPHT1;1*, *-1;12* and *-1;31* genes are putative Pi-uptake genes, their higher expressions in shoots of Suneson suggest their additional functions in distributing Pi in leaves, which remain unexplored. In *Arabidopsis*, AtPHT1;5 was shown to be involved in this process (Nagarajan et al., 2011), although no homoeologs corresponding to this gene were found in *Camelina*. Thus, other CsPHT1 members along with CsPHT4s might compensate for the loss. Moreover, the higher expression of *CsPHT5;8* in shoots, and *CsPHT5;1* in roots of Suneson might maintain vacuolar Pi homeostasis better than CS-CROO under low-P (Liu et al., 2015, 2016c; Luan et al., 2019). Additionally, the higher expression of *CsPHT3;7* in shoots of Suneson might be important for respiration processes in mitochondria as observed in *Arabidopsis* (Takabatake et al., 1999; Rausch and Bucher, 2002; Jia et al., 2015).

Further expression analysis is required as we only covered a subset (15/73) of *CsPHT* genes for which we could design gene-specific primers. Global transcriptomic analysis of low-P-tolerant and -sensitive genotypes coupled with GWAS analysis of large *Camelina* varieties under low-P will aid in dissection of the gene-networks involving PHTs, TFs and signaling components that contribute to the higher PUE in *Camelina* such as Suneson. In addition, this global transcript analysis will provide clues to the functional dominance of one homoeolog over the others in response to low-P stress. Further genetic, molecular, biochemical and electrophysiological analyses of the candidate CsPHT transporters will elucidate the biological significance and function of these transporters in Pi acquisition and distribution. These approaches are critical for future breeding efforts to engineer crops with high PUE for sustainable agriculture.

MATERIAL AND METHODS

Genome-wide Identification of CsPHT Families in Camelina

To identify the candidate CsPHT members, the coding sequences of all known AtPHT members of *Arabidopsis thaliana* were individually blasted against *Camelina sativa* database in the National Center for Biotechnology Information (NCBI, https://blast.ncbi.nlm.nih.gov/). The coding sequences of AtPHT members were obtained from The *Arabidopsis* Information Resource (TAIR10) database (https://www.arabidopsis.org/) (Berardini et al., 2015). The choice of candidate PHTs was based on the E-value over $1e^{-10}$ and query over 60%. Various splicing variants of a gene and incomplete genes with short length were discarded. The CsPHT chromosomal locations and their genomic DNA were obtained from NCBI. The coding sequences and deduced protein sequences were retrieved from the Kyoto Encyclopedia of Genes and Genomes/KEGG Genome: *Camelina sativa* (https://www.genome.jp/). The Locus IDs were obtained from “BLAST Database” in The Prairie Gold Novel Industrial Oilseed Crops for Canada Database (http://Camelinadb.ca/). The properties of putative PHT proteins such as number of amino acids, molecular weights (Mw) and isoelectric Point (pI) were calculated using ProtParam (http://web.expasy.org/protparam/). Transmembrane domains (TMDs) were predicted using TMpred (http://www.ch.embnet.org/software/TMPRED_form.html), with a score above 500 considered as significant.

Phylogenetic Analysis and Chromosome Mapping

Multiple amino acid alignment of the CsPHT proteins of *Camelina* and *Arabidopsis* was generated using ClustalW. The *Arabidopsis* AtPHT protein sequences were obtained from TAIR10 database (https://www.arabidopsis.org/) (Berardini et al., 2015).

Phylogenetic tree was constructed using the neighbor-joining method with the 1000 bootstrap in MEGA version X program (Kumar et al., 2018). The chromosomal locations of *CsPHT* genes were mapped using the MapDraw program (Liu and Meng, 2003). Tandem duplicated genes were identified in *EnsemblPlants* (http://plants.ensembl.org/Camelina_sativa/Info/Index) by close examination of the chromosomal regions of all clustered genes found on the three subgenomes (**Figure S1**). Homologous genes in close proximity, (<200 kb) were considered tandem duplicated genes (Liu et al., 2016b; Li et al., 2019).

Gene Structure and Protein Domain Analyses

The gene structures were generated using *Camelina* genome annotation GFF file (downloaded from http://camelinadb.ca/) (Kagale et al., 2014). The conserved protein domains were identified by scanning the protein sequences in the NCBI Conserved Domain Database (NCBI-CDD) search (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) (Marchler-Bauer et al., 2015). The graphics of both gene structures and conserved protein domains were created using TBtools (Chen et al., 2020).

Cis-Regulatory Element Identification

Promoter sequences located 2-kb upstream of the transcription start sites (ATG codons) of *CsPHT* genes were downloaded from NCBI (https://blast.ncbi.nlm.nih.gov/). The multiple promoter sequences of *CsPHT* genes were scanned in New PLACE (https://www.dna.affrc.go.jp/PLACE/?action=newplace) (Higo et al., 1999) to identify the abundance of 231 *cis*-regulatory elements (**Figure S2**). The subset of data representing Pi-starvation-related *cis* elements was plotted using TBtools (Chen et al., 2020).

In-silico Expression Analysis of CsPHT Genes

The RNA-Seq data (Log2 Ratio) of *CsPHT* genes across 12 different tissues covering 4 developmental stages of *Camelina* life cycle were extracted from *Camelina* eFP browser (http://bar.utoronto.ca/efp_camelina/cgi-bin/efpWeb.cgi) (Kagale et al., 2016). The developmental stages included early growth (germinating seed and cotyledon), vegetative growth (young leaf, root, stem and senescing leaf), flower development (buds and flower) and seed development (early, early-mid, late-mid and late). The heatmap of gene expressions was generated using the MORPHEUS program (https://software.broadinstitute.org/morpheus/).

Plant Materials and Growth Conditions

The sources of seeds for the two *Camelina* accessions, CS-CROO and Suneson, are listed in **Table S2**. The seeds were sterilized in 20% bleach, and washed three times with water. The sterilized seeds were stratified in water at 4 °C for 15 days before plating on the medium. Seeds were germinated on ¼ Murashige and Skoog (MS) Medium (Ref: MSP09-100LT, Caisson Labs, Smithfield, UT, USA) with 1% sucrose, 0.8% Phytoblend for 5 days, then seedlings were transferred to hydroponic solution containing ¼ MS for 9 days. The two-weeks-old plants were divided into 2 groups, and grown in low-P (-Pi, 10 μM) and the control (CK, 312 μM) hydroponic culture for 4 weeks. The plants were grown in a greenhouse with 12h/12h of light/dark cycle at a temperature between 18°C - 22°C. Shoot and root tissues were then separately collected to measure fresh weights. The experiment was repeated three times, with each experiment composed of 5-10 plants per accession for each treatment. The mean and standard deviation (SD) of one representative experiment containing 10 plants per accession for each treatment is presented. The Student's *t* test was used to compare biomass between -Pi and CK conditions for each accession.

RNA Isolation and cDNA Synthesis

Shoot and root tissues were separated, and quickly frozen in liquid nitrogen. Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). After treatment with DNase I (New England Biolabs, Ipswich, MA, USA), 2 μg of total RNA was subjected to reverse transcription reaction using the M-MLV Reverse Transcriptase (Promega, San Luis Obispo, CA, USA) at 42 °C for 1 h. The resulting cDNA was used for PCR amplification with the gene-specific primers. qRT-PCR analysis was performed using the CFX96^{TM} Real-Time System (Bio-Rad, Hercules, CA, USA). Each PCR mixture contained 10 μL PowerUp^{TM} SYBR Green Master Mix (Bio-Rad, Hercules, CA, USA), a pair of 0.5 μM gene-specific primers, and 5μL of the cDNA sample (~100 ng) in a final volume of 20μL. Alpha tubulin (*CsTUA,* accession XM_010481098.1) was used as the internal control (Dixit et al., 2019). The specific primer sequences for the control and *CsPHT* genes are listed in **Table S3**. Primers were designed using NCBI primer-BLAST (Ye et al., 2012) with a desired product size >200bp, and at least 5 total mismatches to unintended targets. Due to the high sequence homology among the close homoeologs/paralogs, the expression analysis was performed on only a subset of genes that show high primer specificity, confirmed by the melting curve analysis. Genes with one single peak in the melting curve analysis were included in the data. The relative expression level of each gene was calculated based on the comparative threshold cycle method using the internal control, and was further normalized to the control expression values measured for the CK condition. The experiment was repeated three times with three technical replicates, and the values represent the mean and SD. Expression

difference in *CsPHTs* between the two *Camelina* accessions after Pi treatments was assessed by the Student's *t* test (*$p < 0.01$ and ** $p < 0.001$).

CONCLUSIONS

We identified and characterized 73 *CsPHT* genes in *Camelina* that share more similar gene structures and protein domains within the same CsPHT family. Polyploidization and tandem duplication events drove the vast expansion of these CsPHT families in *Camelina*. *Cis*-element, P1BS was more specifically conserved in the promoter regions of *CsPHT1* genes to mediate Pi uptake under low-P. *In-silico* RNA-seq analysis suggested the importance of *CsPHT1* genes throughout plant development, *CsPHT2s* and *CsPHT4s* in vegetative growth, *CsPHT5s* in reproductive development, and *CsPHT3s* in early growth and reproductive stages. However, several genes showed dominant expression compared to their close homoeologous genes including *CsPHT1;27, -5;3* and *-5;4*. A popular Camelina variety, Suneson showed better tolerance to low-P than CS-CROO, with higher expression of *CsPHT1;12, -1;31, -4;1, -4;6* and *-5;1* in roots, and *CsPHT1;1, -1;12, -1;31, -3;7*, *-4;1*, *-4;6*, *-4;15* and *-5;8* in shoots. More in-depth analysis at global transcript level and/or GWAS of a large pool of *Camelina* accessions under low-P could provide a more holistic view of specific Pi-transporters and regulators that are involved in providing enhanced low-P tolerance to *Camelina* accessions (e.g. Suneson). This could serve as a better framework for future functional studies of CsPHT transporters in *Camelina*, and as a model for other crop species.

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SUPPLEMENTAL DATA

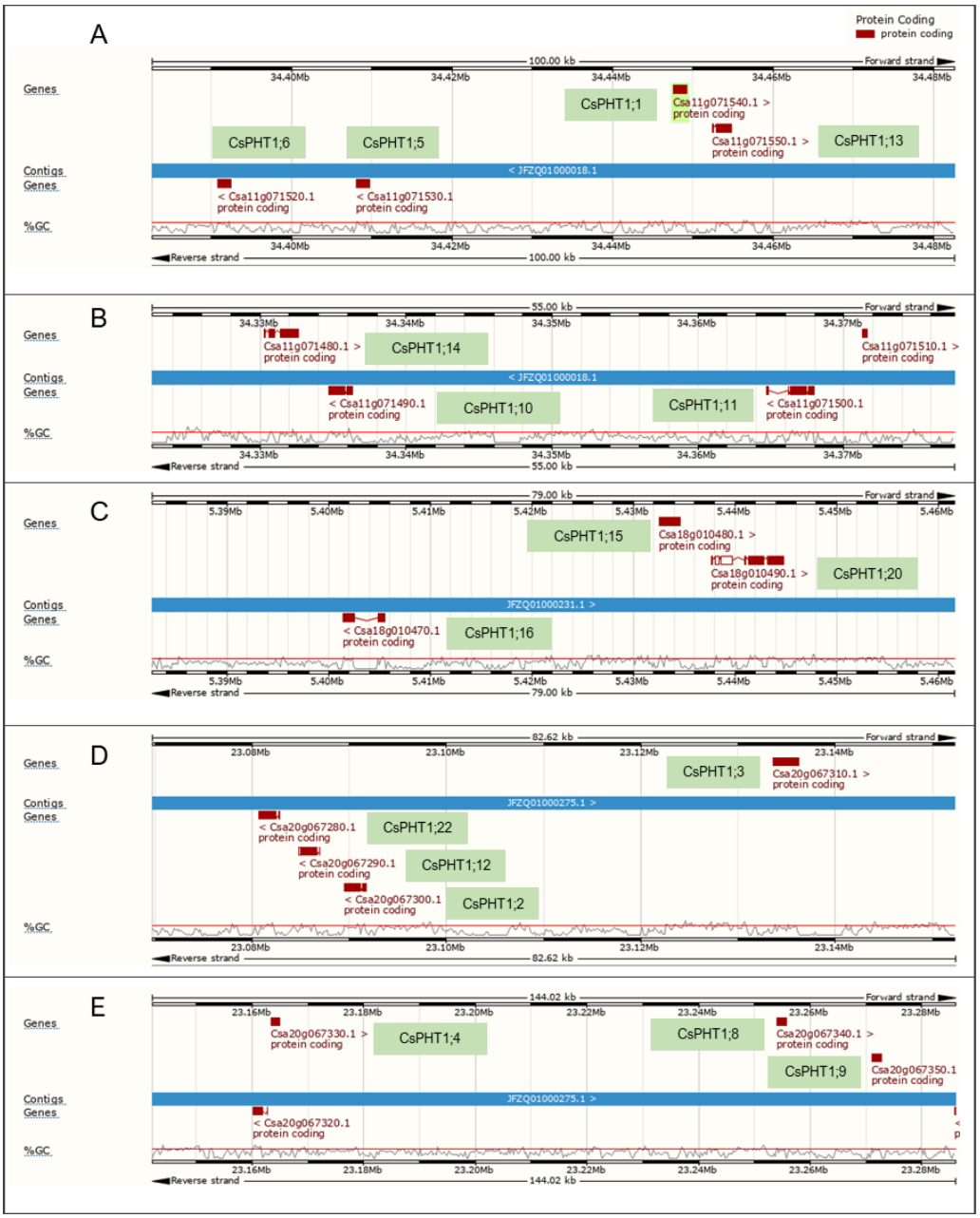

Figure S1. (A-E) Five tandem duplicated regions of *CsPHT1* family genes. The gene regions within the *Camelina* genome were visualized in *EnsemblPlants* (http://plants.ensembl.org/Camelina_sativa/Info/Index).

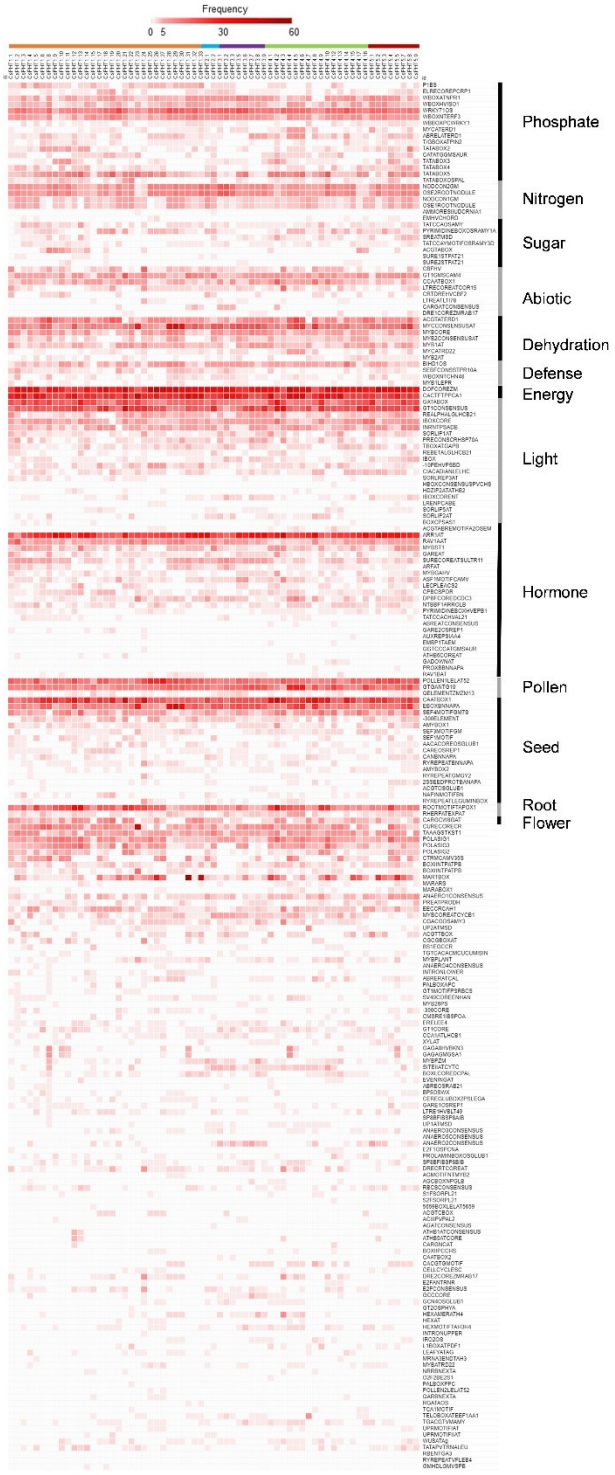

Figure S2. The frequency of 231 cis-regulatory elements in the 2-kb promoter region of CsPHT genes, scanned in New PLACE database.

Table S1. The properties of 73 phosphate transporter (CsPHT) members identified in *C. sativa*

A. thaliana gene	*C. sativa* gene	Locus ID	NCBI gene ID	Genome location	Gene length (bp)	Protein size	Mw (kDa)	pI	TMD
AtPHT1;1 *AtPHT1;2* *AtPHT1;3*	*CsPHT1;1*	Csa11g071540.1	104725125	Chr11: 34447559-34449226	1554	517	56.9	8.9	11
	CsPHT1;2	Csa20g067300.1	104771484	Chr20: 23089482-23091145	1554	517	56.9	8.9	11
	CsPHT1;3	Csa20g067310.1	104771485	Chr20: 23133274-23136312	1554	517	56.8	8.9	11
	CsPHT1;4	Csa20g067330.1	104771486	Chr20: 23163050-23165166	1554	517	56.8	8.9	11
	CsPHT1;5	Csa11g071530.1	104725122	Chr11: 4407992-34409716	1554	517	56.8	8.9	11
	CsPHT1;6	Csa11g071520.1	104725124	Chr11: 34390594-34392799	1554	517	56.8	8.9	11
	CsPHT1;7	Csa18g010450.1	104760616	Chr18: 5356497-5358446	1554	517	56.8	8.9	11
	CsPHT1;8	Csa20g067340.1	104771487	Chr20: 23253748-23255987	1554	517	56.8	8.9	11
	CsPHT1;9	Csa20g067350.1	104771488	Chr20: 23270739-23272750	1554	517	56.8	8.9	11
	CsPHT1;10	Csa11g071490.1	104725120	Chr11: 34334575-34336343	1554	517	56.8	8.9	11
	CsPHT1;11	Csa11g071500.1	104725121	Chr11: 34366281-34367975	1554	517	56.8	8.9	11
	CsPHT1;12	Csa20g067290.1	104771483	Chr20: 23084773-23087017	1575	524	57.7	8.6	12
	CsPHT1;13	Csa11g071550.1	104725126	Chr11: 34452457-34454809	1575	524	57.8	8.8	12
	CsPHT1;14	Csa11g071480.1	104725119	Chr11: 34330271-34332704	1608	535	58.4	8.8	12
	CsPHT1;15	Csa18g010480.1	104763156	Chr18: 5428765-5434620	2043	680	75.2	9.2	13
	CsPHT1;16	Csa18g010470.1	109130617	Chr18: 5401307-5402565	1101	366	40.6	8.3	7
AtPHT1;4	*CsPHT1;17*	Csa05g014350.1	104785556	Chr5: 5014985-5018549	1605	534	58.7	8.4	11
	CsPHT1;18	Csa06g042880.1	104792707	Chr6: 21171205-21174625	1626	541	59.6	8.7	11
	CsPHT1;19	Csa04g052890.1	104784270	Chr4: 25092929-25096715	1161	386	42.0	6.6	9
AtPHT1;6	*CsPHT1;20*	Csa18g010490.1	104760617	Chr18: 5442928-5444835	1554	517	56.5	9.0	12
	CsPHT1;21	Csa13g041920.1	104736909	Chr13: 16476804-16478360	1557	518	56.4	8.8	11

	CsPHT1;22	Csa20g067280.1	104771482	Chr20: 23080792-23082821	1554	517	56.4	9.1	12
	CsPHT1;23	Csa02g023260.1	104749571	Chr2: 7988533-7990089	1557	518	56.2	8.8	11
	CsPHT1;24	Csa08g034850.1	104709547	Chr8: 14895358-14896914	1557	518	56.4	8.8	11
AtPHT1;7	*CsPHT1;25*	Csa06g029160.1	104791742	Chr6: 16352685-16355511	1608	535	58.4	8.7	11
	CsPHT1;26	Csa04g040820.1	104781337	Chr4: 19796700-19799466	1608	535	58.4	8.7	11
	CsPHT1;27	Csa09g064460.1	104712074	Chr9: 24546604-24549170	1608	535	58.4	8.7	11
AtPHT1;8	*CsPHT1;28*	Csa03g024720.1	104776208	Chr3: 9470208-9473149	1611	536	59.1	6.5	12
	CsPHT1;29	Csa14g026060.1	104740779	Chr14: 10079166-10082116	1611	536	59.1	6.5	12
	CsPHT1;30	Csa17g026750.1	104756440	Chr17: 9722033-9725135	1611	536	59.2	6.5	12
AtPHT1;9	*CsPHT1;31*	Csa07g047990.1	104702405	Chr7: 24682226-24685501	1590	529	58.4	8.9	12
	CsPHT1;32	Csa09g081170.1	104713308	Chr9: 30797367-30800766	1593	530	58.5	8.5	12
	CsPHT1;33	Csa16g040570.1	104751540	Chr16: 20731687-20736691	1593	530	58.5	8.5	12
AtPHT2;1	*CsPHT2;1*	Csa06g006270.1	104790285	Chr6: 3480294-3482820	1764	587	61.2	9.3	12
	CsPHT2;2	Csa04g012130.1	104779868	Chr4: 5383632-5386127	1764	587	61.3	9.4	12
	CsPHT2;3	Csa09g010040.1	104710447	Chr9: 4351594-4354039	1764	587	61.2	9.3	12
AtPHT3;1/ AtMPT3	*CsPHT3;1*	Csa08g006050.1	109124421	Chr8: 2089525-2091846	1140	379	40.3	9.3	4
	CsPHT3;2	Csa13g016810.1	104705671	Chr13: 5989032-5992238	1146	381	40.5	9.3	4
	CsPHT3;3	Csa20g020500.1	104769633	Chr20: 6286253-6288550	1146	381	40.5	9.3	4
	CsPHT3;4	Csa10g042780.1	104719447	Chr10: 20121608-20123709	1119	372	39.7	9.3	4
AtPHT3;2/ AtMPT2	*CsPHT3;5*	Csa04g034060.1	104780706	Chr4: 15804679-15806859	1125	374	40.1	9.3	4
	CsPHT3;6	Csa06g022670.1	104791107	Chr6: 12755126-12758457	1125	374	40.4	9.3	4
AtPHT3;3/ AtMPT1	*CsPHT3;7*	Csa19g054110.1	104767030	Chr19: 23808451-23811023	930	309	34.3	9.8	4
	CsPHT3;8	Csa15g074360.1	104747536	Chr15: 25733847-25736413	930	309	34.3	9.8	4

	CsPHT3;9	Csa01g032600.1	104791205	Chr1: 15017664-15019959	930	309	34.3	9.8	4
AtPHT4;1	*CsPHT4;1*	Csa07g014740.1	104700536	Chr7: 6596878-6599540	1530	509	56.2	9.1	11
	CsPHT4;2	Csa16g015150.1	104749907	Chr16: 5712426-5715050	1527	508	56.3	9.1	11
	CsPHT4;3	Csa05g034250.1	104786692	Chr5: 12660802-12665429	1530	509	56.4	9.1	11
AtPHT4;2	*CsPHT4;4*	Csa06g040800.1	104792601	Chr6: 20643470-20647116	1530	509	55.1	9.7	11
	CsPHT4;5	Csa05g015490.1	104785671	Chr5: 5620936-5628866	1530	509	55.0	9.4	11
	CsPHT4;6	Csa04g051740.1	104782239	Chr4: 24496040-24499719	1530	509	55.0	9.7	11
AtPHT4;3	*CsPHT4;7*	Csa09g045340.1	104711167	Chr9: 16771586-16774486	1605	534	58.2	9.7	11
	CsPHT4;8	Csa06g020780.1	104790922	Chr6: 11456453-11460007	1593	530	57.9	9.8	11
	CsPHT4;9	Csa04g029850.2	104780506	Chr4: 14149855-14152770	1593	530	57.7	9.7	11
AtPHT4;4	*CsPHT4;10*	Csa08g001480.1	104705300	Chr8: Chr8: 230348-234133	1677	558	61.6	9.2	10
	CsPHT4;11	Csa13g057010.1	104737737	Chr13: 23830468-23833909	1653	550	60.7	9.1	10
	CsPHT4;12	Csa02g001450.1	104711898	Chr2: 240859-244538	1665	554	61.1	9.3	10
	CsPHT4;13	Csa02g007870.2	104715269	Chr2: 2416948-2420869	1101	366	40.7	8.9	8
AtPHT4;5	*CsPHT4;14*	Csa13g023330.1	104736113	Chr13: 9052413-9057019	1578	525	57.4	6.2	11
	CsPHT4;15	Csa08g014220.1	104706386	Chr8: 5926518-5931342	1578	525	57.6	6.4	11
	CsPHT4;16	Csa20g032330.1	104770418	Chr20: 10882188-10886838	1584	527	57.7	6.2	11
AtPHT4;6	*CsPHT4;17*	Csa11g070280.1	104725004	Chr11: 33280932-33283587	1299	432	47.1	9.4	12
	CsPHT4;18	Csa20g071480.1	104771597	Chr20: 24417095-24419891	1299	432	47.1	9.4	12
	CsPHT4;19	Csa18g009330.1	104760505	Chr18: 4373340-4375925	1299	432	47.1	9.4	12
AtPHT5;1/ AtVPT1	*CsPHT5;1*	Csa07g064340.1	104703914	Chr7: 32705126-32709086	2115	704	78.8	6.5	9
	CsPHT5;2	Csa09g097720.1	104714646	Chr9: 37194004-37198896	2100	699	78.3	6.5	9
	CsPHT5;3	Csa16g055720.1	104752965	Chr16: 28162322-28168131	1905	634	70.4	5.5	9

AtPHT5;2/ AtVPT2	*CsPHT5;4*	Csa13g049020.1	104737184	Chr13: 19601591-19605453	2097	698	78.3	5.8	9
	CsPHT5;5	Csa08g044110.1	104707475	Chr8: 18589381-18594544	2097	698	78.3	6.0	9
	CsPHT5;6	Csa02g016250.1	104748770	Chr2: 5559105-5561998	1938	645	71.8	5.2	9
AtPHT5;3/ AtVPT3	*CsPHT5;7*	Csa10g020090.1	104718005	Chr10: 8699068-8702514	2103	700	78.5	6.0	9
	CsPHT5;8	Csa11g023110.1	104722732	Chr11: 10420297-10423820	2103	700	78.5	6.1	9
	CsPHT5;9	Csa12g033710.1	104731193	Chr12: 10457959-10461455	2103	700	78.4	6.1	9

Table S2. The detailed information on the source of *C. sativa* seeds

Accession	Lot #	Country of origin	Plant Name	Source
PI 650143	06ncai01 SD	Germany	CS-CROO	U.S. National Plant Germplasm System
-	CASU-1	Montana	Suneson	The Experimental Farm Network

Table S3. The sequences of primers used for quantitative Real-Time PCR

C. sativa gene	Locus ID	NCBI gene ID	NCBI Reference Sequence	Forward primer (5' →3')	Reverse primer (5' → 3')	Size (bp)
CsTUA	Csa17g085890.1	104758270	XM_010481098.1	ACTTGGCTTGCTGTTTGATG	CAGTTGGTGGCTGGTAGTTG	159
CsPHT1;1	Csa11g071540.1	104725125	XM_019232427.1	TGCTTCTTCAGGTTTTGGTTG	AAAGCCCCACGAGTCTTCTT	101
CsPHT1;12	Csa20g067290.1	104771483	XM_010496012.2	GGCCAAGACTGACGCAGGA	TGACTATTTCTCGTCCGGGCT	175
CsPHT1;28	Csa03g024720.1	104776208	XM_010500225.1	GACGTTATAAGAATCATTGTCCTGT	GTATTCGAGCTGCATCAAGCG	179
CsPHT1;31	Csa07g047990.1	104702405	XM_010418262.2	GCTAACAAAAGGACGCGTGG	AAACTCCCTCCTCCGACGTT	128
CsPHT1;33	Csa16g040570.1	104751540	XM_010473496.2	TCTTAGGCCACACTTTCAGTCC	CTGTATCTTTGCGGCGGTGT	146
CsPHT2;1	Csa06g006270.1	104790285	XM_010515998.2	ATTTGTGAGGCCCAACCAGAC	AGGTGGAGGTGAGAGGATTGT	162
CsPHT3;1	Csa08g006050.1	109124421	XM_010421723.1	GCAAGCGTGAGATCGGGCTAT	CACCTGGTCGAGGAGGAAAGA	147
CsPHT3;7	Csa19g054110.1	104767030	XM_010491049.2	AGTAGAAGACCTGAAGCAAGC	TCACTGACAAGAATTGGTTTGCC	106
CsPHT4;1	Csa07g014740.1	104700536	XM_010416063.2	AATCGTCCACCTGAAAAATCCG	CGGACCTGTTCCTCGGGTA	91
CsPHT4;6	Csa04g051740.1	104782239	XM_010507114.2	CACACTACGCCAGTCAGTACA	ACGATGTGAAGGTTAGGAGTGG	108
CsPHT4;15	Csa08g014220.1	104706386	XM_010422575.1	TCAAGGGTTAAGTGCTCCGC	CCCCTTTCCGTTTCCTTACTC	146
CsPHT4;17	Csa11g070280.1	104725004	XM_019232187.1	AAACTCATCTCCTTCACCGTGG	AGATTTGGGGGAAAATGACGGA	125
CsPHT5;1	Csa07g064340.1	104703914	XM_010420003.2	CTACTTTGCCTGGGTGGGTT	AGCAGATTACTTGATGTTGTTTCA	139
CsPHt5;4	Csa13g049020.1	104737184	XM_010457294.2	ACACAATCTCTGGAAATCGGA	GGCGACCATTTTCTTTTGACG	130
CsPHT5;8	Csa11g023110.1	104722732	XM_010440947.2	GTTGCAACTCAGAGAGAAGAAGA	GGCGACGTTCAAGATTCCCT	158

Chapter 3: Recent Advances in Genome-wide Analyses of Plant Potassium Transporter Families

The following chapter (excluding protions of the preface) has been accepted for publication as a peer-reviewed research article in the Special Issue, “Genes, Genomes and Germplasm for Climate-Smart Agriculture” of *Current Genomics*, and is reproduced here with permission.

Lhamo, D., Wang, C., Gao, Q., and Luan, S. (2021). Recent advances in genome-wide analyses of plant potassium transporter families. *Curr. Genomics* 22. doi:10.2174/1389202922666210225083634.

Contributions

D. Lhamo provided intellectual contributions, performed bioinformatic analyses, prepared figures and primarily wrote the manuscript. C. Wang, Q. Gao and S. Luan edited the manuscript.

Preface

Like P, K is also a major macronutrient that plants need at a large abundance. These two nutrients come from mineral rocks that are limited in resources. Therefore, there is an urgent need to dissect the key transport systems in different crop species to engineer crops with high nutrient use efficiency. Genome sequencing and transcriptomic analyses of crop species are exponentially increasing each year, but focused studies such as crops’ response to different nutrient stresses are lacking at the gene level. In the previous chapter, all the major Pi transporters and their tissue-specific expressions in different tissues of *Camelina* were identified. In this chapter, the similar studies are performed for all the major K channel and transporter families in *Camelina,* a promising polyploid oilseed crop that may populate marginal soils for biofuel production. In addition, a comprehensive contemporary review on K channel and transporter families in angiosperms are provided, with a major focus on the genome-wide identification, subcellular localization, spatial expression, function and regulation in multiple crop species. Also, new insights and emphasis on the study of K channels and transporters in polyploids are presented in an effort to generate crops with high K utilization efficiency.

INTRODUCTION

Potassium (K^+) is the most abundant mineral nutrient in plants, contributing up to 10% of dry biomass (Leigh and Wyn Jones, 1984; Amrutha et al., 2007; Yang et al., 2009). Plants require 100-200 mM K^+ in cytoplasm (Jones and G, 1983; Leigh and Wyn Jones, 1984; Walker et al., 1996) to carry out many fundamental processes involving membrane transport, protein synthesis, carbohydrate metabolism, enzyme activation, anion neutralization, osmoregulation, stomatal movement and photosynthesis (Wang et al., 2013; Wang and Wu, 2017; Hasanuzzaman et al., 2018). However, the availability of K^+ is limited in soil (> 1mM) (Maathuis, 2009; White, 2013), thus requiring fertilizer application to meet the nutritional demands for plant growth and productivity. The reliance on fertilizers is clearly not a long-term solution as both production and application of fertilizers cause pollution and threaten the sustainability of our environment. Further, K^+ fertilizer comes from potash, a natural resource that will be depleted in the near future (Cordell et al., 2009; Luan et al., 2017). Thus, there is an urgent need to identify new methods to ensure sustainable mineral nutrition for optimal crop production by minimizing or removing fertilizer inputs. An effective strategy to achieve these goals is to breed crops with high K^+ use efficiency (KUE), which requires better understanding of the K^+ transport systems within plants.

In plants, K^+ transport is mediated by four families of typical K^+ transport systems, which include KUP/HAK/KT (K^+ Uptake/High-Affinity K^+/ K^+ Transporter) transporters, *Shaker*-like channels, TPK/KCO (Tandem Pore K^+/K_{ir}-like K^+) channels, and KEA (K^+ Efflux Antiporter) transporters. These K^+ transporters and channels are required to cross the barriers presented by plasma membrane (PM) at the root epidermis for K^+ acquisition, and membranes of the subcellular compartments for K^+ storage and distribution. Our current knowledge of these K^+ transport systems mainly stem from model plant Arabidopsis. It is time for us to apply this knowledge to crop research to engineer high KUE crops. For this purpose, we need to first identify and understand the physiological role of these K^+ transporters and channels in various crop species as their diverse genetic backgrounds allow them to respond to the changing nutrient status differently. Through sequence homology, homologs of some of the key K^+ transporters found in Arabidopsis are identified and functionally characterized in several other plant species. With growing publications on plant genome sequences (Tang et al., 2020a), we need to put more effort in genome-wide search for K^+ transporter families in crop plants to initiate comparative genomic analysis and deduce how K^+ transporters evolve throughout domestication.

Polyploid crops are important groups in crop breeding, and yet little is known about these plant species. The previous work on multiple Arabidopsis accessions confirmed the beneficial effect of the polyploid nature in enhancing K^+ acquisition and salt tolerance (Chao et al., 2013). Therefore, extending our studies to polyploid crops might reveal the underlying genes and regulatory mechanism that allow these crops to adapt to various environmental stresses. Oil-producing crops are important polyploids that support various commodities such as food, feeds and fuels. However, little effort has been made to understand how these polyploids might adapt to low-K^+ stress at the genomic level. To establish such a knowledge base, we first provide a comprehensive review of the four major K^+ transporter family genes in various model and crop species, with reference to other reviews that cover similar topics. We then extend our genome-wide search of the

K^+ transporter family genes in *Camelina sativa* (false flax or gold-of-pleasure), an allohexaploid oilseed crop with a great potential for biofuel production on marginal lands to support sustainable agriculture (Kagale et al., 2014, 2016; Vollmann and Eynck, 2015; Abdullah et al., 2016; Malik et al., 2018). In addition, we evaluated the tissue-specific expression of these K^+ transporter genes in *Camelina* using the publicly available transcriptomic data (Kagale et al., 2016), and compared their expression profiles with other plant species to deduce functional conservation or divergence. Since the *Camelina* genome closely resembles Arabidopsis as members of the same Brassicaceae family (Kagale et al., 2014), it provides a comparable polyploid model to study KUE.

KUP/HAK/KT TRANSPORTERS

Identification of KUP/HAK/KT Family in Plants

KUP/HAK/KT members are K^+ transporters present in fungi, plants and bacteria (Véry et al., 2014). In plants, these K^+ transporters contain 10-14 transmembrane segments consisting of MFS (Major Facilitator Superfamily) domains (Gierth and Mäser, 2007; Rivetta et al., 2013; Li et al., 2018a). The plant KUP/HAK/KT transporters are first identified in barley (Santa-María et al., 1997) and Arabidopsis (Quintero and Blatt, 1997; Fu and Luan, 1998; Kim et al., 1998), from their homology to bacterial KUP and fungal HAK transporters (Schleyer and Bakker, 1993; Banuelos et al., 1995). Since then, KUP/HAK/KT transporters have been cloned from many other plant species especially after genome sequence became available (Garciadeblas et al., 2002; Wang et al., 2002, 2018c; Desbrosses et al., 2004; Martínez-Cordero et al., 2004; Gierth et al., 2005; Davies et al., 2006; Gupta et al., 2008; Yang et al., 2014; Chen et al., 2015; Dai et al., 2019). Genome-wide search by sequence homology has resulted in identification of KUP/HAK/KT transporter family in several diploid and polyploid species as shown in **Table 1**. This K^+ transporter family is further identified in 46 angiosperm plant species, consisting of monocots and dicots (Nieves-Cordones et al., 2016). So far, the genomes of more than 400 plant species (Tang et al., 2020a) including ~50 polyploid plants (Kyriakidou et al., 2018) have been sequenced. However, identification of K^+ transporter families is limited to a relatively small number of species. To facilitate understanding of gene duplication events among the K^+ transporters, we examined the *Camelina* genome and identified 38 KUP/HAK/KT transporters that are divided into five major clades **(Figure 1)**. These include AtKUP1/2/6/8 and their close homoeologs in one large clade; and AtKUP3/4, AtKUP9/10/11, AtKUP5/7/12 and AtHAK5, and their corresponding *Camelina* homoeologs in four separate clades. Genome triplication of *Camelina* has resulted in maintaining triplet homoeologs for the majority of Arabidopsis KUP/HAK/KT orthologs, while further gene duplication resulted in an additional member for AtKUP9 ortholog. Two *Camelina* homoeologs are identified for AtKUP3 and AtKUP11 orthologs. Interestingly, both diploid and polyploids have evolved a large number of KUP/HAK/KT transporters through whole-genome multiplication or gene duplication events, signifying the importance of these K^+ transporters for plant productivity.

Table 1. The Genome-wide Identification of KUP/HAK/KT Family in Plants

Plant species	Common name	Ploidy level	KUP/HAK/KT members	References
Arabidopsis thaliana	Arabidopsis	diploid	13	(Ahn et al., 2004)
Oryza sativa L.	Rice	diploid	27	(Banuelos et al., 2002; Amrutha et al., 2007; Zhang et al., 2012)
Zea mays L.	Maize	diploid	27	(Zhang et al., 2012)
Populus trichocarpa	Poplar	diploid	31	(He et al., 2012)
Solanum lycopersicum L.	Tomato	diploid	19	(Hyun et al., 2014)
Pyrus bretschneideri	Pear	diploid	20-21	(Li et al., 2018b; Wang et al., 2018b)
Setaria italica	Foxtail millet	diploid	29	(Zhang et al., 2018)
Arabidopsis lyrata	Arabidopsis	tetraploid	15	(Nieves-Cordones et al., 2016)
Glycine max	Soybean	Allotetraploid	29-32	(Nieves-Cordones et al., 2016; Rehman et al., 2017)
Manihot esculenta Crantz	Cassava	diploid, autotetraploid	21	(Ou et al., 2018)
Nicotiana tabacum	Tobacco	Allotetraploid	41	(Song et al., 2019)
Saccharum spontaneum	Sugarcane	Autotetraploid	30	(Feng et al., 2020b)
Brassica napus	Rapeseed	Allohexaploid	40	(Zhou et al., 2020)
Triticum aestivum L.	Wheat	Allohexaploid	56	(Cheng et al., 2018)

Subcellular Localization and Expression Pattern of KUP/HAK/KT Transporters

The in-depth reviews on the KUP/HAK/KT transporters in terrestrial photosynthetic organisms (Santa-María et al., 2018), and their function and regulation (Li et al., 2018a) are recently published. Several KUP/HAK/KT transporters are localized to the PM based on experiments using transgenic plants or protoplasts. Other members of this family also localize to various subcellular compartments, including AtKUP4 in endomembrane (Rigas et al., 2013); AtKUP12 in chloroplast (Kleffmann et al., 2004; Peltier et al., 2004); OsHAK10 and potentially AtKUP5/7/12 transporters in vacuole (Banuelos et al., 2002; Jaquinod et al., 2007; Whiteman et al., 2008); and AtKUP9 in endoplasmic reticulum (ER) (Zhang et al., 2020). This suggests the potential roles of these K^+ transporters in not only K^+ acquisition, but also in K^+ distribution via sequestration into or remobilization from various subcellular compartments to maintain K^+ homeostasis.

The diverse roles of KUP/HAK/KT transporters are also implicated by their tissue-specific expression. Generally, *KUP/HAK/KT* genes are expressed in multiple tissues throughout various developmental stages (roots, leaves, flowers, seeds and so forth) in Arabidopsis (Ahn et al., 2004), rice (Gupta et al., 2008), maize (Zhang et al., 2012), wheat (Cheng et al., 2018), pear (Li et al., 2018b), and sugarcane (Feng et al., 2020b). This is also evident in *Camelina*, where *CsKUP* genes are expressed in 12 different tissues covering four developmental stages **(Figure 2A)**. In terms of tissue-specific expression, *AtKUP8*-like genes in *Camelina* are predominantly expressed in leaves; *AtKUP9*- and *AtHAK5*-like genes in roots; *AtKUP1*-like genes in reproductive organs; and *AtKUP6*- and *AtKUP11*-like genes in seeds. While some of their functions are confirmed in these tissues in Arabidopsis such as AtHAK5 in root K^+ uptake (Qi et al., 2008; Nieves-

Cordones et al., 2010) and AtKUP9 (Zhang et al., 2020) in maintaining root meristem activity under low-K^+ stress, the physiological roles of other AtKUP transporters in various tissues remain unresolved.

Function and Regulation of KUP/HAK/KT Transporters

The transport activities of several KUP/HAK/KT transporters have been studied in heterologous systems. AtKUP1 is the first plant KUP/HAK/KT member to be identified using a yeast system (Fu and Luan, 1998). The *in planta* function of some of these transporters have been examined using genetic mutants. The Arabidopsis *AtHAK5*, and its close homologs in barley (*HvHAK1*) (Santa-María et al., 1997), rice (*OsHAK1*) (Banuelos et al., 2002; Yang et al., 2014), tomato (*LeHAK5*) *(Wang et al., 2002; Nieves-Cordones et al., 2008)*, pepper (*CaHAK5*) (Martínez-Cordero et al., 2004), salt cress (*ThHAK5*) (Alemán et al., 2009), wheat (*TaHAK1*) (Cheng et al., 2018), foxtail millet (*SiHAK1*) (Zhang et al., 2018), pear (*PbHAK12*) (Li et al., 2018b), and maize (*ZmHAK1*) (Qin et al., 2019) are highly expressed in roots and serve as PM-localized K^+-uptake transporters under low-K^+ stress. The other KUP/HAK/KT transporters that mediate K^+ transport in Arabidopsis include AtKUP2/6/8 in K^+ efflux from root stele (Osakabe et al., 2013), and AtKUP7 in K^+ uptake from roots and translocation to shoots under low-K^+ stress (Han et al., 2016). Several KUP/HAK/KT transporters in other plant species are also found to function in K^+ uptake under K^+ deficiency, which include lotus *LjKUP* (Desbrosses et al., 2004), rice *OsHAK5/16/19/20/21/27* (Yang et al., 2014; Shen et al., 2015; Okada et al., 2018; Feng et al., 2019), cotton *GhKT2* (Wang et al., 2018c), maize *ZmHAK5* (Qin et al., 2019), Goji *LrKUP8* (Dai et al., 2019), and sugarcane *SsHAK1/21* (Feng et al., 2020b). In rice, OsHAK5 and OsHAK21 might also be involved in long-distance K^+ transport between roots and shoots as they are highly expressed in root vasculature (Yang et al., 2014; Shen et al., 2015). Some KUP/HAK/KT family transporters also appear to promote K^+ uptake when symbiotically associated with Arbuscular mycorrhizal (AM) fungi. For instance, mutation of tomato *SlHAK10* led to reduced mycorrhizal-mediated K^+ uptake and lower AM colonization rate under low-K^+ stress (Liu et al., 2019).

Beyond K^+ acquisition, KUP/HAK/KT transporters are also involved in plant development and in response to various abiotic stresses. For instance, AtKUP4/TRH1 contributes to the polar auxin transport in root apex to generate auxin gradients for gravitropic responses and root hair growth (Rigas et al., 2001, 2013; Vicente-Agullo et al., 2004). AtKUP9 has been shown to control primary root growth and meristematic activity by mediating K^+ and auxin efflux from the ER lumen to the cytoplasm in quiescent center cells under low-K^+ condition (Zhang et al., 2020). AtKUP2 (SHY3) (Elumalai et al., 2002), GhKT1 (Ruan et al., 2001), and VvKUP1/2 (Davies et al., 2006) are involved in cell expansion in Arabidopsis, cotton and grape, respectively. AtKUP2/6/8 negatively affect lateral root formation, but play a positive role in drought response by modulating turgor and hormone signaling in roots and guard cells (Osakabe et al., 2013). Several KUP/HAK/KT transporters in other plant species are shown to be involved in drought tolerance (Chen et al., 2017; Feng et al., 2020a) and salt tolerance (Su et al., 2002;

Mangano et al., 2008; Qi et al., 2008; Nieves-Cordones et al., 2010; Yang et al., 2014; Shen et al., 2015; Dai et al., 2019).

While the functional studies are gradually expanding in Arabidopsis and other plant species, our understanding on the regulation of these KUP/HAK/KT transporters is still at its infancy. At the transcriptional level, studies have shown that *AtHAK5* expression is controlled by several transcriptional factors, including RAP2.11 (AP2/ERF), DDF2 (Dwarf and Delayed Flowering 2), JLO (Jagged Lateral Organs), TFII_A (Transcription initiation Factor II-A gamma chain), bHLH121 (basic Helix-Loop-Helix 121) and ARF2 (Auxin Response Factor 2) (Li et al., 2018a; Santa-María et al., 2018; Ragel et al., 2019). The post-translational regulation of KUP/HAK/KT transporters by several protein kinases have been reported, such as phosphorylation-dependent activation of AtHAK5 and its close homologs in other plant species by the CBL-CIPK (Calcineurin B-Like sensor and CBL-Interacting Protein Kinase) signaling networks (Wang and Wu, 2017; Li et al., 2018a; Santa-María et al., 2018; Ragel et al., 2019). The other potential candidate kinases include SRK2E (SNF1-Related protein Kinase 2E) and ILK1 (INTERGRIN_LINKED KINASE1) that physically interact with AtKUP6 and AtHAK5 in Arabidopsis, respectively, and CrRLK1L receptor kinase with OsHAK1/19/20 in rice (Li et al., 2018a; Santa-María et al., 2018).

SHAKER-LIKE CHANNELS

Identification of *Shaker*-like Family in Plants

Shaker-like channels are K^+-selective voltage-gated channels that are found in both prokaryotes and eukaryotes. Arabidopsis has nine *Shaker*-like channels categorized into several subfamilies based on their differential voltage responses (Pilot et al., 2003). These include inward-rectifying (K^+_{in}) channels such as AtAKT1, AtAKT5, AtSPIK, AtKAT1 and AtKAT2 that are activated by hyperpolarization and mainly mediate K^+ uptake (Hirsch et al., 1998; Pilot et al., 2001, 2003; Mouline et al., 2002; Zhao et al., 2013). Contrarily, the outward-rectifying (K^+_{out}) channels, such as AtSKOR and AtGORK, are activated by depolarization and mediate K^+ efflux (Gaymard et al., 1998; Ache et al., 2000). The weakly rectifying (K^+_{weak}) channels, such as AtAKT2, could be activated by hyperpolarization and may mediate both K^+ uptake and release depending on the membrane potentials (Lacombe et al., 2000; Deeken et al., 2002; Michard et al., 2005a, 2005b). AtKC1 is considered an electrically-silent (K^+_{silent}) channel, but can form heteromeric complexes with K^+_{in} channels to regulate their activities in response to changing environmental conditions (Reintanz et al., 2002; Duby et al., 2008; Geiger et al., 2009; Jeanguenin et al., 2011). These *Shaker*-like channels share a similar hydrophobic core consisting of six transmembrane segments with voltage-sensor and pore loop modules (Pilot et al., 2003; Amrutha et al., 2007). These channels in plants typically contain a long cytoplasmic C-terminal region featuring a putative cyclic nucleotide binding domain and, in some, an ankyrin repeat domain (Pilot et al., 2003; Amrutha et al., 2007).

The first insight in the molecular analysis of membrane K^+ transport in plants comes from the identification of *Shaker*-like channels, AtAKT1 and AtKAT1, in Arabidopsis by

functional complementation of yeast strains defective in K^+ uptake (Anderson et al., 1992; Schachtman et al., 1992; Sentenac et al., 1992). Later, many genes encoding *Shaker*-like channels have been functionally characterized in Arabidopsis and other plant species, discussed in the following section. However, only a few plant species were examined using the genome-wide analysis to identify the complete set of *Shaker*-like channels. These studies revealed 9 members in Arabidopsis (Pilot et al., 2003) and grapevine (Cuéllar et al., 2013), 11 in rice (Pilot et al., 2003; Amrutha et al., 2007) and poplar (Gomez-Porras et al., 2012), 8 in pear, 10 in apple, 6 in peach, strawberry and Chinese plum (Chen et al., 2019). To extend this effort, here we identified 28 *Shaker*-like channels in *Camelina*, with three homoeologs for each Arabidopsis ortholog except AtSPIK1 with an additional member **(Figure 1)**. Similar to Arabidopsis, these channels in *Camelina* are classified into five groups based on the functional diversifications (Pilot et al., 2003).

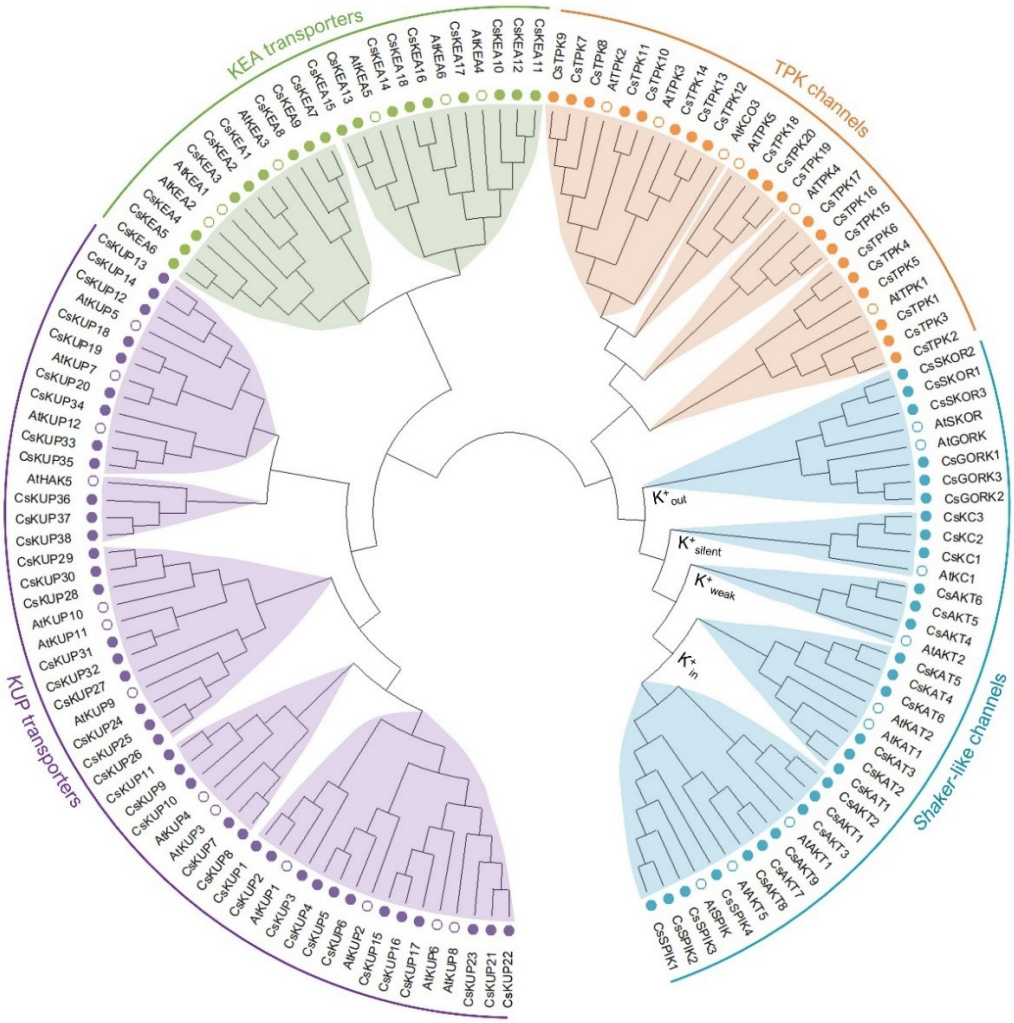

Figure 1. Evolutionary relationship between *Camelina* and Arabidopsis K+ transporters. The four major K+ transporter family proteins are color-coded, with distinct clades. The *Camelina* K+ transporters are represented in closed circles, and Arabidopsis in open circles. The gene IDs are presented in Table S1.

Subcellular Localization, Expression, Function and Regulation of *Shaker*-like Channels in Arabidopsis

In Arabidopsis, the *Shaker*-like family is the most intensively studied among all the K^+ transport systems. The expression pattern of *Shaker*-like genes at tissue and cell-type levels, their functions and regulations have been characterized and previously reviewed (Sharma et al., 2013; Wang and Wu, 2013, 2017; Adams and Shin, 2014; Anschütz et al., 2014; Véry et al., 2014; Luan et al., 2017; Ragel et al., 2019). To briefly summarize, the *Shaker*-like channels are usually localized at the plasma membrane of plant cells and dominate the membrane conductance in most cell types (Véry et al., 2014). *AtAKT1* is highly expressed in root hairs, epidermis and cortex to absorb K^+ from soil under varying K^+ concentrations (Lagarde et al., 1996; Hirsch et al., 1998; Gierth et al., 2005). *AtKC1* is expressed in the similar tissues where it interacts and inhibits the activities of the K^+_{in} channels such as AKT1, AKT2, KAT1 and KAT2 by forming heteromeric complexes (Reintanz et al., 2002; Duby et al., 2008; Geiger et al., 2009; Jeanguenin et al., 2011). After K^+ enters the root cells, *AtSKOR*, highly expressed in root stele (pericycle and xylem parenchyma cells), loads K^+ into the xylem and initiates long-distance transport of K^+ from root to the aerial parts of the plants (Gaymard et al., 1998; Liu et al., 2006). In parallel, the recycling of K^+ from shoot to root may be mediated by AtAKT2 via phloem loading (Marten et al., 1999; Lacombe et al., 2000; Xicluna et al., 2007). *AtGORK* is expressed in guard cells where it contributes to K^+ efflux to control stomatal closure in response to environmental changes (Ache et al., 2000; Ivashikina et al., 2001; Hosy et al., 2003; Demidchik, 2014). *AtKAT1* and *AtKAT2* are also expressed in guard cells where they form heteromeric channels that mediate K^+ influx to promote stomatal opening (Anderson et al., 1992; Nakamura et al., 1995; Kwak et al., 2001; Pilot et al., 2001). The roles of *Shaker*-like channels in stomatal movement and the associated signaling networks are comprehensively reviewed elsewhere (Pandey et al., 2007; Kim et al., 2010; Sharma et al., 2013). Among the *Shaker*-like channels, *AtAKT5 and AtSPIK* display flower-specific expression, with the confirmed role of AtSPIK in K^+ influx into germinating pollen to promote pollen tube growth and development (Lacombe et al., 2000; Mouline et al., 2002; Zhao et al., 2013). However, the physiological role of AtAKT5 has yet to be evaluated. The regulation of *Shaker*-like channels in Arabidopsis by transcriptional factors, hormones, pH, calcium-dependent protein kinases, phosphatases, 14-3-3 proteins and so forth are discussed in detail in recent reviews (Luan et al., 2017; Wang and Wu, 2017; Ragel et al., 2019; Tang et al., 2020b).

Expression and Function of *Shaker*-like Channels in Other Plant Species

The comparison of tissue-specific expression of the *Shaker*-like channels between Arabidopsis and other plant species are reviewed (Véry et al., 2014). Many *Shaker*-like channels have been functionally characterized in various plant species using patch clamp systems (**Table 2**). These channels display overlapping expression and conserved K^+ transport properties as their Arabidopsis counterparts. However, divergence is also observed between and within dicots and monocots because of variations in plant architectures and cell types. For instance, rice OsAKT1.2, another close homolog of

AtAKT1, is shown to be involved in reproduction as it is highly expressed in pollen and the knockout lines display reduced pollen germination rates (Yang et al., 2020). In maize, ZMK1 (AtAKT1-like) is an inward-rectifying K^+ channel whose expression is auxin-induced and implicated in coleoptile growth and gravitropism (Philippar et al., 1999; Bauer et al., 2000; Fuchs et al., 2003).

While Arabidopsis possesses two AtKAT channels, four AtKAT1-like members are found in maize (KZM1-4) (Gao et al., 2017) and three in rice (OsKAT1-3) (Hwang et al., 2013a, 2013b). KZM1/ZmK2.1 functions as a K^+_{in} channel in leaf epidermis and vascular strands, however it cannot mediate K^+ influx in the submillimolar concentration range, unlike its Arabidopsis orthologs (Philippar et al., 2003; Su et al., 2005). *KZM2* and *KZM3* display *AtKAT*-like expression in guard cells and promote K^+ influx in HEK293 cells, but KZM2 did not mediate K^+-inward currents in oocytes, instead inhibited the activity of KZM3 as heteromeric channel (Gao et al., 2017). In rice, OsKAT2 plays the major role in K^+ influx into guard cells, however, its activity is inhibited by OsKAT3 when coexpressed in CHO cells (Hwang et al., 2013a, 2013b).

The GORK function is conserved in both monocots and dicots (**Table 2**). However, GORK channels in monocots are additionally expressed in subsidiary cells that form stomatal complexes along with guard cells. Moreover, OsK5.2/OsGORK is found to perform dual function in stomatal movement and K^+ loading into xylem sap, which requires the action of both AtGORK and AtSKOR in dicots (Nguyen et al., 2017). Similar situation might happen in poplar in which *PTORK* is expressed in both vascular tissues and guard cells (Langer et al., 2002; Arend et al., 2005). Interestingly, single amino acid mutations in AtSKOR and AmGORK can convert these outward-rectifying channels into an inward-rectifying and leak-like channel (AtAKT2/3-like), respectively (Li et al., 2008, 2016), implying a common ancestor of inward and outward channels in the *Shaker*-like family.

The AtAKT2/3-like channels are found in four other plant species, which display similar phloem-specific expressions and conserved functions as weak-rectifying K^+ channels (**Table 2**). However, *VvK3.1* is additionally expressed in pulvinus (a motor organ) that is involved in paraheliotropic leaf movement (Nieves-Cordones et al., 2019; Villette et al., 2019). Barley HvAKT2 is also a weakly-rectifying K^+ channel, but, unlike Arabidopsis, it is more expressed in leaves than roots (Chérel et al., 2002; Boscari et al., 2009). Moreover, maize *ZMK2* is expressed in vascular-enriched coleoptile sections, e.g., the mesocotyl and primary leaves, where it mediates voltage-independent K^+ currents (Philippar et al., 1999; Bauer et al., 2000). Currently, the physiological roles of many of these *Shaker*-like channels in different plant tissues and organs are unknown.

We also examined the expression of *Shaker*-like genes in *Camelina* and have found that they follow the similar pattern as in Arabidopsis **(Figure 2B)**. For instance, *AtAKT1*- and *AtKC1*-like genes in *Camelina* are highly expressed in roots in which they might control K^+ acquisition from the soil. The *AtKAT*- and *AtSKOR*-like genes are highly expressed in leaves with a potential role in regulating stomatal movement. The *AtAKT5*- and *AtSPIK*-like genes are expressed in reproductive organs. However, *AtAKT2*-like genes in *Camelina* are not expressed in roots unlike Arabidopsis. In addition, *AtGORK*-like gene is not expressed in green leaves, but more in root and stem. These differences may suggest divergence in functions between diploid Arabidopsis and hexaploid *Camelina* over the course of evolution. Interestingly, *AtKC1*-like genes in *Camelina* share

overlapping expression patterns with *AtAKT5*-like genes in germinating seeds, *AtAKT1*- and *AtKAT*-like genes in senescing leaves and many in roots. This implies that AtKC1-like silent channels may form heteromeric channels with a broad spectrum of K^+_{in} channels in *Camelina* to fine-tune plant response to the changing K^+ availability and other environmental conditions.

Table 2. The Conserved Roles of *Shaker*-like Channels in Plants

Arabidopsis orthologs	Channel names	Plant species	References
AtAKT1	SKT1	*Solanum tuberosum*	(Müller-Röber et al., 1995; Zimmermann et al., 1998)
	LKT1	*Solanum lycopersicum*	(Hartje et al., 2000)
	NKT1	*Nicotiana tabacum*	(Sano et al., 2007)
	HvAKT1	*Hordeum vulgare*	(Boscari et al., 2009)
	VvK1.1	*Vitis vinifera*	(Cuéllar et al., 2010)
	GhAKT1	*Gossypium hirsutum*	(Xu et al., 2014)
	OsAKT1 (OsK1;1)	*Oryza sativa*	(Li et al., 2014)
AtKAT1/2	KST1	*Solanum tuberosum*	(Müller-Röber et al., 1995)
	SIRK (VvK2.1)	*Vitis vinifera*	(Pratelli et al., 2002)
	KPT1	*Populus tremula*	(Langer et al., 2004)
	AmKAT1	*Ammopiptanthus mongolicus*	(Yang et al., 2015)
	PbrKAT1	*Pyrus bretschneideri*	(Chen et al., 2019)
	KZM2	*Zea mays*	(Gao et al., 2017)
	OsKAT2	*Oryza sativa*	(Hwang et al., 2013a, 2013b)
AtGORK	NTORK	*Nicotiana tabacum*	(Sano et al., 2007)
	AmGORK	*Ammopiptanthus mongolicus*	(Li et al., 2016)
	MtGORK	*Medicago truncatula*	(Drain et al., 2020)
	OsK5.2 (OsGORK)	*Oryza sativa*	(Nguyen et al., 2017)
	ZORK	*Zea mays*	(Büchsenschütz et al., 2005)
AtSKOR	CmSKOR	*Cucumis melo L.*	(Long-Tang et al., 2018)
	Vv5.1	*Vitis vinifera*	(Villette et al., 2019)
AtAKT2	VFK1	*Vicia faba*	(Ache et al., 2001)
	PTK2	*Populus tremula*	*(Langer et al., 2002)*
	VvK3.1	*Vitis vinifera*	*(Nieves-Cordones et al., 2019; Villette et al., 2019)*
	OsAKT2	*Oryza sativa*	*(Shen et al., 2020)*
AtKC1	KDC1	*Daucus carota*	*(Bregante et al., 2008)*

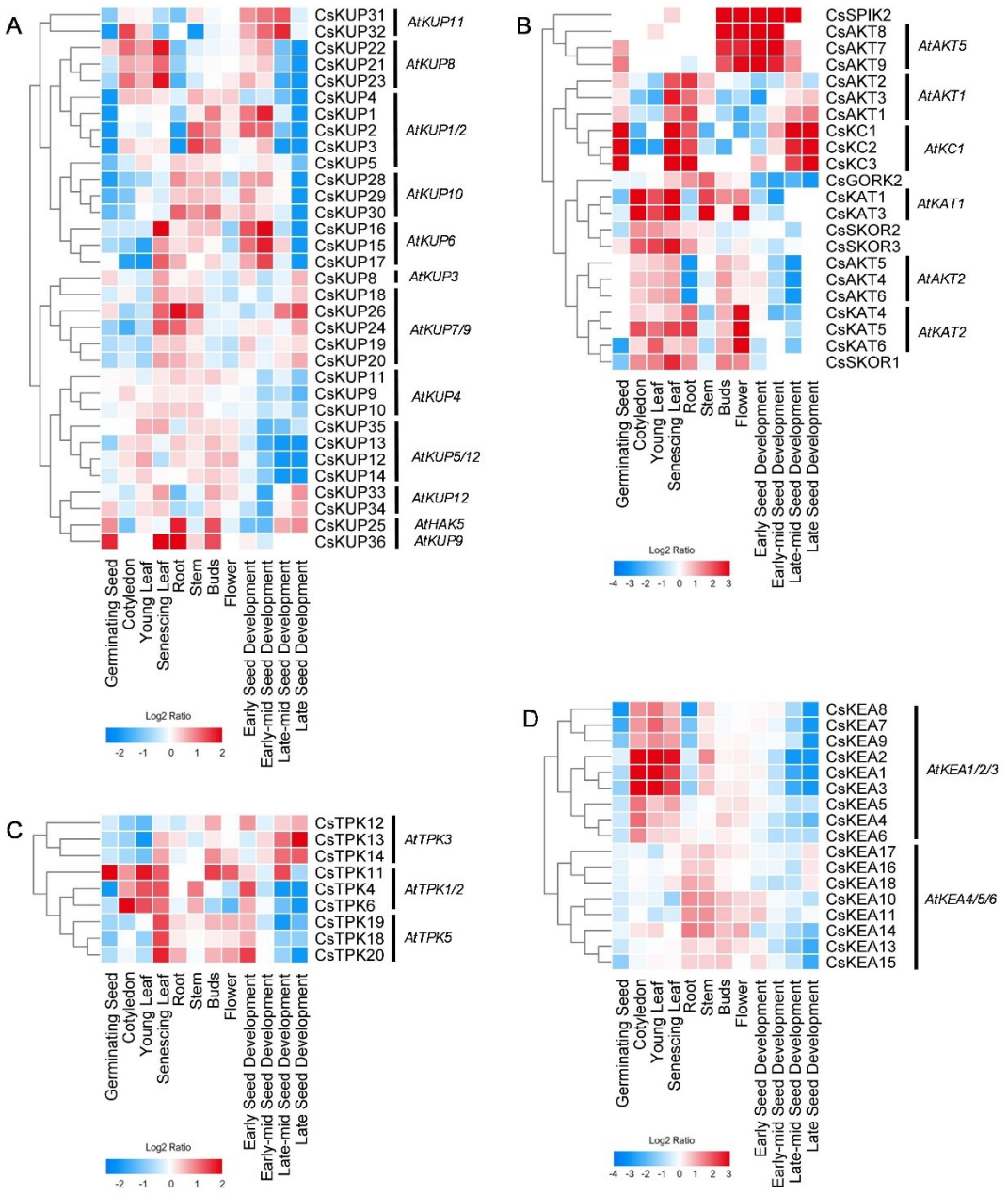

Figure 2. Spatial expressions of the *Camelina* (A) KUP, (B) Shaker-like, (C) TPK and (D) KEA transporter family genes in twelve different tissues covering the whole life cycle. The transcriptomic data are extracted from *Camelina* eFP browser (http://bar.utoronto.ca/efp_camelina/cgi-bin/efpWeb.cgi) (Kagale et al., 2016). Genes without available data or no expression are excluded.

TPK CHANNELS

Identification of TPK Family in Plants

TPK/KCO family proteins are voltage-independent K^+ channels that consist of six members in Arabidopsis. These include five tandem-pore channels, AtTPK1-5, and a single K_{ir}-like channel, AtKCO3. These AtTPK channels possess four transmembrane segments and two pore-loop domains in tandem, whereas AtKCO3 has two transmembrane segments and a single pore-loop domain (Czempinski et al., 1999; Lebaudy et al., 2007; Voelker et al., 2010; Wang and Wu, 2013). Members of TPK/KCO family contain Ca^{2+}-binding EF hands in the cytosolic C-terminal region, and 14-3-3 protein binding sites in the cytosolic N-terminal region (Voelker et al., 2010; Wang and Wu, 2013). AtTPK1 is the first plant TPK channel to be identified via an Arabidopsis EST database search for the conserved K^+ channel pore domain motif TXGYGD (Czempinski et al., 1997; Sharma et al., 2013). Later, its homologs are found and characterized in tobacco (NtTPK1) (Hamamoto et al., 2008) and in strawberry (FaTPK1) (Wang et al., 2018a). Only limited effort has been made to survey the TPK/KCO channels in other plant species using genome-wide analysis, which identified 3 TPK family members in rice (Amrutha et al., 2007; Gomez-Porras et al., 2012), 10 in poplar (Gomez-Porras et al., 2012), and 9 in soybean (Rehman et al., 2017). We additionally identified 20 CsTPK channels in *Camelina*, with triplet homoeologs for AtTPK3/4/5 orthologs, and no homoeolog for AtKCO3 ortholog **(Figure 1)**. Moreover, the *Camelina* genome underwent further expansion in AtTPK1- and AtTPK2-like channels, with 6 and 5 homoeologs, respectively. These TPK/KCO channels in *Camelina* are phylogenetically divided into four major clades, with AtTPK1 and its close homoeologs in one clade, AtTPK2/3 and their close homoeologs in another large clade along with AtKCO3, and AtTPK4 and AtTPK5 with their corresponding homoeologs into two separate clades.

Subcellular Localization and Expression of TPK Channels

All the Arabidopsis TPK/KCO channels are localized to tonoplast except AtTPK4, which is localized to PM (Czempinski et al., 2002; Schönknecht et al., 2002; Becker et al., 2004; Gobert et al., 2007; Dunkel et al., 2008). Tonoplast localization is also observed for strawberry FaTPK1 (Wang et al., 2018a), tobacco NtTPK1 (Hamamoto et al., 2008), and rice OsTPKa (lytic vacuoles) and OsTPKb (protein storage vacuoles) (Isayenkov et al., 2011). The expression patterns of *AtTPK* and *AtKCO3* genes are assessed in-depth using qRT-PCR and promoter-reporter gene (GUS) fusions (Voelker et al., 2006, 2010). The overlapping expression is observed for *AtTPK1/5* and *AtKCO3* genes in vascular tissues, *AtTPK1/3* in root tips, and *AtTPK1/2/3/4* in pollen (Voelker et al., 2006, 2010). Tobacco *NtTPK1*, and rice *OsTPKa* and *OsTPKb* are ubiquitously expressed, similar to *AtTPK1* (Hamamoto et al., 2008; Isayenkov et al., 2011). Soybean *GmKCO1* (Glyma02g46930.1) and *GmKCO2* (Glyma19g40890.1) genes are differentially expressed in nodulated root hairs, implying their roles in nodulation (Rehman et al., 2017). In *Camelina*, almost all *CsTPK* genes are expressed in senescing leaves, with *AtTPK1*- and *AtTPK2*-like genes displaying the predominant expression in leaves throughout the

vegetative growth **(Figure 2C)**. This suggests the potential roles of these CsTPKs in K^+ remobilization from vacuoles of source to sink during the aging process. In addition, many *CsTPK* genes are expressed in early-seed development, suggesting these genes might be involved in seed maturation. Interestingly, *AtTPK3*-like genes in *Camelina* are expressed predominantly in late-seed development, implying their possible roles in K^+ storage into seed vacuoles to support the next growth cycle.

Function and Regulation of TPK Channels

The physiological roles of a few AtTPK channels have been examined in Arabidopsis. AtTPK1 mediates K^+ release from vacuoles under low-K^+ stress to maintain K^+ homeostasis, and plays a role in stomatal closure and seed germination (Czempinski et al., 2002; Bihler et al., 2005; Voelker et al., 2006; Gobert et al., 2007; Latz et al., 2007; Tang et al., 2020c). The tonoplast-localized AtTPK2 and AtTPK5 channels are shown to form functional K^+ transport systems in *E. coli* (Isayenkov and Maathuis, 2013), but their physiological roles in plants are yet to be elucidated. AtTPK3 is also present in the vacuole and may function in K^+ remobilization (Voelker et al., 2006; Dunkel et al., 2008; Höhner et al., 2019). On the other hand, AtTPK4 is an open rectifier that contributes to the K^+ conductance across the PM of pollen tubes (Becker et al., 2004).

Aside from studies in Arabidopsis, TPK homologues have also been analyzed in other plants. For example, tobacco NtTPK1 complimented K^+ uptake-deficient *E. coli* and exhibited high selectivity for K^+ over Na^+ in patch-clamp studies (Hamamoto et al., 2008). In rice, the transgenic lines overexpressing *OsTPKb* display higher K^+ content and better growth under low-K^+ and drought stress (Ahmad et al., 2016). In addition, vacuolar TPKs have been shown to be mechanosensitive and osmosensitive when examined using patch clamp experiments (Maathuis, 2011). In strawberry, vacuolar FaTPK1 is associated with fruit ripening, linking K^+ homeostasis to fruit quality and commercial value (Wang et al., 2018a).

In terms of regulation, the activity of plant TPK channels are dependent on cytosolic factors such as calcium (Ca^{2+}), 14-3-3 proteins and pH (Czempinski et al., 1999; Becker et al., 2004; Bihler et al., 2005; Gobert et al., 2007; Latz et al., 2007, 2013; Carraretto et al., 2013; Tang et al., 2020c). The maintenance of proper cytosolic pH has been shown to be critical for the channel activities of AtTPK1/4 in Arabidopsis (Becker et al., 2004; Gobert et al., 2007) and NtTPK1 in tobacco (Hamamoto et al., 2008). The interaction between 14-3-3 proteins and TPKs are confirmed in barley (HvTPK1) (Sinnige et al., 2005), rice (OsTPKb) (Isayenkov et al., 2011) and Arabidopsis (AtTPK1 and AtTPK5) (Latz et al., 2007; Voelker et al., 2010). Because TPKs harbor EF-hand motifs for Ca^{2+} binding, cytosolic Ca^{2+} elevation have been shown to activate AtTPK1 (Czempinski et al., 1999; Bihler et al., 2005; Latz et al., 2007), AtTPK3 (Carraretto et al., 2013), OsTPKb (Isayenkov et al., 2011), and NtTPK1 (Hamamoto et al., 2008). In addition, a Ca^{2+}-dependent protein kinase, CPK3, has been shown to phosphorylate and activate the AtTPK1 channel in response to salt stress (Latz et al., 2013). Further, Ca^{2+}-dependent CBL2/3-CIPK3/9/23/26 complexes also promote AtTPK1 channel activity to maintain K^+ homeostasis under low-K^+ stress (Tang et al., 2020c). Because these vacuolar channels are essential for plant adaptation to multiple stresses, more in-depth

analysis is required in order to fully understand the function of TPK channels (e.g., AtTPK2/3/5 and AtKCO3) at the physiological and mechanistic levels in Arabidopsis and other plant species.

KEA TRANSPORTERS

Identification of KEA Family in Plants

The KEA transporter family is a subgroup of the large Cation/Proton Antiporter (CPA) superfamily. These transporters are highly homologous to the bacterial K^+/H^+ antiporters KefB and KefC (Chanroj et al., 2012; Han et al., 2015). Six KEA members are encoded in the Arabidopsis genome, and phylogenetically, they diverge into two clades with AtKEA1/2/3 in clade I and AtKEA4/5/6 in clade II (Mäser et al., 2001; Chanroj et al., 2012). Both clades contain an N-terminal Na^+/H^+ exchange domain, but only clade I contains C-terminal KTN (K^+ transport, nucleotide binding) domain (Chanroj et al., 2012). Sequence homology to Arabidopsis AtKEAs resulted in finding 6 members in maize (Ye et al., 2013), 4 in rice (Ye et al., 2013), 12 in pear (Zhou et al., 2016) and soybean (Rehman et al., 2017), and 24 in wheat (Sharma et al., 2020). In *Camelina*, 18 CsKEA members are identified that form two major clades, with three homoeologs for each Arabidopsis ortholog **(Figure 1)**.

Expression Patterns of KEA Transporter Genes

In Arabidopsis, the expression patterns of all six *AtKEA* genes are evaluated in detail (Han et al., 2015). The promoter-GUS fusions reveal overlapping expressions of *AtKEA2/4/5/6* in root steles, with *AtKEA4* additionally expressed in root tips (Han et al., 2015). In leaves, all *AtKEA* genes are expressed in guard cells except *AtKEA6* (Han et al., 2015). *AtKEA1/2/3* genes are also expressed in leaf vasculature and petioles, whereas *AtKEA4/5/6* in trichomes (Aranda-Sicilia et al., 2012; Han et al., 2015; Höhner et al., 2019). In flowers, all *AtKEA* genes are expressed in the sepals, but only *AtKEA1* in pollen grains (Han et al., 2015). In wheat, the majority of *AtKEA1/2/3*-like genes are expressed in leaves, whereas *AtKEA4/5/6*-like genes are more broadly expressed in stem, spike, leaves and roots (Sharma et al., 2020). Similarly, *AtKEA1/2/3*-like genes in *Camelina* are also predominantly expressed in leaves, with *AtKEA1*-like genes displaying the highest levels **(Figure 2D)**. On the other hand, *AtKEA4/5/6*-like genes in *Camelina* are all expressed in root and stem during vegetative growth, and several are expressed at lower level in floral organs. These similar expression patterns in different plants suggest conserved functions of KEAs.

Subcellular Localization, Function and Regulation of KEA Transporters

AtKEA1 and AtKEA3 peptides are first detected in Arabidopsis chloroplast proteome (Zybailov et al., 2008). AtKEA1 and AtKEA2 proteins are later found to localize

to the inner envelope membrane of chloroplasts, whereas AtKEA3 is targeted to the thylakoid membrane (Kunz et al., 2014). The first functional study of the AtKEA transporter family came across by reconstituting AtKEA2 transporter in liposomes, which demonstrated cation/H^+ antiport activity with preference for K^+ (Aranda-Sicilia et al., 2012). Later, clade I AtKEA1/2/3 transporters are found to be critical for photosynthesis, chloroplast structure, osmoregulation, and K^+/pH homeostasis (Kunz et al., 2014). These plastidial AtKEA transporters are also important for stress-induced Ca^{2+} elevation (Stephan et al.). In addition, AtKEA3 transporter has been shown to increase photosynthetic efficiency under fluctuating light by reducing pH-dependent energy dissipation (Armbruster et al., 2014; Kunz et al., 2014; Höhner et al., 2019). The role of KEA transporters in chloroplast development is also conserved in rice as the *Osam1* (*Atkea2*-like) mutants display similar phenotypes with impaired photosynthetic activity and abnormal plastid structure (Sheng et al., 2014).

Clade II AtKEA4/5/6 transporters are found in Golgi, Trans-golgi network and prevacuolar compartment (Zhu et al., 2018; Wang et al., 2019). These AtKEA transporters are crucial for maintaining K^+/H^+ homeostasis in the endomembrane networks to support plant growth and adapt to various abiotic stresses. The disruption of these genes in Arabidopsis (*Atkea4 Atkea5 Atkea6* triple mutants) resulted in a broad spectrum of defects in the mutant plants, including hypersensitivity to low-pH, low- and high-K^+, high-Na^+ and high Li^+ stresses (Zhu et al., 2018; Wang et al., 2019). The mutants also show more acidic pH in the endomembranes and growth defects in skotomorphogenesis (Zhu et al., 2018; Wang et al., 2019). However, the precise and specific physiological roles of AtKEA transporters and their underlying regulatory mechanisms in both chloroplast and endomembranes remain unexplored. In addition, there is minimal studies of KEA transporter in other plant species, which is worth further investigation.

CONCLUSIONS

In this review, we provide the current knowledge of K^+ transport systems in angiosperms. **Figure 3** summarizes the physiological roles of known K^+ transport systems in Arabidopsis and other plant species. Essentially, plants possess elaborate K^+ transport systems to facilitate K^+ uptake from the rhizosphere and further transport from roots to the aerial parts. At a cellular level, K^+ needs to be distributed into various cell-types and subcellular compartments to support numerous physiological functions. While KUP/HAK/KT transporters and *Shaker*-like channels play major roles in K^+ uptake from soil and translocation to various tissues and organs, TPK channels and KEA transporters mediate K^+ transport in and out of different subcellular compartments. *Shaker*-like and TPK channels share complementary functions in stomatal movement and pollen tube growth.

Genome-wide query and transcriptomic analysis of the K^+ transporter genes can open a gateway for comparative genomic analysis and functional studies. In general, we find that the K^+ transporter family genes have expanded in both diploid and polyploid crops over the course of evolution, implicating the need to efficiently acquire and distribute K^+ to support plant growth under the changing nutrient status. Using *Camelina* as a potential polyploid model, we show how the polyploidization led to the expansion of

specific K^+ transporter genes. For example, the copy numbers of *AtTPK1* and *AtTPK2*-like genes encoding vacuolar K-remobilizing transporters have doubled beyond its ploidy norm, which may contribute to enhanced K^+ remobilization in response to K^+-deficiency. In addition, the comparison of the expression profile of the K^+ transporter genes in *Camelina* with other plant species provides us with additional insights to the functional conservation and divergence in a specific tissue. Leveraging such publicly-available datasets will initiate further functional studies in economically important crops.

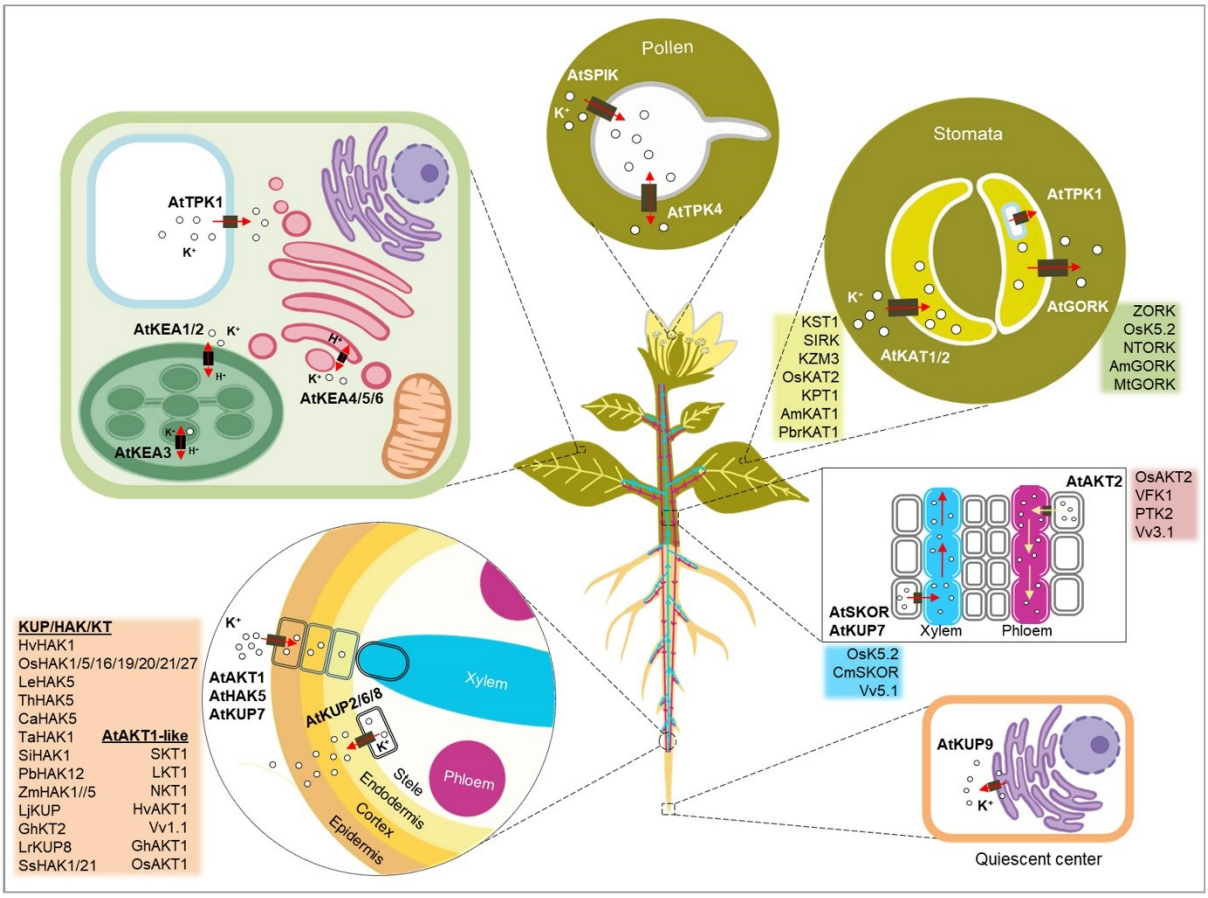

Figure 3. Physiological roles of the K+ transporters and channels in Arabidopsis and other plant species discussed in this review. For simplicity, the Arabidopsis genes are placed within the diagram and their closely-related genes from the other plant species are listed in the colored boxes.

While we find the functional conservation of several key K^+ transporters across multiple plant species, some variations exist due to the difference in genetic composition and plant architecture, as observed in monocots and dicots. Therefore, transitioning our studies to different crop species is critical to find novel K^+ transporters that evolved to function in a specific cell type, organ or tissue. However, the increased genetic complexity of crops imposes challenges in the functional analysis as the expanded genes resulting from genome or gene duplication events present high genetic redundancies. Genome-wide association studies and transcriptomic analyses may reveal natural genetic diversities present in a crop species under low-K^+ stress and will help identify important

genetic loci and genes that contribute to high KUE. Further genetic analysis using the CRISPR-Cas9 system to generate single and higher order mutants of these candidate K^+ transporters will be needed to study the dosage-effect and the functional dominance among the closely related genes. Moreover, a large effort is required to dissect the regulatory mechanisms of the K^+ transporters and channels, even in Arabidopsis. Such studies will aid in unlocking the complexity of the K^+ transport in crops and ultimately lead to the genetic engineering of high KUE crops to support sustainable agriculture.

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SUPPLEMENTAL DATA

Table S1. The K^+ transporter family genes are identified by blasting Arabidopsis genes against the *Camelina* genome in National Center for Biotechnology Information/NCBI Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The Genome Locus IDs are obtained from The Prairie Gold Novel Industrial Oilseed Crops for Canada Database (http://Camelinadb.ca/).

KUP transporters

C. sativa gene	NCBI gene ID	Locus ID
CsKUP1	104786666	Csa05g033830.1
CsKUP2	104700492	Csa07g013260.1
CsKUP3	104749889	Csa16g014860.1
CsKUP4	104792922	Csa06g044970.1
CsKUP5	104785347	Csa05g012200.1
CsKUP6	104782535	Csa04g054920.1
CsKUP7	104773919	Csa00765s030.1
CsKUP8	104773486	Csa00555s040.1
CsKUP9	104717940	Csa10g019510.1
CsKUP10	104785736	Csa05g016130.1
CsKUP11	104722648	Csa11g021270.1
CsKUP12	104729903	Csa12g010290.1
CsKUP13	104716803	Csa10g008710.1
CsKUP14	104721463	Csa11g009580.1
CsKUP15	104701670	Csa07g037130.1
CsKUP16	104712574	Csa09g071520.1
CsKUP17	104750873	Csa16g031900.1
CsKUP18	104734930	Csa13g011060.1
CsKUP19	104708178	Csa08g055240.1
CsKUP20	104769124	Csa20g012550.1
CsKUP21	104705763	Csa08g006910.1
CsKUP22	104735506	Csa13g017680.1
CsKUP23	104769726	Csa20g021370.1
CsKUP24	104731547	Csa12g039460.1
CsKUP25	104718319	Csa10g023260.1
CsKUP26	104723040	Csa11g026300.1
CsKUP27	104732243	Csa12g057800.1
CsKUP28	104757506	Csa17g048650.1
CsKUP29	104741707	Csa14g038890.1
CsKUP30	104777150	Csa03g034560.1
CsKUP31	104781906	Csa04g047350.1
CsKUP32	104786046	Csa05g020170.1
CsKUP33	104703617	Csa07g061490.1
CsKUP34	104787142	Csa05g048310.1
CsKUP35	104752700	Csa16g052160.1
CsKUP36	104723858	Csa11g041660.1
CsKUP37	104719114	Csa10g033400.1
CsKUP38	104732373	Csa12g063140.1

***Shaker*-like channels**

C. sativa gene	NCBI gene ID	Locus ID
CsKAT1	104760275	Csa18g003810.1
CsKAT2	104773143	Csa00470s010.1
CsKAT3	104724759	Csa11g065760.1
CsKAT4	104723242	Csa11g030180.1
CsKAT5	104718492	Csa10g026890.1
CsKAT6	104792984	Csa06g046620.1
CsKC1	104716910	Csa10g009740.1
CsKC2	104730005	Csa12g013340.1
CsKC3	104721563	Csa11g010590.1
CsAKT1	104703477	Csa07g060200.1
CsAKT2	104754062	Csa16g050840.1
CsAKT3	104787078	Csa05g044590.1
CsAKT4	104722816	Csa11g023970.1
CsAKT5	104731293	Csa12g034730.1
CsAKT6	104718095	Csa10g020970.1
CsAKT7	104716929	Csa10g009960.1
CsAKT8	104730022	Csa12g013520.1
CsAKT9	104727609	Csa11g010760.1
CsSPIK1	104708830	Csa08g061540.1
CsSPIK2	104752193	Csa16g047230.1
CsSPIK3	104714024	Csa09g091780.1
CsSPIK4	104705047	Csa07g056630.1
CsSKOR1	104763904	Csa19g004960.1
CsSKOR2	104744366	Csa15g002540.1
CsSKOR3	104779325	Csa01g002460.1
CsGORK1	104719368	Csa10g041020.1
CsGORK2	104732640	Csa12g074910.1
CsGORK3	104724113	Csa11g051270.2

TPK channels

C. sativa gene	NCBI gene ID	Locus ID
CsTPK1	104732132	Csa02g058240.1
CsTPK2	104726111	Csa11g088860.2
CsTPK3	104761611	Csa18g027180.1
CsTPK4	104726113	Csa11g088880.1
CsTPK5	104761614	Csa18g027170.2
CsTPK6	104732168	Csa02g058270.1
CsTPK7	104760260	Csa18g003660.1
CsTPK8	104771899	Csa20g079510.1
CsTPK9	104728525	Csa11g065600.1
CsTPK10	104771898	Csa20g079500.1
CsTPK11	104724744	Csa11g065610.1
CsTPK12	104723266	Csa11g030410.1
CsTPK13	104718510	Csa10g027060.1
CsTPK14	104731722	Csa12g044220.1
CsTPK15	104743114	Csa14g002560.1
CsTPK16	104759825	Csa03g002540.1
CsTPK17	104754223	Csa17g001290.1
CsTPK18	104707925	Csa08g052700.1
CsTPK19	104737605	Csa13g055470.1
CsTPK20	104713412	Csa02g004960.1

KEA transporters

C. sativa gene	NCBI gene ID	Locus ID
CsKEA1	104758559	Csa03g001430.1
CsKEA2	104738613	Csa14g001340.1
CsKEA3	104754337	Csa17g002310.1
CsKEA4	104712144	Csa02g001700.1
CsKEA5	104705328	Csa08g001760.1
CsKEA6	104737713	Csa13g056760.1
CsKEA7	104707637	Csa08g048850.1
CsKEA8	109124494	Csa13g052690.1
CsKEA9	104737337	Csa02g011190.1
CsKEA10	104773076	Csa00441s170.1
CsKEA11	104766876	Csa19g049260.1
CsKEA12	104702640	Csa01g044850.1
CsKEA13	104726430	Csa02g044560.1
CsKEA14	104761171	Csa18g022560.1
CsKEA15	104725674	Csa11g083230.1
CsKEA16	104769416	Csa20g017390.1
CsKEA17	104735193	Csa13g014660.1
CsKEA18	104705446	Csa08g002990.1

Chapter 4: Rice SKS1 is a Transcriptional Regulator in Response to Low-K Stress

Contributions

D. Lhamo performed all the bioinformatic analyses and contributed to the writing of the manuscript.

Preface

The two previous chapters represent the first effort in characterizing P and K channels/transporters in *Camelina* at genome and transcriptome levels. These studies showed that the transcription of nutrient channels and transporters are subjected to changes during the plant life cycle as well as in response to the changing nutrient status. These transcriptional regulations are under the control of transcriptional factors (TFs), however, only few TFs were identified in Arabidopsis that are known to control the expression of low-K responsive genes including K channels and transporters. Attempts to identify novel TFs are ongoing, with the aim to decode the complex regulatory networks including nutrient transport and signaling genes. This chapter explores the potential involvement of such TF in rice crop, known as SKS1 (Sensitive to K Starvation 1). It is a homolog of Arabidopsis AtSTOP1 (Sensitivity to proton rhizotoxicity) TF that is known to provide tolerance to various abiotic stresses. Rice was chosen because the T-DNA insertional mutants were readily available to evaluate the functional conservation. Bioinformatic analysis of RNA-seq data generated in the present study unravels the major low-K responsive genes that are transcriptionally regulated by SKS1, including signaling and nutrient transporter genes. In addition, SKS1 may be involved in signal integrations between K and P macronutrients, and between root and shoot tissues.

INTRODUCTION

Potassium (K) macronutrient is essential for many physiological processes, including osmoregulation, anion balance, pH homeostasis, enzyme activation, stomatal movement, photosynthesis and pollen tube growth (Marschner and Rengel, 2012; Luan et al., 2017; Wang and Wu, 2017; Lhamo et al., 2021). However, crops in agricultural lands are often challenged with K deficiency, requiring complex signaling networks to decode the low-K signals by cytosolic sensors and kinases that regulate the downstream transcriptional and posttranslational responses for adaptation (Wang and Wu, 2013).

In *Arabidopsis thaliana*, low-K induces Reactive Oxygen Species (ROS) production via NADPH oxidase RHD2 and peroxidase RCI3 in root cells that regulates the transcription of low-K responsive genes including *AtHAK5* (Shin and Schachtman, 2004; Kim et al., 2010). ROS also activates calcium (Ca) channels, elevating the cytosolic Ca to transduce signals to Ca sensors and kinases that post-translationally regulated K channels and transporters (Li et al., 2006; Luan, 2009; Laohavisit et al., 2012; Tang et al., 2020a). For instance, AtCBL1/9-AtCIPK23 (Calcineurin B-like proteins and their interacting kinase) complex activates the K uptake transporters (AtAKT1 and AtHAK5) in the plasma membrane (PM) to enhance K acquisition; and in parallel AtCBL2/3-AtCIPK3/9/23/26 targets the tonoplast K channels (AtTPK1) to release K from vacuole into the cytoplasm; both of which facilitate cellular K homeostasis in response to low-K conditions (Li et al., 2006; Xu et al., 2006; Cheong et al., 2007; Ragel et al., 2015; Tang et al., 2020a, 2020b). This CBL1-CIPK23-AKT1 pathway is also conserved in rice (Li et al., 2014), implicating the efficacy of translational research from Arabidopsis to crops.

In Arabidopsis, AtCIPK23 is shown to be transcriptionally regulated by AtSTOP1 (Sensitivity to proton rhizotoxicity), a zinc finger transcriptional factor (TF) in response to different abiotic stresses (Sawaki et al., 2009; Sadhukhan et al., 2019; Ojeda-Rivera et al., 2020). Because the expression of AtCIPK23 is important for low-K adaptation, we evaluated the phenotype of *stop1* mutants under low-K stress and found it to be hypersensitive to K deficiency (data not shown). A recent study also reported AtSTOP1 regulation of CIPK23 to be essential in conferring salt and drought tolerance (Sadhukhan et al., 2019). AtSTOP1 appears to be a master regulator involved in regulating genes encoding different signaling and transport processes that allow adaptation to multiple stresses including Aluminum (Al) and proton rhizotoxicities (Iuchi et al., 2008; Sawaki et al., 2009; Ojeda-Rivera et al., 2020), low-phosphate with excess Fe (iron) or Al (Balzergue et al., 2017; Mora-Macías et al., 2017; Godon et al., 2019), hypoxia (Enomoto et al., 2019), salinity and drought (Sadhukhan et al., 2019).

To better understand how AtSTOP1 might play a role in low-K adaptation, we used the translational biology approach to dissect its transcriptional regulatory mechanism in rice (*Oryza sativa*). We first isolated T-DNA insertion rice mutants denoted as *sensitive to K starvation 1* (*sks1*) that have a mutation in Cys_2-His_2-type zinc finger protein, the homolog of AtSTOP1. SKS1 as a putative transcriptional factor was found to be localized to the nucleus. Our study revealed *sks1* to be hypersensitive to low-K stress, and K and P (Phosphorus) composition in the mutants were altered under low-K and the control conditions. Further transcriptomic analyses of *sks1* and wild type (WT, Hwayoung) roots and shoots in response to low-K and the control conditions revealed the roles of SKS1 in

modulating expression of genes with potential functions in signal transduction, ROS production, immune response, cell wall synthesis, ion transport and homeostasis. This work together with previous reports implicate SKS1 in integrating multiple stress signals via regulation of diverse kinases including the nutrient sensing CIPKs that fine-tune various nutrient transporter activities.

RESULTS

Rice *sks1* mutant plants are hypersensitive to low-K stress

In Arabidopsis, we identified a transcription factor, AtSTOP1 that regulates the expression of CIPK23, a key component involved in plant low-K response. In an effort to investigate whether the mechanism is conserved in other plant species, we used the monocot plant rice as an alternative model. To pursue such a goal, we isolated a T-DNA insertional mutant (PFG_A_23757) from the Korean rice T-DNA library (http://cbi.khu.ac.kr/RISD_DB.html), and designated it as "*sks1*" (*sensitive to K starvation 1*) that is potentially defective in K nutrition (**Figure 1**). The T-DNA insertion sites were identified by PCR-based genotyping to isolate the homozygous *sks1* mutants (**Figure 1A-B**). Sequencing analysis revealed a short DNA fragment of the gene is shifted into the middle of two T-DNAs.

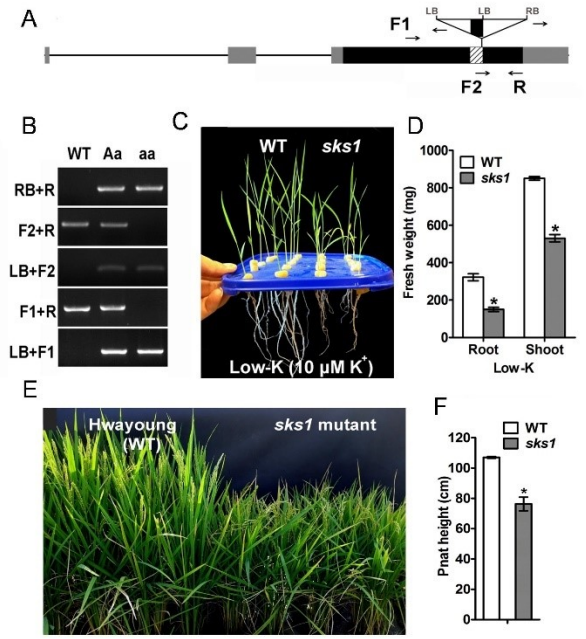

Figure 1. Identification of the *sks1* T-DNA insertional mutants with hypersensitivity to low-K conditions. (A) Schematic diagram of T-DNA insertion in *sks1*. Exons and introns are depicted to scale by boxes and lines, respectively. The coding region of the gene is shown as black boxes while the 5′ and 3′ UTR of the cDNA is shown as light-shaded boxes. The position of the T-DNA is indicated by the triangle, and the deleted genomic region by the hatched box. **(B)** PCR-based genotyping of WT, heterozygous (Aa) and homozygous *sks1* mutant (aa) plants. LB, left border;

RB, right border. Primers are indicated in (A). **(C)** Phenotype of *sks1* mutants under low-K (10 μM) hydroponic condition. Four-day old rice seedlings were treated with low-K for 12 days. **(D)** Biomass of the low-K-treated *sks1* mutants. **(E)** Phenotype of 5-months-old *sks1* mutants in the field, without additional K supply. **(F)** Quantitative measurement of the plant height in the field test. Data are mean ± standard error (SD) from four independent experiments. Asterisk indicates statistically significant difference (P<0.01).

We then performed phenotypic analysis of WT (Hwayoung) and *sks1* under low-K (10 μM) hydroponic condition to evaluate the potential role of SKS1 in low-K response. Indeed, *sks1* displayed hypersensitive response to low-K compared to WT plants, with shorter stature and reduction in shoot and root biomass (**Figure 1C-D**). The field studies also revealed compromised plant growth of 5-month-old *sks1* compared to WT, with a significant reduction in height when no additional K was supplied to the natural soil (**Figure 1E-F**). These data suggest the involvement of SKS1 in adaptive response to low-K stress.

SKS1 is a nuclear-localized transcriptional factor

SKS1 (LOC_Os01g65080) is predicted to encode a putative C_2H_2 zinc finger protein with 523 amino acids. The alignment of SKS1 protein sequence to AtSTOP1 revealed 55.5% identity (**Figure S1**). To determine the transcriptional regulatory function of SKS1, we first evaluated the subcellular localization of SKS1 protein by a transient expression assay (**Figure 2A**). Yellow fluorescent protein (YFP)-tagged SKS1 was expressed in rice mesophyll protoplasts. As expected, the fluorescence of SKS1-YFP was detected in the nucleus, which coincided with HMGB1-CFP, a nuclear protein marker. To further evaluate the role of SKS1 in transcriptional regulation, we quantified the expression of *OsCIPK23* (LOC_Os07g05620) in *sks1* since its Arabidopsis ortholog, AtSTOP1 was previously reported to induce the expression of AtCIPK23 (Sawaki et al., 2009; Sadhukhan et al., 2019; Ojeda-Rivera et al., 2020). OsCIPK23 was significantly down-regulated in both roots and shoots of *sks1* compared to WT (**Figure 2B**). This suggests SKS1 positively regulates the expression of OsCIPK23, which can activate the K channels and transporters to promote K acquisition.

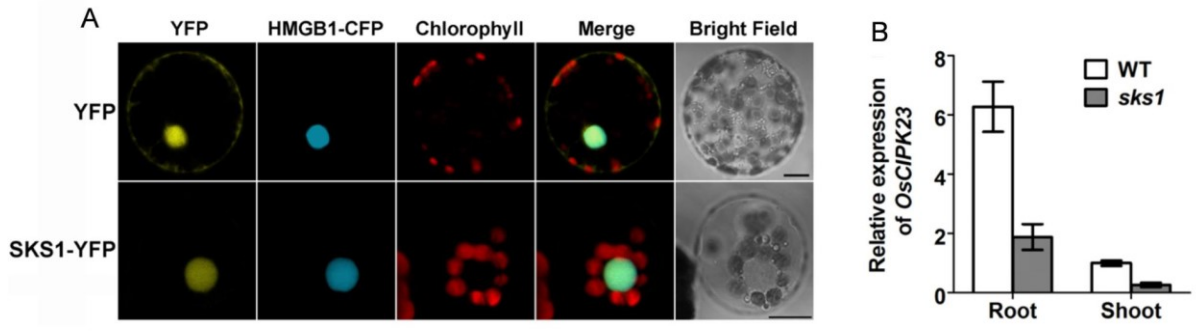

Figure 2. SKS1 is localized to the nucleus and regulates *OsCIPK23* expression.

(A) Fluorescent signals from rice mesophyll protoplasts transiently expressing SKS1-YFP and HMGB1-CFP. Scale bar = 10 μm. **(B)** Relative expression of *OsCIPK23* gene in *sks1*. Data are derived from quantitative real-time PCR analysis and depicted as mean ± SD (n=3).

Distinct transcriptomic profiles in root vs shoot of *sks1* mutant plants in response to low-K

To elucidate the function of SKS1 in transcriptional regulation in response to low-K, we performed RNA-sequencing analyses of WT and *sks1* plants grown under low-K (0 mM) and the control (3 mM K) conditions (**Figure 3A**). We first evaluated differently expressed (DE) genes [$-1.5 \geq \log_2(FC) \geq 1.5$; $P_{adj} \leq 0.05$] in roots since it is the main tissue responsible for nutrient acquisition (**Table S1-S3**). A total of 1,119 DE genes were found to be responsive to low-K (-K/+K) in the WT roots, whereas 1,681 and 816 genes in the *sks1* in response to low-K (*sks1*/WT -K) and the control (*sks1*/WT +K) conditions, respectively (**Figure 3B**). While a larger number of genes in WT roots were induced (700) than repressed (419), more genes in *sks1* were found to be repressed (1,300) than induced (381) under low-K. Less DE genes (383 down and 433 up) were found in *sks1* roots under the control codition compared to low-K stress, suggesting a more significant role of SKS1 in root response to low-K. Overlapping of these DE genes revealed that 18% (126/700) of low-K inducible genes in WT were downregulated in *sks1* roots under low-K, among which, only four genes were also repressed in *sks1* under the control condition (**Figure 3C**). In contrast, the substantial portion (72%, 942/1,300) of *sks1* root genes were uniquely repressed under low-K, whereas 38% (147/383) under the control condition. Among the low-K-repressed genes in WT, only 1% (5/419) were induced in the *sks1* roots in response to low-K (**Figure 3D**). 53% (202/381) of *sks1* root genes were uniquely expressed in response to low-K, whereas 60% (688/1,306) under the control condition. The overlapping genes between WT and *sks1* are likely under the transcriptional control of SKS1 in response to low-K (**Figure3E, Table S4**). These include a number of TFs that belong to putative zinc finger proteins (LOC_Os02g52870, LOC_Os03g60560, LOC_Os03g60570, LOC_Os06g09310, LOC_Os06g34620 and LOC_Os06g34860), MYBs (LOC_Os05g04210 and LOC_Os09g23620) and WRKY5/24/70 (LOC_Os05g04640, LOC_Os01g61080 and LOC_Os05g39720). Misregulation of these TFs might also impact the expression of many DE genes found in the *sks1* roots in response to low-K.

In shoots, 349 (287 up and 62 down) genes were responsive to low-K in WT, 1,325 (499 down and 826 up) and 1,797 (491 down and 1,306 up) in *sks1* in response to low-K and the control conditions, respectively (**Figure 3F, Table S5-S7**). Among these,15% (44/287) of WT low-K inducible genes were downregulated in *sks1* under low-K and only 2% (5/287) under the control condition (**Figure 3G**). 25% (125/499) of *sks1* shoot genes were uniquely repressed in response to low-K, 32% (156/491) genes under the control condition, with 330 genes overlapping between the two conditions. Among the low-K repressed genes in WT shoots, 23% (14/62) were upregulated in *sks1*, with only 5% (3/62) shared with *sks1* under the control condition (**Figure 3H**). A massive portion of genes (74%, 615/826) upregulated in *sks1* under low-K were shared with the control condition, while 24% (197/826) and 53% (688/1306) were uniquely expressed in low-K and the control conditions, respectively. These data indicated that a large portion of shoot

genes were repressed by SKS1 independent of K status. However, there were several low-K responsive genes in WT shoots that were controlled by SKS1 specifically under low-K stress (**Figure 3I, Table S8**). Many of them encode enzymes especially transferase family proteins, and several ubiquitin-related proteins were found. The expression of genes encoding enzymes involved in protein synthesis, and one putative MYB TF (LOC_Os01g50110) were induced by SKS1 in response to low-K. Putative MATE (LOC_Os10g20350) and magnesium transporters (LOC_Os04g35160) were upregulated in WT and downregulated in *sks1* under low-K stress. These data suggest SKS1 mainly regulates shoot metabolic processes in response to low-K stress.

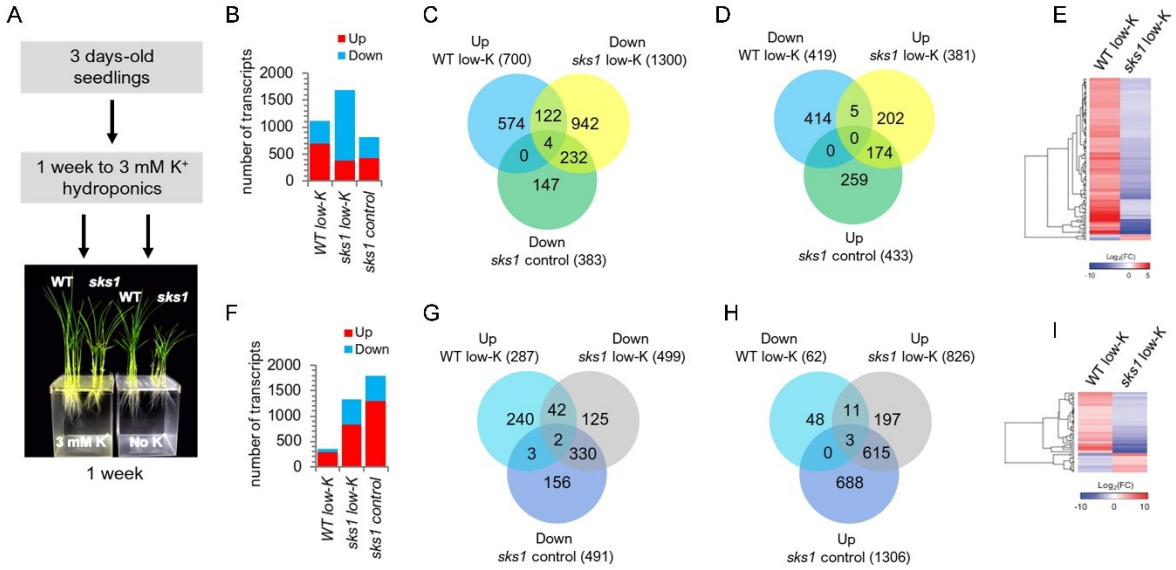

Figure 3. Transcriptomic profiling revealed discrete root and shoot response of SKS1 to low-K and the control conditions. (A) Experimental design for RNA-sequencing analysis. Details are found in the method section. **(B, F)** The number of root and shoot DE genes [-1.5 ≥ logFC ≥ 1.5; P_{adj} ≤ 0.05] in WT (-K/+K) and *sks1* (*sks1*/WT -K) that are responsive to low-K stress, and *sks1* under the control (*sks1*/WT +K) condition. **(C, G)** Venn diagrams displaying the overlap of the low-K inducible genes in WT and *sks1* under low-K and the control conditions in roots and shoots, respectively. **(D, H)** Venn diagrams displaying the overlap of low-K repressed genes in WT and *sks1* under low-K and the control conditions in roots and shoots, respectively. (**E, I**) Heatmaps of overlapping genes between WT and *sks1* in response to low-K in roots and shoots, respectively.

Differential expression profiles of redox and immune response genes in roots and shoots of *sks1* mutants

To identify the biological processes regulated by SKS1 in response to K^+ availability, we performed gene ontology (GO) analyses using the public GO database agriGO v2.0 (Tian et al., 2017). In response to low-K, genes encoding proteins involved in response to stimulus/stress, oxidation reduction (redox), cell death and carbohydrate metabolic processes were significantly enriched (FDR < 0.05) in WT and more so in *sks1*

roots. In addition, many low-K responsive genes in WT roots were involved in photosynthesis and generation of precursor metabolites and energy (**Figure 4A**). On the other hand, low-K responsive genes in *sks1* roots were mainly involved in macromolecule modification and cell wall organization or biogenesis. These biological processes were also enriched in *sks1* shoots under both conditions, in addition to cell death and chitin metabolic process (**Figure 4B**). Redox and response to stress were significantly enriched in WT shoots, but more DE genes in *sks1* shoots were involved in these processes irrespective of the conditions. Reproductive process was specifically enriched in *sks1* shoots under normal growth. Detailed GO enrichment analyses of WT and *sks1* DE genes in roots and shoots responsive to low-K and the control conditions are presented in the supplementals (**Table S9-S14**).

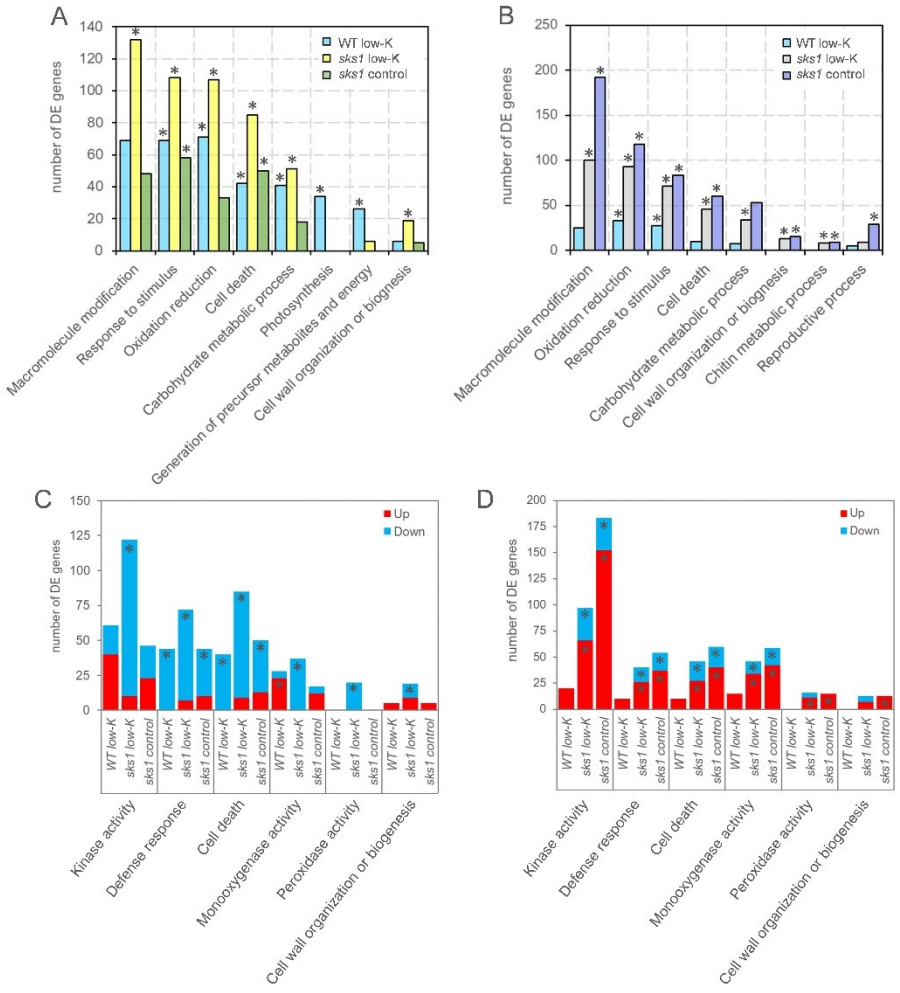

Figure 4. Gene ontology (GO) enrichment analyses of WT and *sks1* DE genes in response to low-K and the control conditions. (**A, B**) Biological processes and (**C, D**) molecular processes affected in roots and shoots, respectively. Asterisk represents overrepresented categories with FDR<0.05.

Analysis of molecular processes revealed that the expression of genes encoding kinases, monooxygenases and cell wall organization or biogenesis were largely induced in WT roots, whereas these genes were highly downregulated in *sks1* roots especially in response to low-K (**Figure 4C**). Other redox producing genes that encode peroxidases were specifically downregulated in *sks1* roots in response to low-K. On the other hand, WT and *sks1* plants shut down a large number of defense response and cell death genes in response to low-K, although several genes were found to be induced in *sks1* roots under both conditions. Contrarily, the genes involved in these immune responses were low-K inducible in WT shoots, whereas more were induced and few repressed in *sks1* shoots (**Figure 4D**). In contrast to roots, kinase, Monooxygenase and peroxidase genes were largely upregulated in *sks1* shoots in response to both conditions. Genes involved in cell wall organization or biogenesis were more upregulated in the *sks1* shoots under the control than low-K stress. These data suggest the contrasting roles of SKS1 in transcriptional regulation of large numbers of kinases, redox and immune response genes in roots and shoots.

Mis-regulation of protein kinase genes in *sks1* mutants

Because the kinase activity was the most overrepresented group in response to low-K, we compared the expression patterns of kinase genes between WT and *sks1* to assess gene overlaps and differences. Intriguingly, we found different kinase genes to be affected in *sks1* compared to WT in response to low-K, with 10% (6/62) of low-K responsive kinases in WT downregulated in *sks1* roots (**Figure 5A, Table S15**). These include OsWAK125 (LOC_Os12g29430), putative TKL_IRAK_DUF26-lf.1 (LOC_Os10g34220), CRINKLY4 (LOC_Os08g28710), STE_MAPK3K.19 (LOC_Os05g46760), ITPK4 (LOC_Os02g26720) and receptor-like kinase (LOC_Os02g13780) that were upregulated by SKS1 in roots specifically under low-K except CRINKLY4, which was also induced under the control condition. A large number of kinase genes were uniquely expressed in *sks1* roots in response to low-K, which might be caused by the mis-regulation of other low-K responsive TFs in the mutants. Compared to WT, *sks1* roots shared more similar expression patterns (7 up and 18 down) between the two conditions, suggesting that these kinases might be regulated by SKS1 independent of the K status. In shoots, 6 low-K responsive kinase genes (23) in WT overlapped in similar expression patterns with *sks1* under low-K stress and 15 under the control condition. Among these, only one leucine-rich repeat family protein (LOC_Os02g13410) showed contrasting expression between WT and *sks1* shoots specifically in response to low-K. A large portion of shoot *sks1* genes (79%, 77/97) in response to low-K were similarly mis-regulated under the control condition.

To better understand kinase groups that are responsive to low-K in WT and *sks1*, we further examined the number of kinase genes that belong to a specific group or family. This analysis revealed that TKL (Tyrosine Kinase-like), STE (homolog of yeast Sterile7/11/20), CAMK (calcium/calmodulin-dependent protein kinases), WAK (Wall-associated kinases) and Serine/Threonine (S/T) kinases were the major groups responsive to low-K in WT roots (**Figure 5B**). However, more kinases in these groups except STE were affected in the *sks1* roots in response to low-K, with additional kinases

in Cysteine-rich, Lectin-like and SHR5 receptor groups. In contrast to roots, S/T and lectin-like kinases were more responsive to low-K in WT shoots compared to the other groups (**Figure 5C**). Between the two, lectin-like receptor kinases were mis-regulated at a greater extent in *sks1* shoots under both conditions, along with WAK kinases. TKL and S/T kinases were most affected in *sks1* shoots under normal growth. These data show distinct kinase groups responsive to low-K between WT shoots and roots, and between WT and *sks1*. Overall, a larger number of kinases were affected in *sks1* roots specifically in response to low-K, whereas more in shoots under the control condition. This suggests SKS1 modulates transcripts of different kinase groups in roots to respond to low-K stress, and shoots under the control condition to support normal plant development.

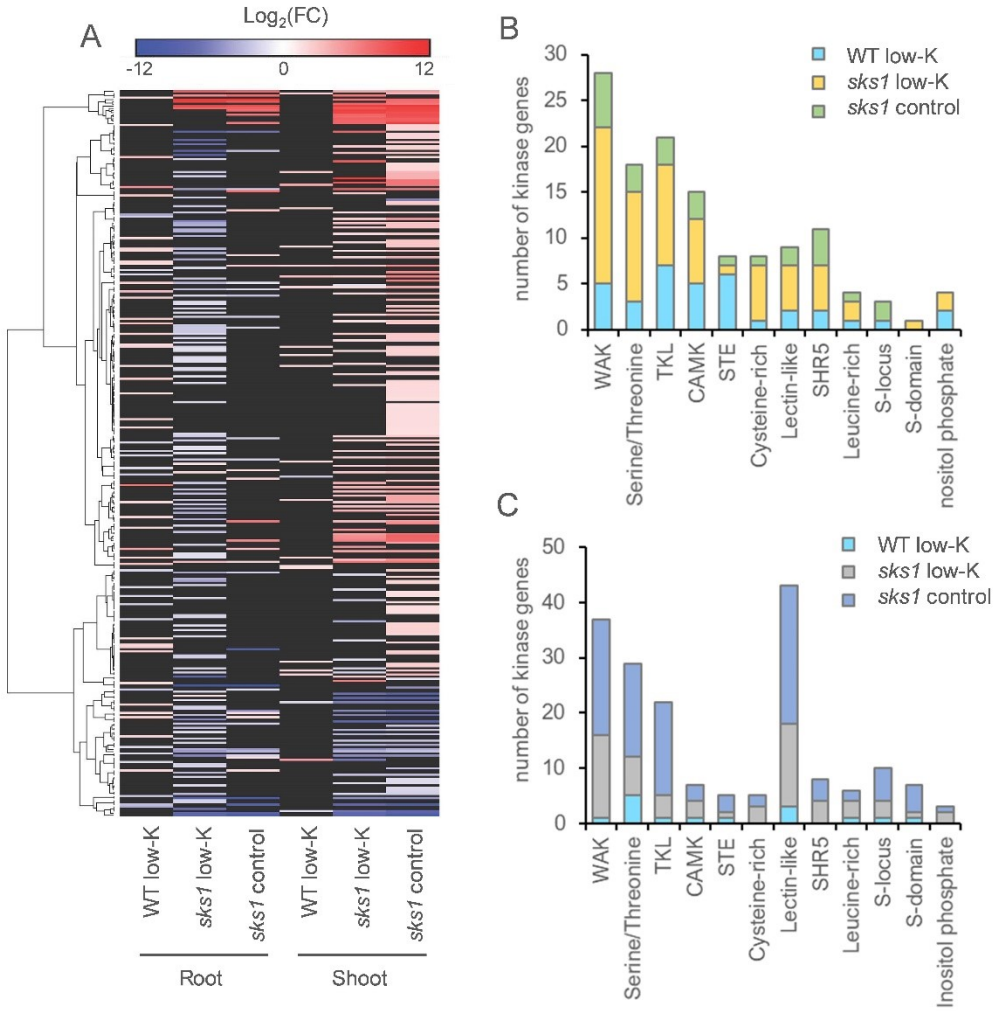

Figure 5. *sks1* displayed distinct expression patterns of various kinase groups in roots and shoots. **(A)** Heatmap with gene expression of kinases in WT and *sks1* roots and shoots responsive to low-K and the control conditions. **(B, C)** The number of genes belonging to different kinase groups responsive to low-K and the control conditions in WT and *sks1* roots and shoots, respectively.

Altered nutrient compositions and transporter gene expression in *sks1* mutants

Plants respond to low-nutrient stresses primarily via transcriptional regulation of nutrient transporter genes, which are activated at the post-translational level by kinases (Luan et al., 2017; Wang and Wu, 2017; Brumbarova and Ivanov, 2019; Saito and Uozumi, 2020; Tang et al., 2020a). To understand how the mis-regulation of kinases in *sks1* might impact nutrient accumulation, we quantified K and P contents in WT and *sks1* roots and shoots under low-K and the control conditions (**Figure 6A**). P was measured because it is one of the essential macronutrients, and AtSTOP1 was previously shown to control P starvation response (Balzergue et al., 2017; Mora-Macías et al., 2017). Surprisingly, we found no changes in K content in both shoot and root tissues of *sks1* under low-K despite a significant impact on plant growth (**Figure 6B**). Instead, *sks1* compared to WT, accumulated less K in roots and more in shoots under the control condition. Astonishingly, P content was more altered than K in both conditions. Higher level of P was found in both tissues of *sks1* under the control condition, and only in shoot under low-K stress (**Figure 6C**). These quantitative analyses of nutrients suggest the possible involvement of SKS1 in signal integration between K and P to maintain cellular ion homeostasis, which is essential for plants to adapt to low-K stress and support normal plant development.

We further examined the expression profile of DE nutrient transporters in WT and *sks1* roots and shoots in response to low-K and the control conditions to elucidate their impacts on K and P accumulation (**Figure 6D-6E**). Several K transporter genes were found to be low-K inducible in WT roots, which included putative SKOR (LOC_Os06g14030), 2 members of CHX (LOC_Os12g42200 and LOC_Os05g02240) and other putative K transporters/channels (LOC_Os08g36340 and LOC_Os02g39910). Only one CHX (LOC_Os05g02240) was found to be differentially expressed in *sks1* roots under the control condition, while several different K transporters were downregulated in *sks1* under low-K stress including a putative AKT1 (LOC_Os01g45990) and CHX (LOC_Os12g42200) (**Figure 6D, Table S16**). However, few sodium transporters were upregulated in *sks1* roots such as OsHKT1;5 (LOC_Os01g20160) under both conditions and a putative NHX transporter (LOC_Os05g31730) under low-K, which might be capable of transporting K. Ammonium (NH_4) transporter genes were downregulated in WT and *sks1* roots in response to low-K, but upregulated in *sks1* under the control condition, which might disrupt K transport in roots.

Compared to WT, P transporter genes were mostly down-regulated in *sks1* in response to low-K, but expressed under the control condition (**Figure 6D**). In addition, more citrate efflux transporter genes were expressed in *sks1* under the control condition, but repressed under low-K stress. These putative MATE transporters release organic acids that might enhance P uptake in *sks1* roots under the control condition. These data

are consistent with the higher P accumulation in *sks1* roots under the control condition compared to WT. More sulfate (S) and peptide/nitrate (NO_3) transporter genes were downregulated in *sks1* roots especially in response to low-K stress. The mis-regulation of many of these transporters might disrupt ion homeostasis in roots, which might affect nutrient transport and balance in shoots.

The expression of K, Na, P and organic acid transporter genes were induced in WT, but less were expressed in *sks1* shoots in response to low-K (**Figure 6E**). Several nutrient transporters were repressed in *sks1* shoots, more frequently under low-K and the control condition (**Figure 6E**). K accumulation observed in *sks1* shoots under the control condition might be contributed by the higher expression of a putative HAK5 (LOC_Os01g70490) compared to WT under low-K stress (**Table S16**). Interestingly, HAK5 in rice was previously shown to enhance K acquisition and translocation to shoot, and impacted root architecture by modulating auxin distribution (Yang et al., 2014, 2020). In addition, a high frequency of peptide/nitrate transporter genes (putative PTR2 and PTR3) were upregulated in *sks1* under the control condition (**Figure 6E, Table S16**), which might contribute to K accumulation as some nitrate transporters are capable of transporting K in Arabidopsis as previously reported (Drechsler et al., 2015; Li et al., 2017; Fang et al., 2020). However, how P accumulated in *sks1* shoots under low-K was less predictable from gene expression, although altered root anion balance and/or crosstalk between K and P via SKS1 might have some contributions.

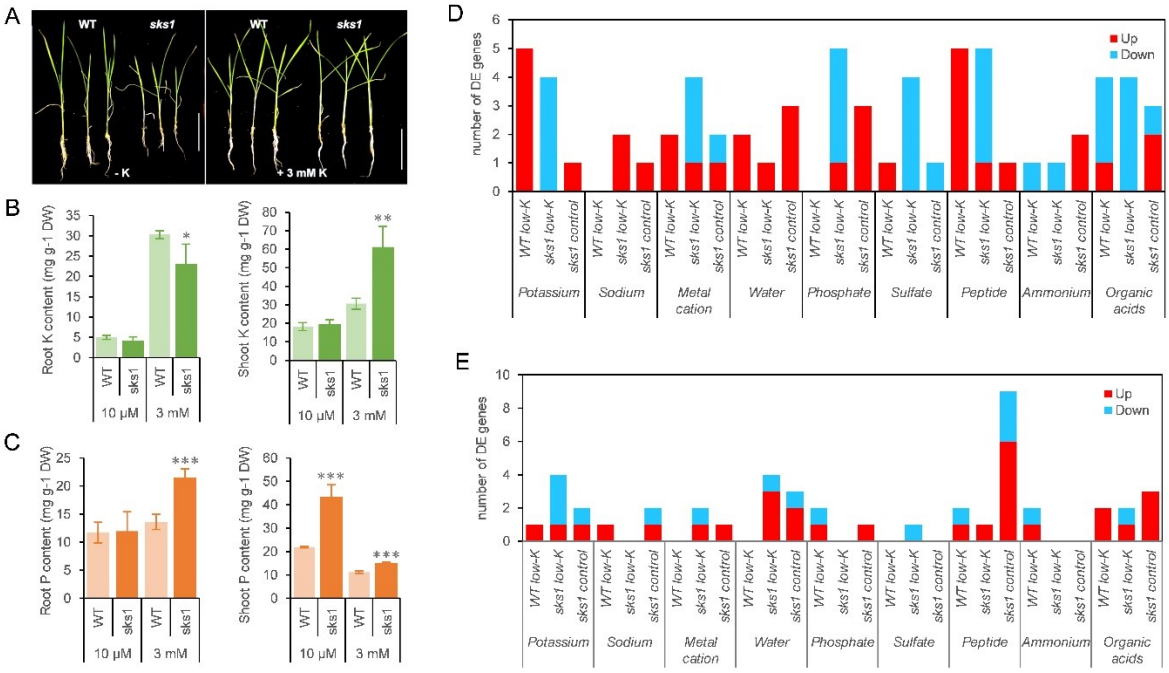

Figure 6. *sks1* with changes in K and P contents and transporter gene expressions.
(A) Growth phenotype of 3-weeks-old WT and *sks1* under low-K and the control hydroponic conditions. **(B)** K and **(C)** P content in WT and *sks1* roots and shoots under -K (10 µM) and +K (3 mM) hydroponic conditions. Data are represented as mean ± SD (n=3-4) with statistically significant difference of **P*<0.05, ***P*<0.005, ****P*<0.0005. **(D, E)** Frequency of DE genes

encoding specific nutrient transporters responsive to low-K and the control conditions in WT and *sks1* roots and shoots, respectively.

DISCUSSION

The current study identified rice SKS1 as a homolog of AtSTOP1 in Arabidopsis, which has been shown to be critical for plant response to various abiotic stresses (Ojeda-Rivera et al., 2020). AtSTOP1 provides tolerance to acidic environments, which is detrimental to plants because of toxic Al and Fe accumulation that can react with P, making it inaccessible for uptake (von Uexküll and Mutert, 1995; Iuchi et al., 2008; Sawaki et al., 2009; Daspute et al., 2017; Ojeda-Rivera et al., 2020). These stresses trigger systemic and local responses via AtSTOP1, which activates AtALMT1 that mediates malate efflux and chelates the toxic Al or Fe at the root apex, leading to ROS production and callose deposition that blocks SHR TF movement and ultimately inhibit primary root growth (Balzergue et al., 2017; Mora-Macías et al., 2017; Godon et al., 2019). The transcription of MATE1 (citrate efflux transporter) and ALS3 (ABC transporter that sequesters Al into vacuole) were regulated by AtSTOP1 in response to Al rhizotoxicity (Larsen et al., 2005; Liu et al., 2009; Sawaki et al., 2009). The transcriptional regulatory mechanism of AtSTOP1 suggests its significance in ionic homeostasis under various abiotic stresses. Indeed, transcriptomic analysis of *Atstop1* mutants under those rhizotoxic conditions revealed that genes encoding proteins with kinase activity, detoxification, transport, P starvation response and cell wall-related processes were largely mis-regulated (Ojeda-Rivera et al., 2020). Among these, AtCIPK23 expression is also found to be regulated by AtSTOP1 (Sawaki et al., 2009; Sadhukhan et al., 2019; Ojeda-Rivera et al., 2020). Because this kinase is the key regulator of AtAKT1 and AtHAK5 (Li et al., 2006; Xu et al., 2006; Cheong et al., 2007; Ragel et al., 2015; Tang et al., 2020a), it prompted us to determine whether AtSTOP1 is also involved in low-K tolerance.

We initially found that *Atstop1* mutants were hypersensitive to low-K stress (data not shown). To assess its functional conservation in other plant species, we transitioned our study to rice crop. Similar to Arabidopsis, rice *sks1* mutants were also sensitive to low-K (**Figure 1**). Analysis of subcellular localization revealed SKS1 to be a nuclear protein, which can induce the expression of OsCIPK23 (**Figure 2**). We then dissected the transcriptional program of SKS1 in response to low-K stress, and found similar biological and molecular processes to be affected as AtSTOP1, including kinase activity, response to stress, redox and cell wall synthesis (**Figure 4**). However, genes encoding those proteins were differently regulated in shoots and roots by SKS1 in response to low-K. In roots, SKS1 promotes expression of kinase, immune response, peroxidase genes, whereas mostly repressed in shoots. These contrasting transcriptional profiles suggest SKS1 in integrating root and shoot signaling in response to low-K stress, which appears to be lost in *sks1* mutants. Perhaps this might be because SKS1 is a positive regulator of

several low-K responsive TFs such as other putative zinc finger proteins, WRKYs and MYCs in roots (**Figure 3, Table S4**). In contrast, SKS1 appears to regulate low-K responsive genes involved in metabolic processes, probably because K is an important cofactor for many enzymatic reactions in shoots.

Protein kinases are important signaling components that regulate diverse targets involved in plant development and response to abiotic and biotic stresses (De Smet et al., 2009; Vaid et al., 2013; Gao et al., 2014; Kohorn, 2016; Wang and Bouwmeester, 2017; Sun et al., 2020; Tang et al., 2020a). A previous transcriptomic analysis of rice under low-K reported that a large number of kinase genes were responsive to K deficiency (Ma et al., 2012). Our study identified many kinase genes whose expressions were modulated by SKS1 in response to different K status (**Figure 4**). In fact, SKS1 regulates distinct groups of kinases in root and shoot tissues. Diverse kinase groups (WAK, S/T, TKL, CAMK) were mis-regulated in *sks1* roots, whereas many Lectin-rich and WAK receptor-like kinase genes in *sks1* shoots in response to low-K stress (**Figure 5**). In addition, SKS1 appears to also regulate many kinases in shoots under the control condition, suggesting it might be important for plant development, especially during flower development, where many genes were significantly enriched in pollen-pistil interaction (**Table S14**, FDR < 4.30E-11).

Among the kinases transcriptionally altered by SKS1, we narrowed our focus to ones that were responsive to low-K in WT. Among the six found, one was ITPK4 [Inositol 1, 3, 4-trisphosphate 5/6-kinase (LOC_Os02g26720)] (**Table S15**). Earlier studies in Arabidopsis have demonstrated the importance of this inositol polyphosphate kinases in Pi sensing and signaling, which can modify the expression of P starvation response genes including P transporters via regulation of PHR1 activity and chromatin remodeling (Kuo et al., 2014, 2018; Dong et al., 2019). We also observed altered expression of phosphate and citrate transporter genes in *sks1* in response to low-K and the control conditions, which might have resulted in increased P level in the mutant roots and shoots (**Figure 6**). We hypothesize this phenotype might be caused by the misregulation of ITPK4 in *sks1* mutants. However, the mechanism is unclear because Arabidopsis *Atiptk4* (Kuo et al., 2018) and rice *sks1* mutants both accumulated more P in shoots, but ITPK4 expression was upregulated in *sks1* mutants (**Table S15**). It would be interesting to perform genetic analysis of ITPK4 in rice to determine functional conservation or divergence. In addition, there were several other phosphatidylinositol-4-phosphate 5-Kinase (PI4P5K) genes that were differentially expressed in response to low-K, and might be potential candidates for P signaling and transport regulations. Some of their transcriptions might be under the control of SKS1, which has been shown to be essential in P starvation responses (Balzergue et al., 2017; Mora-Macías et al., 2017; Ojeda-Rivera et al., 2020). In addition to P, K level was also accumulated in *sks1* shoots under the control condition, which correlated with the high expression of *OsHAK5* (**Table S15**). In Arabidopsis, *AtHAK5* was only induced and function in roots under low-K stress, but rice O*sHAK5* was expressed

in both roots and shoots, and function in K uptake and shoot translocation (Nieves-Cordones et al., 2010; Yang et al., 2014; Ragel et al., 2015). This divergence in function might explain the lower K and *OsHAK5* expression in *sks1* roots, and higher in shoots under the control condition.

At the upstream of kinase-transporter regulation, there also exists Ca signaling that can bind and activate multiple kinases in response to low-K stress, as observed for CBL-CIPK-AKT1/HAK5 (Li et al., 2006, 2014; Xu et al., 2006; Ragel et al., 2015). Evaluation of low-K response genes in WT revealed that Ca sensors such as OsCML15/16/31 (LOC_Os05g31620, LOC_Os01g04330 and LOC_Os01g72530), which might trigger the activation low-K responsive kinases described earlier (**Table S4**). Low-K also promotes the expression of a putative Ca channel, GLR2.7 (LOC_Os06g08880), which might be involved in cytosolic Ca accumulation to transduce low-K signal to downstream CML-kinases. All these Ca signaling genes were transcriptionally regulated by SKS1 in response to low-K.

In conclusion, SKS1 is involved in low-K tolerance by transcriptionally regulating different low-K responsive genes that encode Ca channels and sensors, kinases, and ion/molecule transporters. It is a central hub for signaling integrations between nutrients, and between root and shoot tissues. SKS1 is also important for plant development and immunity. Future research on how SKS1 integrates different stress signals will dissect the plant adaptive response to multiple stresses, allowing us to generate multi-stress-tolerant crops. More specifically, elucidating the interaction and signaling integration among NPK macronutrients are of interest since their resources are limited, and SKS1 appears to regulate nutrient signaling and transport. The homolog, AtSTOP1 in Arabidopsis was shown to be post-translationally regulated by RAE1 (Regulation of ALMT1 Expression), F-box E3-type ubiquitin ligase (Zhang et al., 2019). Whether the similar regulation exists in rice remains to be explored. Since kinases were the major group affected in SKS1, it might be possible that kinases also feedback control SKS1 via phosphorylation.

METHODS AND MATERIALS

Isolation and phenotypic analysis of *sks1*

We collected seeds of T-DNA inserted *sks1* mutants (PFG_A_23757) from the Korean rice T-DNA library (http://cbi.khu.ac.kr/RISD_DB.html). We generated T-DNA insertional and activation tagging lines in *japonica* rice *Oryza sativa* cv. Dongjin or Hwayoung. The vectors used are pGA2707 (GUS trapping vector), pGA2717 (GUS and GFP trapping vector), and activation tagging vectors pGA2715 and pGA2772 (Jeon et al., 2000, Jeong et al., 2002). Flanking sequences of each T-DNA were determined by inverse-PCR or TAIL PCR (An et al., 2003, Jeong et al., 2006). The database can be searched at SIGnAL or RABDB site. Following are the genotype primers used:

LB primer: 5'AACGTCCGCAATGTGTTATTAAG 3',
RB primer: 5'TTCCCACCAACGCTGATCAATTC 3'
F1 primer: 5'AAGGAGAGTGATGATGGTGGTGAG 3'
F2 primer: 5'GTGGCTATGCTCGTGTGGAACTAC 3'
R primer: 5'CCCGAAGCTGCATGATCCTGAG 3'

For phenotype, we first sterilized the seeds in 75% ethanol for 5 minutes and then in bleach for another 10 minutes. After rinsing with sterile water, we plated in Murashige and Skoog (MS) medium for four days. Seedlings of WT and mutants were directly transferred to the hydroponic medium (Yoshida medium, , pH 6.0) in which the K^+ concentration was modified to 10 μM. Plants were grown at 25-28 °C, 12h light/12h dark photoperiod in a growth chamber. Field tests were performed by our collaborators in China. Plants were grown in fields without any fertilizers for five months, and height measured from four independent experiments.

Subcellular localization of SKS1

To determine the subcellular localization of OsSKS1 protein, the coding gene sequence without the stop codon was in-frame fused upstream to the YFP sequence in the pA7-YFP vector. The resulting fusion construct or the pA7-YFP alone was co-transfected into rice mesophyll protoplasts with CFP-HMGB1. Fluorescence of YFP and CFP in the transformed protoplasts was imagined using a confocal laser scanning microscope (LSM510, Carl Zeiss) after the protoplasts were incubated at 23 ºC for 12 hours.

RNA Isolation and Quantitative RT-PCR for *OsCIPK23*

Total RNA was extracted from rice seedlings (*Oryza.Sativa L. spp. japonica*，*var Nipponbare*) using TRIZOL Reagent. cDNA was synthesized via reverse transcription. *SKS1* gene was amplified from cDNA using the primers:
SKS1-F: 5'ATGGACAGTGGCTTGGGAAGAAG 3' and
SKS1-R: 5'TCAGCTGTCTCCGTTCTGCTGC 3'.

RNA-seq analysis

Three-day old seedlings of WT and mutants were first transferred to a hydroponic medium (Yoshida) with sufficient-K (3mM KCl). 10-day-seedlings were then transferred to hydroponic solutions with (3 mM KCl) and without K (0 mM) for 1 week. Root and shoot tissues were separately collected and frozen in liquid nitrogen. Total RNA was extracted from frozen tissues, with three biological replicates. RNA-seq was performed on the Illumina HiSeqTM 2500 system by Shanghai OE Biotech Co., LTD using the

manufacturer's guidelines. Clean pair-end reads were obtained by removing low quality reads, and were aligned to the reference genome using TopHat2 (Kim et al., 2013). HTSeq (Anders et al., 2014) was used to count the read numbers mapped to each gene. The FPKM (fragments per kilobase of exon per million fragments mapped) of each gene was calculated based on the length of the gene and the read counts mapped to this. Differential expression analysis was performed using the DESeq (Anders and Huber, 2012). Genes with adjusted P value ($P \leq 0.05$) found by DESeq and $-1.5 \geq Log_2(FC) \geq 1.5$ were assigned as differentially expressed. Three biological replicates were used for each sample. The public GO database, AgriGO2 (Tian et al., 2017) was used to analyze the functional categories of the DE genes. Venn diagrams were generated using the R package, ggvenn (version 0.1.8). Heatmaps were generated using the MORPHEUS program (https://software.broadinstitute.org/morpheus/).

K and P Content

Three-day old seedlings of WT and mutants were first transferred to a hydroponic medium (Yoshida) with sufficient-K (3mM KCl). After growing for 10 days, WT and mutant plants were transferred to low-K (10 uM) and the control (3 mM KCl) conditions. After 3-weeks of K treatments, root and shoot tissue samples were collected and dried for 48 h at 80 °C, milled to fine powder, weighed, and digested with ultrapure HNO_3 (Sigma-Aldrich). K and P contents were determined using inductively coupled plasma optical emission spectroscopy (PerkinElmer).

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SUPPLEMENTAL DATA

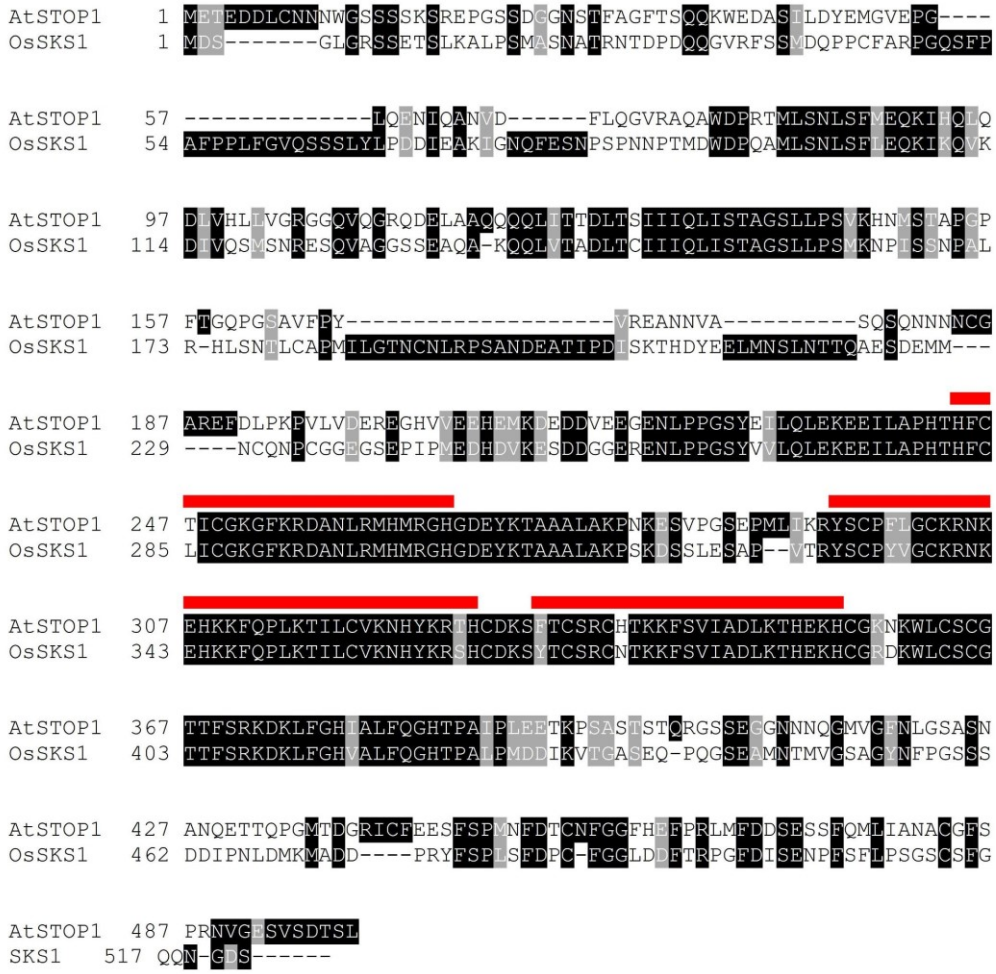

Figure S1. Alignment of AtSTOP1 and OsSKS1 protein sequences. Red lines indicate predicted C2H2 zinc finger domain.

Chapter 5: Potential Networks of NPK Channels and Transporters in Arabidopsis Roots at a Single Cell Resolution

Contributions

D. Lhamo performed the literature searches, bioinformatic analysis, and wrote the manuscript.

Preface

The last three chapters indicated that the expression of nutrient transporters are prone to changes during the course of plant development or in response to nutrient stresses. However, the transcriptional profiles of nutrient transporters are also dynamic in different cell-types of a specific tissue. Generation of promoter-reporter transgenic plants have been utilized for decades to specify the specific tissues and cell-types in which the nutrient channels and transporters may function. However, this approach is time-consuming, with the ability to determine the expression of one gene at a time. Recent advances in single-cell transcriptomics using microfluidic devices and barcode systems provide the high-throughput platform to measure the transcription of all genes at a single cell resolution in a single experiment. The present chapter utilizes the recently-generated Arabidopsis root altas from >110 K single cells to understand how nutrient channels and transporters may function collaboratively to acquire and distribute nutrients (NPK) throughout the root system for a long-distance transport to shoots and roots. This study provides functional predictions on many uncharacterized NPK channels and transporters based on their expression patterns in specific cell-types under the normal growth conditions.

INTRODUCTION

Plant growth and development depend on the constant supply of mineral nutrients through the root system. Therefore, a deficiency in macronutrients such as NPK (Nitrogen, phosphorus and potassium) can have a profound impact on root growth and architecture (**Figure 1**), which in turn can alter the efficiency of nutrient acquisition and transport (López-Bucio et al., 2003; Gruber et al., 2013; Giehl and von Wiren, 2014; Luan et al., 2017). Plants possess large sets of nutrient transporters to perform these functions, among which many remain uncharacterized. Because the levels of these macronutrients in natural soils are often limiting plant growth, crop production often relies on heavy use of chemical fertilizers, which is not sustainable and pollutes the environment (Poirier and Bucher, 2002; Luan et al., 2017). To support sustainable agriculture, there is an urgent need to enhance their nutrient use efficiency (NUE), which will require decoding the complex genetic and biochemical components of nutrient transport.

Decades of research have advanced our understanding on the physiological roles of many nutrient transporters. One important aspect of transporter function is its site of activity. By combining genetic approaches with expression pattern analysis using promoter-reporter transgenic plant, studies can link the phenotype of a mutant to the tissue or cell-type where a particular transporter is expressed to make a functional prediction. This approach has identified a number of nutrient transporters that function in nutrient uptake in roots, root-to-shoot translocation, stomatal movement, pollen tube elongation and reproductive development (Wang and Wu, 2013; Gu et al., 2016; Wang et al., 2018). Although highly effective, this approach is time-consuming, labor-intensive, and limited by the number of genes and cells it can assess at a time. Recent breakthrough in single cell RNA-sequencing (scRNA-seq) provides a high-throughput platform to assess global gene expression at a single cell resolution, with the capacity to measure thousands of cells of different types and developmental stages simultaneously in a single experiment (Macosko et al., 2015; Ziegenhain et al., 2017). In the context of nutrient transport, scRNAseq can map the cell-type profiles of a large number of transporters so that we can predict where they may function and how they may be connected in a systematic process for nutrient handling at whole root or whole plant level.

The scRNAseq technology has been successfully applied to *Arabidopsis thaliana* roots for spatiotemporal characterization of distinct root cell-types, their developmental trajectories, and their transcriptional regulatory pathways (Jean-Baptiste et al.; Denyer et al., 2019; Ryu et al., 2019; Shulse et al., 2019; Zhang et al., 2019). An Arabidopsis root atlas is generated by integrating two of the previously published datasets (Denyer et al., 2019; Ryu et al., 2019), and newly produced datasets from total of 110,427 single cells to profile gene expression dynamics in eleven different root cell-types (Shahan et al., 2020). Here, we leveraged this enormous dataset to map the cell-type expression pattern of NPK transporters in the Arabidopsis roots. Such analyses identified the putative transport map that allow nutrient transport longitudinally from the root cap (lateral root cap/LRC and columella) to the quiescent center (QC), and radially from the root epidermis (trichoblast and atrichoblast) to cortex and endodermis to reach stellar cells (pericycle, xylem, procambium and phloem) for root-to-shoot translocation. We first examined the expression patterns of known nutrient transporters in each family. This allowed us to gain more insights into the known NPK transporters at the root single cell resolution, which

has not been fully achieved before. Then, we profile the expression patterns of uncharacterized nutrient transporter genes to predict their functional roles, considering their membrane localizations and phylogenetic relationship to the functionally-characterized NPK transporters. However, this single cell profiling is currently limited to roots of young seedlings (5-7 days-old) under the normal growth conditions, which may not remain the same throughout the plant life cycle and under the changing nutrient status. For instance, 5-days-old seedlings respond to low-nutrient (N, P or K) stresses differently, with a different degree of modification to the root architectures and shoot growth (**Figure 1**). The transcriptional profiles of nutrient transporters under these stresses may be different compared to the controls and between the treatments. Therefore, low-nutrient-induced transporters may not be fully captured under the normal growth conditions presented in this study. However, these provide future opportunities to generate more detailed Arabidopsis root atlas that cover dynamic transcriptional changes of nutrient transporters over the course of plant development under changing nutrient status. This coupled with the physiological characterization of nutrient transporters will decipher the complexity of nutrient transport processes in plants. Our study identifies nutrient transporters that may function in collaborative fashion to distribute nutrients throughout the root system in concentric manners under non-stress conditions to support normal plant growth. In addition, it aims to lay the foundation for future research to dissect the complex networks of nutrient transport systems at a single cell resolution under varieties of biotic and abiotic stresses of different plant tissues and organs.

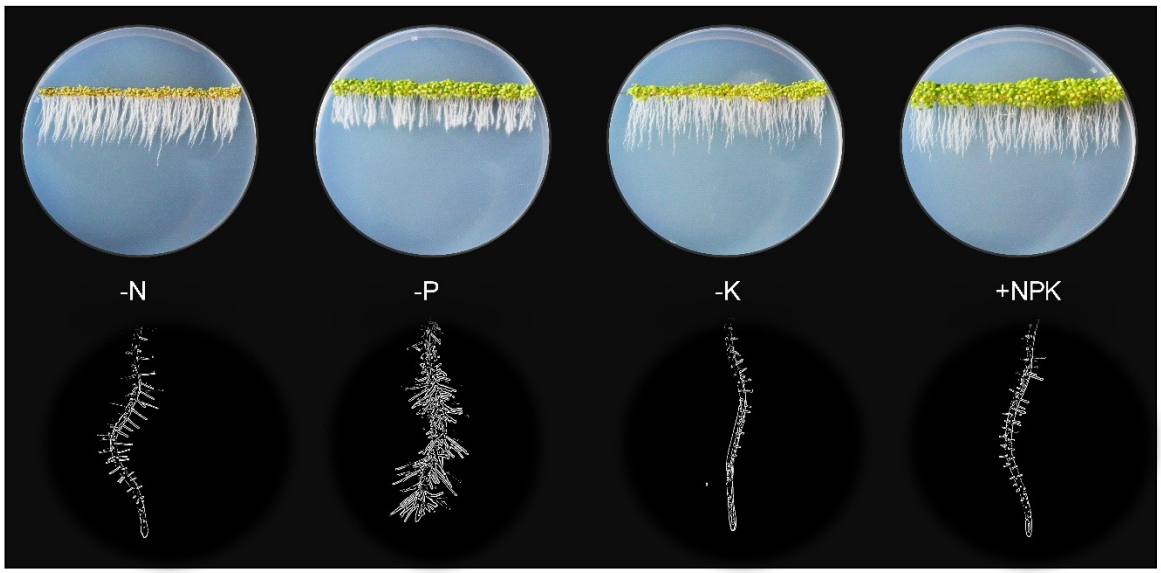

Figure 1. **Plant growth highly depends on the continual nutrient supplies.** Without N, P or K (0 mM, 1/6 MS), five-days old Arabidopsis seedlings displayed stunted shoot growth (top rows) and modified root architectures including primary root and root hair growth (bottom rows) compared to the sufficient-nutrient (1/6 MS) conditions. MS for Murashige and Skoog media.

RESULTS AND DISCUSSION

Nitrate Transport Systems

Nitrate (NO_3^-) is the predominant form of N available in the soil and absorbed by plants for the synthesis of amino acids, nucleic acids, and many secondary metabolites (Noguero and Lacombe, 2016; Bellegarde et al., 2017; Fan et al., 2017). NO_3^- not only serves as a major macronutrient but also acts as a signaling molecule that regulates plant developmental processes including seed germination, lateral root development, and flowering time (Bellegarde et al., 2017; Fan et al., 2017; Fredes et al., 2019). However, NO_3^- is often limited in natural soils (< 1mM) and crop production relies on heavy fertilizer application that can reach up to 70 mM in agricultural lands, leading to runoff and pollution of aquatic ecological systems (Reisenauer, 1966; Krapp et al., 2014). To combat the constantly changing NO_3^- status in the environment, plants have evolved large sets of NO_3^- transporters for efficient uptake and distribution throughout plant cells and tissues (Krapp et al., 2014). These processes are mediated by four major NO_3^- transporter families, which include NPF (Nitrate Transporter 1 (NRT1)/ Peptide Transporter (PTR) Family), NRT2 (Nitrate Transporter 2), CLC (Chloride Channel), and SLAC/SLAH (Slow Anion Channel/SLAC1 homolog) (Krapp et al., 2014; Fan et al., 2017; Wang et al., 2018). While some of these NO_3^- transporters in each family have been functionally characterized, the majority of them remains to be evaluated, especially those in the large NPF family. We examined the expression of these NO_3^- transporters at a single cell resolution to understand how plants spatially distribute these transporters in the root system to charge the uptake, and transport of NO_3^- from the external environment to the xylem vessels for long-distance translocation. The potential network we presented here of NO_3^- channels and transporters is mainly applicable to young seedlings under the sufficient nutrient supply. It is solely based on the transcriptional profiles, which needs to be backed up by sufficient physiological studies in the future.

NPF Transporters

The Arabidopsis NPF family includes 53 members that display high sequence homology with the SLC15/PepT/PTR/POT family of peptide transporters in animals (Léran et al., 2014; O'Brien et al., 2016; Wang et al., 2018). NPF transporters are mostly located at the plasma membrane (PM) and serve as low-affinity transporters that transport NO_3^- and other diverse metabolites and hormones (Krapp et al., 2014; Léran et al., 2014; Wang et al., 2018). NPFs are divided into eight subfamilies based on their phylogenetic relationships (Léran et al., 2014). NPF1 contains three members, among which the PM-localized NPF1.1 (NRT1.12) and NPF1.2 (NRT1.11) were involved in transferring root-derived NO_3^- into phloem of mature leaves for redistribution to young leaves (Hsu and Tsay, 2013). Root single cell transcriptomes indicated these NPF1s were broadly expressed in stellar cells, with the highest expression in the procambium (**Figure 2A**), the meristematic tissue that give rise to xylem and phloem (Jouannet et al., 2015; De Rybel et al., 2016). This expression pattern suggests NPF1.1/1.2 may function in NO_3^- loading

to both meristematic and mature phloem and xylem cells in developing roots under the normal growth conditions. The function of NPF1.3 has not been previously characterized. Based on its single cell profile, NPF1.3 was expressed at a much lower level as compared to NPF1.1/1.2, mainly in endodermal and xylem cells (**Figure 2A**), possibly to translocate NO_3^- to shoots. Its expression may amplify during the later stage of development or under a specific nutrient stress condition. The broad expression of NPF1.1 in other cell-types except root cap suggests its potential contribution to the radial transport and distribution of NO_3^- to or from stellar cells.

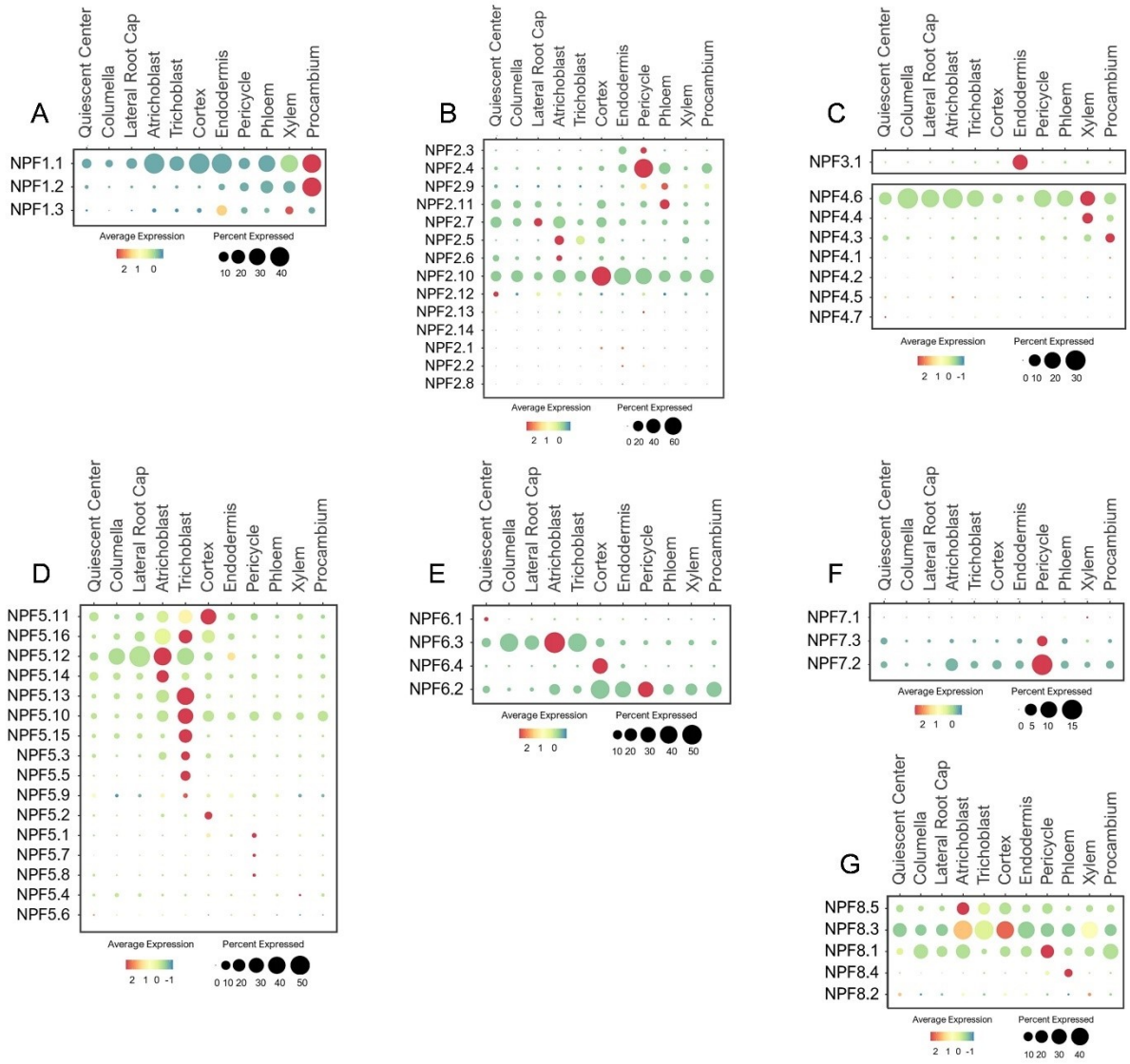

Figure 2. Expression of the low-affinity NPF transporters in Arabidopsis root single cells, (A-B) NPF1-2, **(C)** NPF3/4, and **(D-G)** NPF5-8. Dot plot shows proportion of cells (circle size) and mean expression (circle color).

The NPF2 subfamily contains 14 members, several of which have been functionally characterized. For example, the PM-localized NPF2.3 (NAXT2) mediates

NO_3^- efflux from pericycle to xylem for root-to-shoot transport in response to salt stress (Taochy et al., 2015). As expected, NPF2.3 was mainly expressed in the pericycle, but at a low level under the normal conditions (**Figure 2B**), suggesting it may be more stress-responsive. NPF2.4 was reported to specifically mediate chloride (Cl^-) loading to xylem in response to salt stress (Li et al., 2016). Consistent with this function, NPF2.4 was expressed in the pericycle (**Figure 2B**), but its high expression under the normal growth conditions suggests it may also mediate Cl^- transport under the non-stress conditions. NPF2.9 (NRT1.9) was shown to recirculate NO_3^- from aerial tissues to roots via phloem loading (Wang and Tsay, 2011). As expected, NPF2.9 was expressed in phloem at a low level (**Figure 2B**), implying NO_3^- recirculation may not be a high priority under the nutrient sufficiency. NPF2.11 (GTR2) can transport NO_3^-/gibberellin/glucosinolate, and mediated source-to-sink transport of glucosinolate via phloem loading (Nour-Eldin et al., 2012; Andersen et al., 2013; Saito et al., 2015; Wang et al., 2018). Consistent with these studies, NPF2.11 was expressed in phloem (**Figure 2B**). Its function may overlap with NPF2.9 in NO_3^- loading to phloem based on their similar expression patterns. NPF2.7 (NAXT1) extrudes NO_3^- from root cells under the acidic environments (Segonzac et al., 2007). In compliance with its physiology, NPF2.7 was expressed in the cortex, QC, epidermis, and root cap (**Figure 2B**). NPF2.5/2.6 are closely-related to NPF2.7, and may share similar functions in NO_3^- secretion from root epidermis to maintain ionic balance at the root-soil interface. A previous study reported the involvement of NPF2.5 in Cl^- extrusion from roots under salt stress (Li et al., 2017a), however, its NO_3^- transport activity needs further evaluation. Among all the NPF2s, NPF2.10 displayed a ubiquitous expression, signifying its potential function in NO_3^- distribution across different root cell layers. Studies have shown that NPF2.13 (NRT1.7) recycles NO_3^- from old to developing leaves by directly loading NO_3^- to the phloem (Fan et al., 2009), and NPF2.12 (NRT1.6) delivers NO_3^- from source leaves to developing seeds (Almagro et al., 2008). As a result of specific expression and function in leaf and seed developments, these two transporters were barely expressed in roots (**Figure 2B**).

As the only member in the NPF3 clade, NPF3.1 was reported to be a PM-localized transporter involved in nitrite and NO_3^- transport in leaves, and GA transport to root endodermis (Sugiura et al., 2007; Pike et al., 2014; Tal et al., 2016). Consistently, it exhibited endodermis-specific expression in the developing roots under the normal conditions (**Figure 2C**). This suggests that NPF3.1 may enable NO_3^-/nitrite/GA transport across the casparian strip barrier.

Seven NPF4 members are present in Arabidopsis, among which, only NPF4;6 (NRT1.2/AIT1) has been characterized to function as a low-affinity NO_3^- uptake transporter (Huang et al., 1999). As anticipated, NPF4.6 was expressed in epidermis and root tip (QC, columella and LRC) to initiate NO_3^- acquisition (**Figure 2C**). In addition, its high expression in stellar cells, especially xylem, suggests a potential role in NO_3^- loading to xylem. We hypothesize that NPF4.4 may also be involved in xylem loading because its expression was exclusively associated with xylem. NPF4.3 was preferentially expressed in procambium, suggesting its potential role in xylem and/or phloem loading. Their potential functions in long-distance transport needs to be evaluated experimentally.

While the majority of NPFs are found at the PM, NPF5.11/5.12/5.16, among the sixteen NPF5 members, display the tonoplast localization to remobilize NO_3^- from the vacuoles of stellar cells, especially pericycle (He et al., 2017). However, these

transporters were mainly expressed in root surface and cortical cells based on the single cell profiles of young seedlings under the normal growth conditions (**Figure 2D**), necessitating a more detailed examination of their function in the future. Intriguingly, the other NPF5s were also largely expressed in the epidermis, especially trichoblast, suggesting a role in soil-root exchange. Because the subcellular locations of these NPF5s remain to be determined, there is a gap of knowledge regarding their physiological functions. The single cell expression patterns here provide a reference point for functional analysis using genetic procedures. Among the NPF5 subfamily, NPF5.10 was expressed more broadly in stellar cells, and NPF5.1/5.7/5.8 specifically in a few pericycle cells. Depending on their subcellular locations and transport activities, these transporters could serve as candidates for NO_3^- transfer or remobilization from stellar cells to xylem vessels for long-distance transport. In addition, some of their expressions may be more responsive to nutrient stresses or during later-stages of the plant development.

NPF6 has four members, among which, NPF6.3 (NRT1.1/CHL1) functions as a dual-affinity transporter that mediate NO_3^- uptake and nitrate-dependent auxin transport (Huang et al., 1996; Wang et al., 1998; Liu et al., 1999; Krouk et al., 2010). As the key transporter in NO_3^- acquisition, NPF6.3 was highly expressed in epidermis and root cap (**Figure 2E**). Root cap cells, located at the root tip are essential for nutrient sensing and uptake, root penetration in soils, root growth, gravitropic and hydrotropic responses, which are mostly controlled by polar auxin transport (Svistoonoff et al., 2007; Arnaud et al., 2010; Ruiz Herrera et al., 2015). Studies have shown NPF6.3 at the root tip is able to sense the changes in soil NO_3^- status, and transduces NO_3^- signaling to regulate nitrate uptake and auxin distribution for lateral root development (Liu and Tsay, 2003; Ho et al., 2009; Krouk et al., 2010; Gojon et al., 2011; Mounier et al., 2014; O'Brien et al., 2016). Other members in the same subfamily displayed divergent expression patterns. For example, NPF6.4 (NRT1.3) showed cortex-specific expression while NPF6.2 (NRT1.4) was broadly expressed in cortical and stellar cells. The more distant member, NPF6.1 expression was not detected in roots under the conditions provided. These NPF6s may have evolved to perform distinct functions in transporting NO_3^- radially from root surfaces to inner vasculature.

Among the three NPF7 transporters, the closely-related NPF7.3 (NRT1.5) and NPF7.2 (NRT1.8) mediate opposite functions in long-distance transport. NPF7.3 has been shown to export NO_3^- and K^+ from pericycle to xylem for shoot translocation, whereas NPF7.2 retrieves NO_3^- from xylem and retain NO_3^- in root cells (Lin et al., 2008; Li et al., 2010, 2017b; Drechsler et al., 2015). Interestingly, these two homologs shared high expression in the pericycle (**Figure 2F**), suggesting that their transport mode may be opposite, one mediating influx and one efflux in the same cell-type.

The five NPF8 proteins are not well understood concerning their NO_3^- transport properties. NPF8.1/8.2/8.3 (PTR1/5/2) are shown to transport dipeptide, hormones and/or amino acid (Corratgé-Faillie and Lacombe, 2017). NPF8.2 is preferentially expressed in flowers, where it transported dipeptide across the PM of germinating pollen (Komarova et al., 2008; Hammes et al., 2010). Thus, its expression was barely detected in roots (**Figure 2G**). The tissue- and cell-specific expression for other NPF8 transporters have not been previously reported. In this study, we found that these NPF8.1/8.3/8.5 were broadly expressed in multiple root cell-types. If these transporters are localized to the PM and capable of transporting NO_3^-, they may function with NPF6.3 and NPF4.6 in NO_3^-

uptake at the root surfaces. In addition, NPF8.1/8.3 were also expressed in stellar cells, suggesting their involvement in long-distance transport. NPF8.4 may have more specific function in NO_3^- loading to phloem based on its phloem-specific expression. While these expression patterns provide a valuable information regarding their potential functions, analyses of their subcellular localizations, transport properties and physiological roles are necessary for validations.

NRT2 Transporters

Unlike NPF, the NRT2 family comprises seven proteins that are considered high-affinity NO_3^- transporters (Orsel et al., 2002; Krapp et al., 2014). These proteins were identified by sequence homology to NRT2.1, which was isolated by a differential display approach due to highly NO_3^-inducible expression (Filleur and Daniel-Vedele, 1999; Orsel et al., 2002). NRT2.1/2.2/2.4/2.5 were shown to be PM-localized transporters, involved in NO_3^- uptake especially under low-NO_3^- stress (Cerezo et al., 2001; Filleur et al., 2001; Kiba et al., 2012; Lezhneva et al., 2014). Because their expression is highly induced by low-NO_3^-, the single cell profiling detected only low levels of expression in roots because the plants were grown under the normal conditions. Nevertheless, they were expressed in root epidermis (**Figure 3**), consistent with their uptake functions. NRT2.7 is the only transporter located at the tonoplast, where it is shown to import NO_3^- into vacuoles of seeds (Chopin et al., 2007). However, it was also broadly expressed in various cell-types in young roots compared to the other NRT2s (**Figure 3**). This suggests that NRT2.7 may serve as a housekeeping function to sequester excess NO_3^- into the vacuole in multiple tissues.

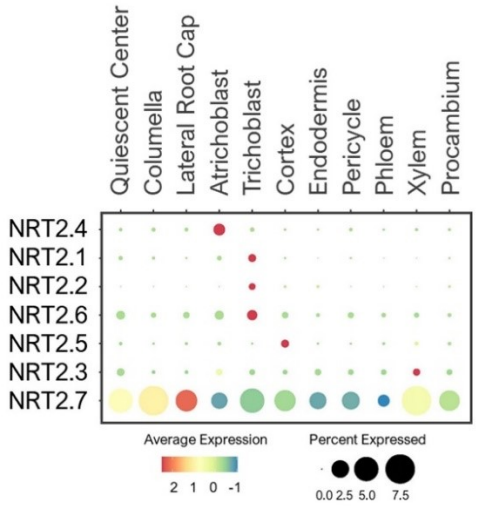

Figure 3. Expression of the high-affinity NRT transporters in Arabidopsis root single cells. Dot plot shows proportion of cells (circle size) and mean expression (circle color).

CLC Antiporters

Arabidopsis CLCs were identified by the sequence homology to voltage-gated chloride channels in animals (Hechenberger et al., 1996; Lv et al., 2009). The plant CLC family contains both channels and antiporters that transport anions including NO_3^- (Fan et al., 2017). The Arabidopsis genome encodes 7 CLC members. CLCa-c, and CLCg were localized to tonoplast, CLCd/f to Golgi, and CLCe in the chloroplast (Fecht-Bartenbach et al., 2007; Marmagne et al., 2007; Lv et al., 2009; Barbier-Brygoo et al., 2011). Because CLCe is shown to be specifically expressed in green tissues (Monachello et al., 2009), its expression in roots was undetected (**Figure 4A**). CLCa is a major component that drives NO_3^- accumulation into vacuoles (Geelen et al., 2000; De Angeli et al., 2006). Its functional significance was evident from the ubiquitous expression in all cell-types in developing roots under the normal conditions (**Figure 4A**). Likewise, CLCc was widely expressed, thus may share similar tasks as CLCa in vacuolar NO_3^- sequestration. The expression of CLCb was specifically detected in the cortex, suggesting a specific function of this CLC in storing NO_3^- in the vacuole of the cortical cells. Additionally, two Golgi-localized transporters, CLCd and -f showed ubiquitous expression in all cell-types, implicating them in the housekeeping function of anion distribution in Golgi apparatus of all cell-types.

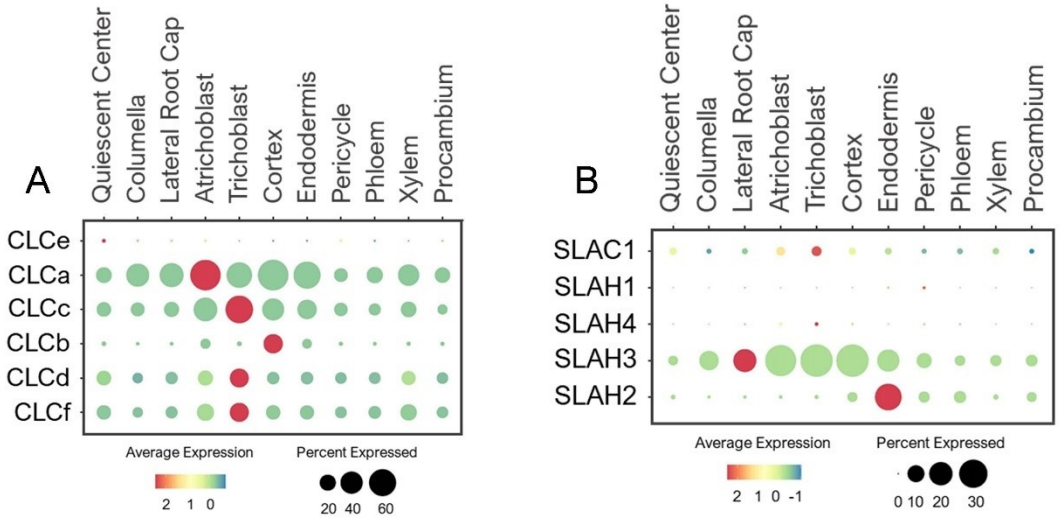

Figure 4. Expression of the (A) CLC antiporters and (B) SLAC1 channels in Arabidopsis root single cells. Dot plot shows proportion of cells (circle size) and mean expression (circle color).

SLAC Channels

SLAC1 shares homology to TehA transporters in bacteria, archaea and fungi (Chen et al., 2010; Dreyer et al., 2012). SLAC1 and its four homologs, SLAH1-4, are anion channels that conduct NO_3^- and Cl^- transport across the PM (Negi et al., 2008;

Barbier-Brygoo et al., 2011; Fan et al., 2017). SLAC1 and SLAH3 were shown to mediate NO_3^-/Cl^- efflux and regulate stomatal closure (Schmidt and Schroeder, 1994; Vahisalu et al., 2008; Geiger et al., 2009b, 2011; Chen et al., 2010; Zheng et al., 2015). Because SLAC1 is preferentially expressed in guard cells (Negi et al., 2008), it was not detected in roots (**Figure 4B**). SLAH1/3 are earlier reported to be expressed in root pericycle to mediate Cl^- efflux to xylem (Cubero-Font et al., 2016). SLAH2 is shown to be a NO_3^--specific channel that may facilitate root-to-shoot NO_3^- transport (Maierhofer et al., 2014). Based on their expressions in stellar cells, especially pericycle, SLAH1-3 have been speculated to drive NO_3^- loading to xylem (Hedrich and Geiger, 2017). We examined their expressions based on single cell profiles and found that the closely-related SLAH1/4 were barely detected in the developing roots under the normal conditions (**Figure 4B**). These genes may require specific growth conditions or plant developmental stages for induction. On the other hand, SLAH2/3 showed distinct expression patterns. While SLAH3 was broadly expressed in epidermis, root cap, cortical and endodermal cells, SLAH2 displayed predominant expression in endodermis (**Figure 4B**), suggesting an endodermis-specific function for SLAH2. The broad expression pattern of SLAH3 is consistent with its functions in diverse processes (Gutermuth et al., 2013; Zheng et al., 2015; Cubero-Font et al., 2016; Zhang et al., 2016; Hedrich and Geiger, 2017).

Potential Network of Nitrate Channels and Transporters in Roots

In Arabidopsis, 73 NO_3^- transporters belonging to the NPF, NRT2, CLC and SLAC1/SLAH families are identified, among which, only several have been characterized to function as NO_3^- transporters in roots (Krapp et al., 2014; Wang et al., 2018). Through single cell transcriptomic analysis, we were able to expand this list, and map the potential network of NO_3^- channels and transporters in the root system of young seedlings under the normal growth conditions (**Figure 5**). The previous studies reported the roles of NPF4.6/6.3 and NRT2.1/2.2/2.4/2.5 in NO_3^- uptake from root surface cells (Huang et al., 1996, 1999; Wang et al., 1998; Liu et al., 1999; Cerezo et al., 2001; Filleur et al., 2001; Kiba et al., 2012). We additionally found several NPF2/5/8, CLCs, NRT2.7 and SLAH3 in the root tip and epidermis, where they might participate in NO_3^- uptake, efflux or storage/distribution to adjacent cells (**Figure 5**). Many of these transporters were also expressed in the cortex and endodermis to distribute NO_3^- towards stellar cells. Among these families, the putative PM-localized NPF2/4/6/8 members are likely involved in NO_3^- uptake, transfer and long-distance transport; SLAH3 in NO_3^- distribution; CLCs in NO_3^- sequestration into vacuoles (also NRT2.7) and Golgi; and the putative vacuolar NPF5s in NO_3^- remobilization.

In addition, we also found few transporters with cell-type specific expressions such as NPF6.4 and CLCb in cortex, and NPF3.1 and SLAH2 in endodermis. These cortex-specific transporters may be the major contributors to the higher NO_3^- concentrations observed in the cortical cells compared to the epidermal cells, as reported in the previous study using the NO_3^--selective microelectrodes (Zhen et al., 1991). Such single cell profiling of nitrate concentrations in NO_3^- transporter mutants compared to the wild type may determine the function of a nutrient transporter in a specific cell type.

NPF2.3/7.3 was previously reported to export NO_3^- from pericycle to xylem (Lin et

al., 2008; Taochy et al., 2015; Li et al., 2017b), NPF7.2 in NO_3^- retrieval from xylem (Lin et al., 2008; Li et al., 2010, 2017b; Drechsler et al., 2015), and NPF2.9 in NO_3^- loading to phloem (Wang and Tsay, 2011). We additionally found several NPF1/2/4/5/6/8, NRT2.7 and CLC transporters expressed in stellar cells that might participate in long-distance NO_3^- transport and distribution (**Figure 5**). Among these, several showed cell-type specific expressions, such as NPF5.1/5.7/5.8 in pericycle, NPF4.3 in procambium, NPF4.4 xylem and NPF8.4 in phloem. These transcriptional profiles provide functional predictions of these NO_3^- transporters in specific cell-types of small root tissues, grown under the normal condition. More in-depth analysis of these genes under diverse nutritional and developmental conditions are required to reflect their main sites of action. Also. it is critical to determine the subcellular localizations and transport activities of these uncharacterized transporters to understand their mode of transport for NO_3^- distribution. So far, the majority of the functionally-known NO_3^- transporters were PM-localized, with few found at the tonoplast and Golgi membranes. There still exists numerous novel NO_3^- transporters that might localize to different subcellular compartments such as plastids, mitochondria, and ER in addition to vacuole and Golgi.

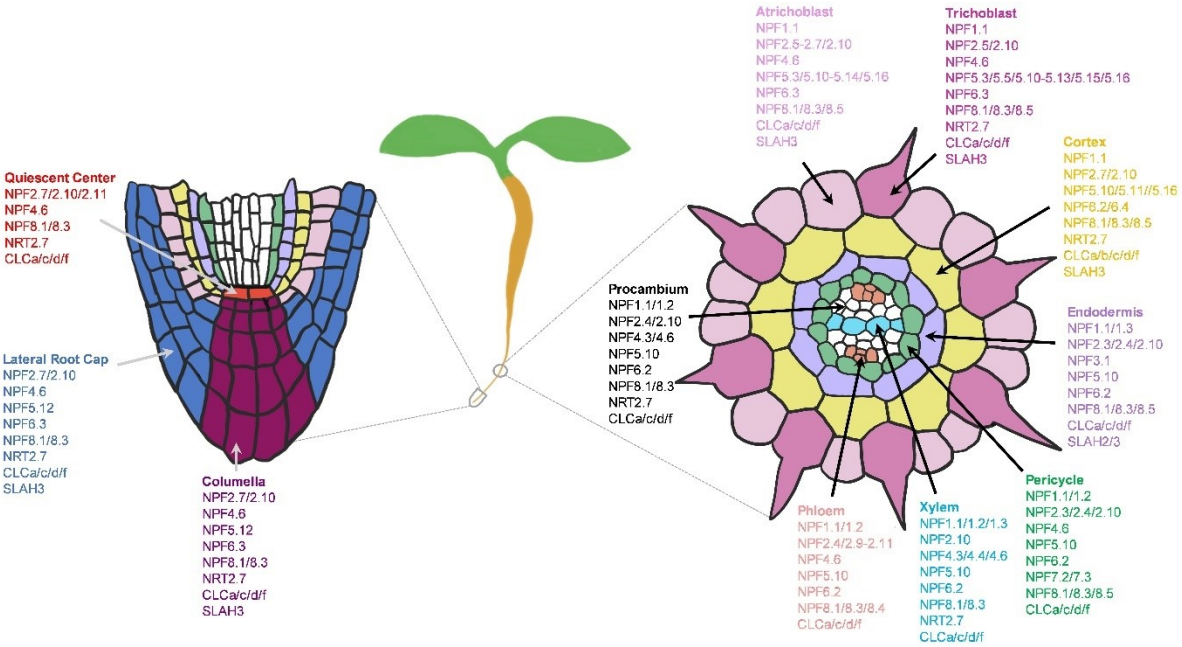

Figure 5. Potential network of nitrate (NO_3^-) channels and transporters in the root system of 5-7 days old Arabidopsis seedlings grown under the normal growth conditions. Expressed NO_3^- transporters involved in longitudinal transport (left) at the meristematic zone and radial transport (right) at the elongation/maturation zones were shown. Nitrate transporter genes not expressed under the normal growth conditions were excluded.

Phosphate Transport Systems

P is absorbed by plants in the form of inorganic phosphate (Pi) that serves as an integral component of nucleic acids, phospholipids and ATP, and participates in energy metabolism and signaling transduction (Poirier and Bucher, 2002; Chiou and Lin, 2011).

Plants require 60-80 μM of cytosolic Pi to sustain growth, but the typical Pi concentration in the natural soil is extremely low (<10 μM) (Pratt et al., 2009; Plaxton and Tran, 2011; Luan et al., 2017). This is largely compensated by the intensive use of P-rock fertilizers, compromising the sustainability of natural resources and negatively impacting the environment (Chiou and Lin, 2011; López-Arredondo et al., 2014; Luan et al., 2017). In response to the changing Pi status in soils, plants have developed large families of Pi transporters for efficient Pi acquisition, translocation, storage and distribution throughout cells and tissues (Gu et al., 2016; Luan et al., 2017). These include five families of Phosphate Transporters (PHT1-5), Glycerol 3-phosphate permease (G3Pp) and Phosphate1 (PHO1) (Stefanovic et al., 2007; Gu et al., 2016; Xu et al., 2019; Lhamo et al., 2020). The membrane localizations of many of these Pi transporters have been elucidated, allowing us to better predict their functions in the root system using single cell transcriptomic datasets. This *in silico* approach has provided us the opportunity to create a potential network of Pi transporters that together direct the Pi flow from the epidermis to the vasculature. However, this is purely based on transcriptional profiling of Pi transporters at a single time-point (5-7 days old seedlings) and one condition (normal). While the present study serves as a starting point, the responses of Pi transporters during different plant life stages and stress conditions need to be evaluated in more details, backed up by sufficient genetic and physiological studies to support the putative model.

PHT1 Transporters

The PHT1 family contains nine members that share sequence homology to the yeast high-affinity H^+/Pi symporter, PHO84 (Poirier and Bucher, 2002; Nussaume, 2011). PHT1s are located at the PM of root surface cells to mediate Pi uptake and translocation (Mudge et al., 2002; Nussaume, 2011). PHT1;1 and PHT1;4 are the dominant players in Pi absorption under a wide-range of Pi concentrations (Misson et al., 2004; Shin et al., 2004). Consistently, single cell profiles showed the high expression of these two transporters in root epidermis under the normal conditions (**Figure 6**). PHT1;4 was additionally expressed in LRC. This cell-type is shown to be important for sensing low-Pi stress to promote root growth arrest (Svistoonoff et al., 2007), as observed in **Figure 1**. In addition, a study using high-resolution real-time ^{33}P imaging indicated that the root cap cells contribute 20% of the total seedling Pi uptake (Kanno et al., 2016). These suggest the role of PHT1;4 in Pi sensing and uptake at the root tip. This together with PHT1;1 at the epidermis makeup the largest portion of absorbed Pi in Arabidopsis seedlings (Shin et al., 2004).

PHT1;8 and PHT1;9 were shown to mediate Pi uptake from roots and translocation to shoots especially under low-Pi (Remy et al., 2012; Lapis-Gaza et al., 2014). In line with these studies, the single cell profiles revealed a low expression of PHT1;9 in epidermis and PHT1;8 in xylem of developing roots under the normal conditions (**Figure 6**). PHT1;7 was also lowly detected in xylem, and may share overlapping functions with PHT1;8 in translocating Pi from root to shoot. Because no definitive Pi transporters at the PM of stellar cells have been reported, we speculate that some of the PHT1 members such as PHT1;7/1;8 may be present to promote xylem loading, and their expression may be more responsive to low-Pi than the normal conditions provided in this study.

Other PHT1 members were lowly expressed in epidermis of young seedlings under the normal conditions (**Figure 6**), and might be more responsive to Pi starvation to initiate Pi uptake (Mudge et al., 2002; Nussaume, 2011). In addition to the condition requirement, some may function in later stages of plant development. For instance, PHT1;5 was shown to recycle Pi from source-to-sink (senescing leaves to young leaves and roots) in response to low-Pi (Mudge et al., 2002; Nagarajan et al., 2011; Nussaume, 2011). These indicate plants may not need to activate the transcription of all PHT1 members during the early growth stage when the Pi supply is sufficient.

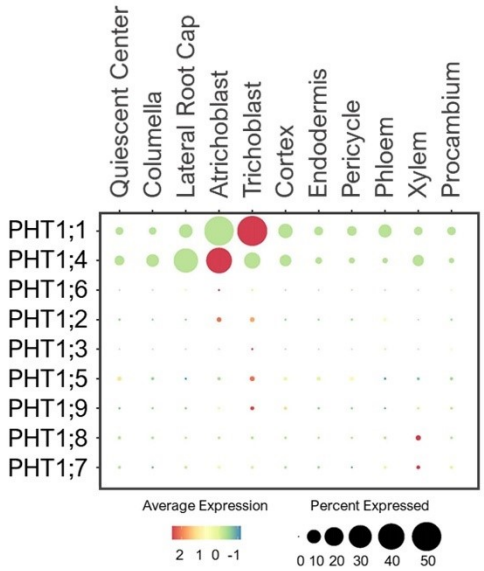

Figure 6. Expression of the high-affinity PHT1 transporters in Arabidopsis root single cells. Dot plot shows proportion of cells (circle size) and mean expression (circle color).

PHT2/3/4 Transporters

PHT2/3/4/5 family transporters are located at different subcellular compartments where they participate in Pi storage and distribution throughout the plant (Gu et al., 2016; Luan et al., 2017). Several of these transporters have been characterized and shown to have function in green tissues. However, their expression and physiology in the root system are not well understood. For example, PHT2;1, a single member of the PHT2 family was shown to transport Pi into chloroplasts in leaves (Daram et al., 1999; Versaw and Harrison, 2002; Rausch et al., 2004). We detected PHT2;1 expression exclusively in root endodermis (**Figure 7A**), raising a question about its intriguing function in root Pi transport. We hypothesize that PHT2;1 in endodermis may contribute to Pi translocation to shoots, where its function is more prominent.

Similar to PHT2;1, five PHT4 members (PHT4;1-4;5) are targeted to plastids, and one (PHT4;6) in Golgi (Guo et al., 2008a, 2008b). These PHT4s were shown to mediate Pi transport when expressed in yeast Pi transport mutants (Guo et al., 2008b). PHT4;1, PHT4;4 and PHT4;5 were reported to be expressed in leaves, PHT4;2 exclusively in

roots, and PHT4;3 and PHT4;6 in both tissues (Guo et al., 2008a, 2008b). Among these, PHT4;1 was shown to compartmentalize Pi in chloroplasts for ATP synthesis and photosynthesis (Guo et al., 2008a; Karlsson et al., 2015). PHT4;4 was reported to transport ascorbate into chloroplasts, and capable of transporting both Pi and ascorbate in liposomes (Miyaji et al., 2015). PHT4;2 was shown to perform reversible Na^+-dependent Pi transport in root plastids (Irigoyen et al., 2011). Expression analysis of these PHT4 members in root single cells revealed broad expression in multiple cell-types of young roots under the normal growth conditions (**Figure 7A**), likely to support their housekeeping functions in plastids. However, these transporters had distinct preferential expressions. For example, PHT4;3 and PHT4;5 were highly expressed in QC and PHT4;1 in procambium, suggesting their roles in balancing plastid Pi in meristematic cells of the root tip and the vasculature, respectively. Also, PHT4;3/4;4/4;6 were highly expressed in epidermis, PHT4;5 in cortex, and PHT4;2 in endodermis. These PHT4s may coordinate with each other to control Pi fluxes in plastids throughout the root system, which is essential for the synthesis and distribution of amino acids, fatty acids and secondary compounds (Flügge et al., 2003). PHT4;6 is the only PHT4 member localized to the Golgi, where it was shown to mediate Pi efflux from Golgi to cytosol (Cubero et al., 2009; Hassler et al., 2012, 2016). In a single cell profile of developing roots, PHT4;6 was largely expressed in epidermis and cortex (**Figure 7A**). This suggests a potential function of PHT4;6 in maintaining Pi homeostasis in Golgi of these transit cell-types. Currently, the function of many PHT4 members in Pi transport and distribution in the root system and whether these may participate in long-distance transport are vaguely understood. We provided assumptions based on the known subcellular localization and expression profiling of these Pi transporters at one time point and growth condition. Their functions in the root system need to be verified experimentally.

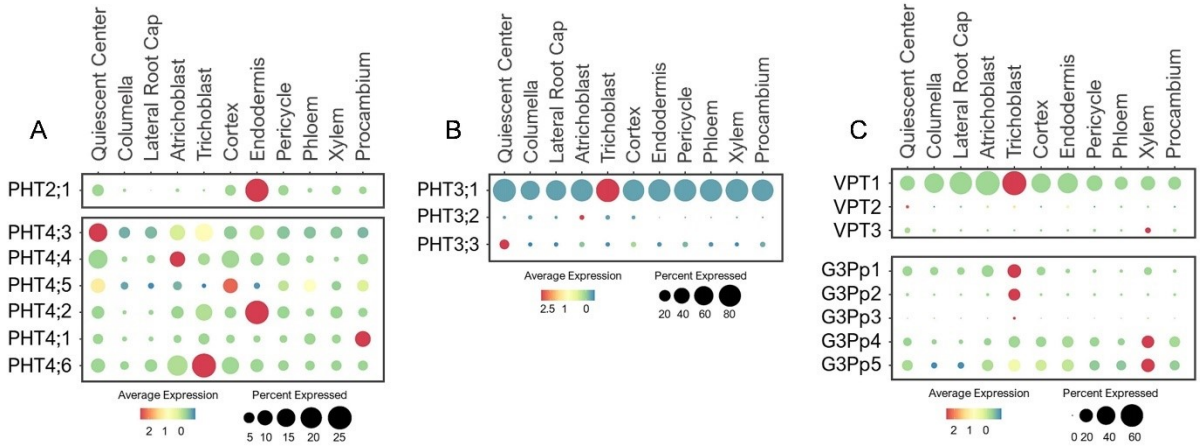

Figure 7. Expression of the (A) PHT2/4, (B) PHT3 and (C) VPT and G3Pp transporters in Arabidopsis root single cells. Dot plot shows proportion of cells (circle size) and mean expression (circle color).

The PHT3 family contains three members in Arabidopsis that show sequence homology to the first mitochondrial Pi translocator (MPT1) cloned from birch plants and

yeast MIR1 (Kiiskinen et al., 1997; Takabatake et al., 1999; Hamel et al., 2004). These PHT3s transport Pi from cytosol into the mitochondrial matrix to support ATP synthesis and redox homeostasis (Zhu et al., 2012; Jia et al., 2015). PHT3;1 (MPT3) was reported to be ubiquitously expressed in multiple tissues, while PHT3;2 (MPT2) and PHT3;3 (MPT1) were only expressed in the green and/or reproductive tissues (Zhu et al., 2012). Among these, PHT3;1 had the largest contribution to the mitochondrial Pi homeostasis, which was shown to be indispensable for plant development (Jia et al., 2015). Consistent with these studies, PHT3;1 displayed ubiquitous expression in all root cell-types, while the other two were barely detected in developing roots under the normal growth conditions (**Figure 7B**). This emphasizes the essential role of PHT3;1 in supporting mitochondrial functions throughout the root system. Further studies of these mitochondrial transporters have been compromised by the lethality of their homozygous mutants (Jia et al., 2015).

VPT and G3Pp Transporters

In Arabidopsis, three members of the PHT5 family are responsible for Pi storage into vacuoles (Liu et al., 2015, 2016). VPT1 (PHT5;1) is the major player in vacuolar Pi sequestration (Liu et al., 2015, 2016; Luan et al., 2019). Single cell profiling revealed a ubiquitous expression of VPT1 in all root cell-types, while VPT2 (PHT5;2) and VPT3 (PHT5;3) were expressed at low levels in roots of young seedlings under the normal growth conditions (**Figure 7C**). This is consistent with the earlier functional analysis (Liu et al., 2015; 2016). However, the expressions of VPT2/3 may become more noticeable in roots of later stages or in response to a specific stress, such as Pi toxicity. In addition, these VPTs may function in other tissues or organs. For example, VPT3 along with VPT1 are shown to play role in the later stage of plant development for systemic allocation of Pi between leaves and flowers (Luan et al., 2019).

The stored Pi in vacuoles is remobilized by the G3Pp family transporters (Xu et al., 2019). This family contains five G3Pp members in Arabidopsis, among which, G3Pp1-3 (VPE1-3) are localized to tonoplast (Ramaiah et al., 2011; Luan and Lan, 2019; Xu et al., 2019). Only G3Pp2 has been confirmed to be a functional vacuolar Pi exporter (Xu et al., 2019). G3Pp4 is localized to the plastid envelope and mediated lipid accumulation in seeds (Kawai et al., 2014). The membrane localization of G3Pp5 remains uncharacterized, so are the functions of the majority of G3Pps. Single cell profiling of young seedlings under the nutrient sufficiency displayed G3Pp1 expression in root tip, epidermis and cortex, and G3Pp2 exclusively in trichoblast, whereas G3Pp3 was barely detected (**Figure 7C**). Considering these G3Pps as efflux transporters, G3Pp1/2 may work concurrently with VPT1 in controlling Pi fluxes between vacuoles and cytosol of epidermal cells to maintain stable cytosolic Pi concentration, and might aid in Pi distribution to the adjacent cells under the normal conditions. Their roles in vacuolar Pi remobilization are critical for low-Pi adaptation, and as such, these genes were shown to be highly induced by low-Pi stress (Ramaiah et al., 2011). On the other hand, the G3Pp3 expression was highly upregulated under various low-nutrient (Pi, K^+, NO_3^- and Iron) stresses (Ramaiah et al., 2011), suggesting the specific role of this transporter in stress-response. Based on their putative functions, we anticipate a wider expression of these

G3Pps in different root cell-types upon nutrient-stress conditions. G3Pp4 and its close homolog, G3Pp5, shared broad expression in multiple cell-types including xylem during normal growth conditions (**Figure 7C**). These G3Pps may contribute to Pi distribution via Pi efflux from plastids, however their Pi transport mechanisms and physiological functions need to be confirmed.

PHO1 Transporters

Plants transfer Pi from root-to-shoot via xylem loading. This process requires Pi efflux from root pericycle to xylem, which is mediated by the Golgi-localized Pi exporter, PHO1 (Poirier et al., 1991; Hamburger et al., 2002; Stefanovic et al., 2011; Arpat et al., 2012). It contains 10 additional homologs in the Arabidopsis genome, referred to as PHO1;H1-H10 (Wang et al., 2004). Studies have shown their tissue- and organ-specific expressions (Hamburger et al., 2002; Wang et al., 2004; Stefanovic et al., 2007; Khan et al., 2014), but their cell-type-specific expression in the root system remains unknown. PHO1;H1 is closely-related to PHO1, and shares a redundant function in xylem loading (Stefanovic et al., 2007). Consistent with their functions, single cell profiling revealed the overlapping expression of PHO1 and PHO1;H1 in pericycle (**Figure 8**). However, PHO1;H1 was expressed at a low level under the normal conditions because its function is more prominent in response to low-Pi (Stefanovic et al., 2007).

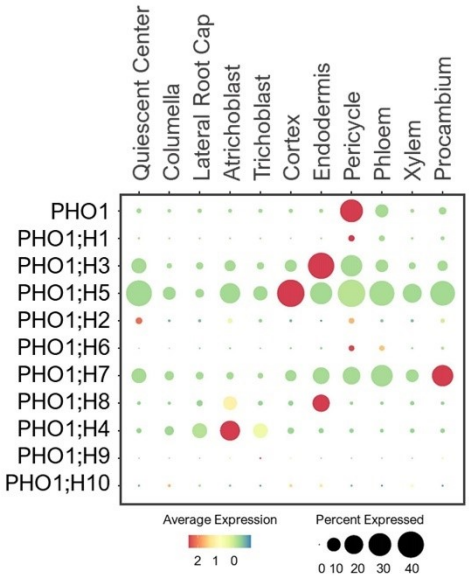

Figure 8. Expression of the PHO1 transporters in Arabidopsis root single cells. Dot plot shows proportion of cells (circle size) and mean expression (circle color).

PHO1;H3 shared a similar expression pattern and subcellular localization to PHO1, but it had the opposite function in long-distance transport (Hamburger et al., 2002; Khan et al., 2014). It was shown to restrict root-to-shoot Pi transport under zinc deficiency (Khan et al., 2014). In root single cell profile, PHO1;H3 was largely expressed in

endodermis and pericycle (**Figure 8**). This implies PHO1;H3 may retrieve Pi from the xylem back to pericycle and endodermis. PHO1;H2, PHO1;H5 and PHO1;H6 are close homologs of PHO1;H3 (Wang et al., 2004), and shared overlapping expressions in pericycle (**Figure 8**). However, PHO1;H2 and PHO1;H6 were expressed at low level and may require specific environmental conditions to induce their expression and function in long-distance transport. In contrast, PHO1;H5 was broadly expressed in all cell-types except root cap, suggesting its potential role in radial Pi distribution from root epidermis to stellar cells or vice versa. PHO1;H4, PHO1;H7 and PHO1;H8 are closely-related (Wang et al., 2004), but they showed distinct expression patterns. For example, PHO1;H4 was largely expressed in epidermis and lateral root cap (**Figure 8**), and may mediate root-soil Pi exchange. PHO1;H8 was highly expressed in endodermis, and likely transport Pi to pericycle for xylem loading. PHO1;H7 was more broadly expressed in endodermis and stellar cells, suggesting its role in xylem and/or phloem loading. However, these are putative functions based on their expression patterns, and need to be validated experimentally. Whether all these PHO1 homologs localize to Golgi, and how Golgi might engage in long-distance transport remain unknown.

Potential Network of Phosphate Transporters in Roots

Pi transporters are found at the PM and membranes of different subcellular compartments, and most of their tissue expressions have been elucidated (Wang et al., 2004; Ramaiah et al., 2011; Gu et al., 2016; Luan et al., 2017; Xu et al., 2019). However, many of their cell-type specific expressions, physiology and Pi transport network in the root system are not well understood. By examining the expression patterns of Pi transporters, we generated a potential network of Pi transporters involved in longitudinal and radial Pi transport in the root system of young Arabidopsis seedlings under the normal growth conditions (**Figure 9**). In general, PHT1s (esp. PHT1;1/1;4) mainly absorbed Pi from the root tip and epidermis, while other subcellular-localized PHT, G3Pp and PHO1;H transporters might distribute Pi further to the adjacent cells. Many of these subcellular Pi transporters that are expressed under the normal conditions showed widespread expression to distribute Pi throughout the root system to support housekeeping functions. These include mitochondrial PHT3;1, vacuolar PHT5;1, most plastid/Golgi PHT4s, putative plastid G3Pp4/5 and putative Golgi PHO1;H5.

Several PI transporters showed cell-type specific expressions, including G3Pp2 and PHO1;H4 in epidermis, PHT2;1 in endodermis, and PHO1 in pericycle. Among these, G3Pp2 is shown to be involved in vacuolar Pi remobilization (Xu et al., 2019), PHT2;1 in Pi transport into plastids (Daram et al., 1999; Versaw and Harrison, 2002; Rausch et al., 2004), and PHO1 in root-to-shoot Pi translocation (Poirier et al., 1991; Hamburger et al., 2002). Except for PHO1, their contribution in specific root cell-types remain obscure, and may be subjected to modifications upon the changing environmental stresses and developmental stages. We additionally found several PHO1 homologs especially PHO1;H5/H7 that are most likely involved in long-distance transport during plant development (**Figure 9**). These are potential candidates for both xylem and/or phloem loading or unloading, which need to be confirmed genetically and physiologically.

To understand how these Pi transporters may contribute to the low-Pi adaptation,

we also profiled their expression patterns from scRNA-seq analyzed under low-Pi (Wendrich et al., 2020). PHT1;1/1;4, PHT2;1, VPT1, G3Pp2, PHO1 and PHO1;H3/H5 were differentially expressed in response to low-Pi (**Figure S1**), with similar expression patterns to our analysis. These Pi transporters may contribute to low-Pi adaptation, probably by enhancing Pi uptake, fine-tuning Pi fluxes from subcellular compartments, and redistributing Pi between shoots and roots. Therefore, these Pi transporters are worth further investigations especially PHT2;1, G3Pp2 and PHO1;H5, whose roles in the root system have not been studied yet. In addition, we need to identify novel Pi transporters that transfer Pi across the PM of different root cell-types besides PHT1s.

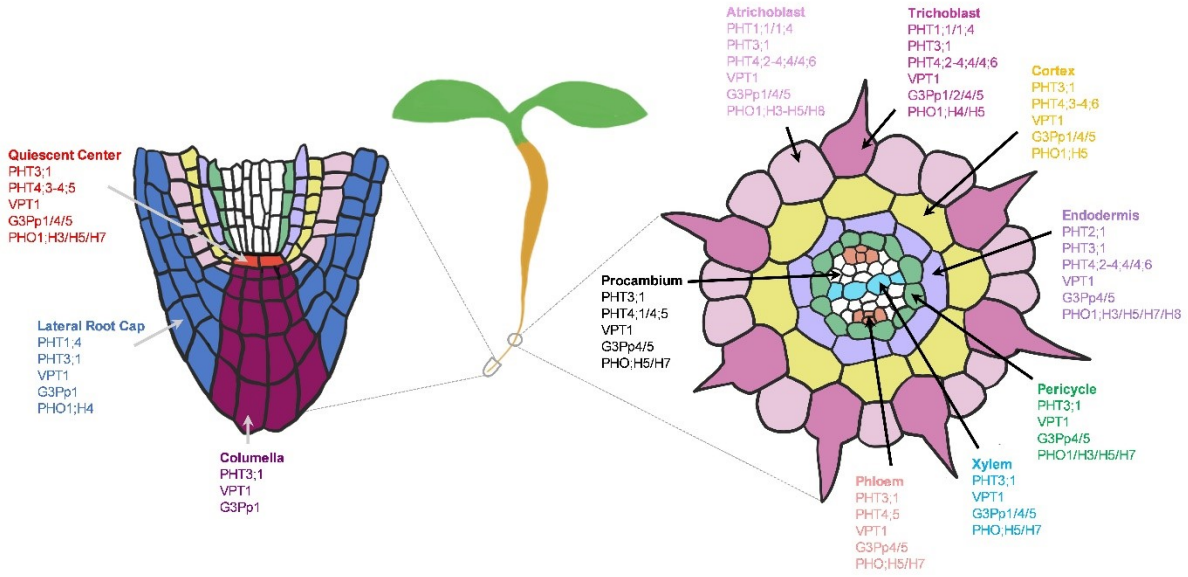

Figure 9. Potential network of phosphate (Pi) transporters in the root system of 5-7 days old Arabidopsis seedlings grown under the normal growth conditions. Expressed Pi transporters involved in longitudinal transport (left) at the meristematic zone and radial transport (right) at the elongation/maturation zones were shown. Pi transporter genes not expressed under the normal growth conditions were excluded.

Potassium Transport Systems

K^+ is the most abundant cation in plants, required for the membrane potential maintenance, protein synthesis, enzyme activation, stomatal movement and photosynthesis (Wang et al., 2013; Luan et al., 2017; Wang and Wu, 2017; Hasanuzzaman et al., 2018). Plants need 100-200 mM of cytosolic K^+ to sustain cell metabolism (Walker et al., 1996; Tester and Leigh, 2001; Sharma et al., 2013; Luan et al., 2017). However, K^+ is often limited in the natural environment (Maathuis, 2009; White, 2013), leading to heavy fertilizer application that raises sustainability issues. To combat the large fluctuations in the soil K^+, plants have evolved elaborate transport systems to maximize K^+ absorption and distribution. These include *Shaker*-type and TPK (Tandem Pore K^+) channels, KUP/HAK/KT (K^+ Uptake/High-Affinity K^+ Transporter) transporters, and four families of Cation/Proton Antiporters (CPA), including CHX (Cation/H^+

Exchangers), KEA (K^+ Efflux Antiporters) and NHX (Na^+/H^+ Exchangers) (Mäser et al., 2001; Gomez-Porras et al., 2012; Wang and Wu, 2013; Santa-María et al., 2018; Lhamo et al., 2021). Decades of research have elucidated the importance of these K^+ channels and transporters in different plant tissues and organs. However, how these diverse K^+ channels and transporters coordinate in the root system to translocate K^+ to aerial tissues is unclear. We utilize the single cell profiles of to provide a potential network of K^+ channels and transporters that transport K^+ from the root epidermis to the xylem vessels using young Arabidopsis seedlings grown under the normal conditions.

Shaker-type Channels

The Arabidopsis *Shaker*-type family is a well-studied group of voltage-gated K^+ channels, with nine members that are classified into three categories based on their voltage dependence (Pilot et al., 2003; Lebaudy et al., 2007; Wang and Wu, 2013). These include K^+ inward-rectifying channels such as AKT1/5, KAT1/2 and SPIK (Hirsch et al., 1998; Pilot et al., 2001; Mouline et al., 2002), and weak-rectifying channel, AKT2 (Lacombe et al., 2000; Michard et al., 2005) that are activated by membrane hyperpolarization; and outward-rectifying channels such as SKOR and GORK that are activated by membrane depolarization (Gaymard et al., 1998; Ache et al., 2000). These *Shaker*-type channels are mainly localized to PM and participate in different physiological processes (Wang and Wu, 2013; Véry et al., 2014; Luan et al., 2017; Lhamo et al., 2021). For example, AKT1 channel is known to mediate K^+ uptake from the soil (Lagarde et al., 1996; Hirsch et al., 1998; Gierth et al., 2005), and its activity is fine-tuned by KC1 channel via heteromerization (Reintanz et al., 2002; Duby et al., 2008; Geiger et al., 2009a). As such, these two K^+ channels shared overlapping expression patterns in the root tip and epidermis (**Figure 10A**), where changes in K^+ level can be sensed to initiate or prevent K^+ uptake. AKT1 was additionally expressed in large portions of xylem and procambium, suggesting its additional role in K^+ translocation. This is in line with a previous study reporting AKT1 involvement in K^+ retrieval from the xylem sap under the sufficient-K^+ condition (Nieves-Cordones et al., 2019).

For shoot translocation, K^+ efflux channel, SKOR mediates K^+ release from pericycle to xylem (Gaymard et al., 1998; Liu et al., 2006). As expected, SKOR was expressed specifically in pericycle (**Figure 10A**). However, its low expression level implies the existence of other transporters that may also function in K^+ root-to-shoot translocation under the normal conditions since the K^+ concentrations in shoots were previously reported to be twice the roots in different plant species using the K^+-selective microelectrodes (Tester and Leigh, 2001). Once K^+ is present in leaves, it can be recirculated back to roots by AKT2 via phloem loading (Marten et al., 1999; Lacombe et al., 2000). However, AKT2 was lowly detected in root single cells (**Figure 10A**), suggesting a minimal requirement for K^+ recycling from shoot-to-root under the normal conditions. This recycling process may be activated during low-K^+ stress to support root metabolic processes.

Several *Shaker*-type channels were shown to be specifically expressed and function in green tissues and floral organs. For example, AKT5 and SPIK displayed flower-specific expression, where SPIK channel was reported to conduct K^+ influx into

pollen for pollen tube growth (Lacombe et al., 2000; Mouline et al., 2002; Zhao et al., 2013). KAT1/2 are expressed in leaves and flowers, where they mediate K^+ influx to control stomatal opening (Nakamura et al., 1995; Kwak et al., 2001; Pilot et al., 2001). Since these four *Shaker*-type channels are specifically expressed in aerial tissues, their expressions in root single cells were barely detected (**Figure 10A**). GORK is also expressed in leaf stomata and mediates K^+ efflux to induce stomatal closure (Ache et al., 2000; Hosy et al., 2003). Studies have also revealed GORK expression in root epidermis, where it mediates K^+ efflux from root cells to maintain osmotic balance (Ivashikina et al., 2001; Osakabe et al., 2013; Demidchik, 2014). We also found GORK to be expressed in large portions of epidermal, LRC and QC cells (**Figure 10A**), suggesting GORK may export K^+ radially and longitudinally from the root surfaces. Because AKT1 and GORK expressions overlap, their coordination at the root surface might balance K^+ influx and efflux, which may prevent epidermal and LRC cells from bursting under the nutrient-rich conditions.

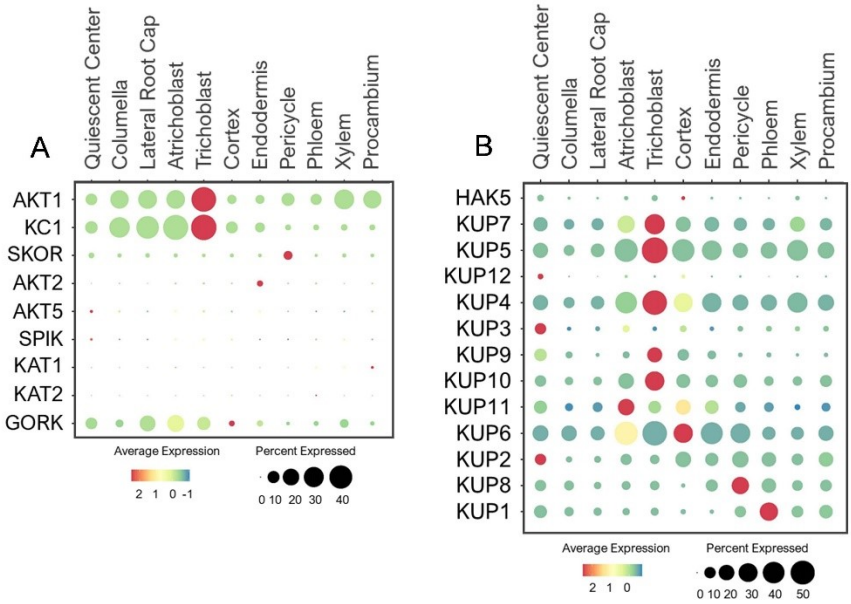

Figure 10. Expression of the (A) *Shaker*-type channels (B) KUP transporters in Arabidopsis root single cells. Dot plot shows proportion of cells (circle size) and mean expression (circle color).

KUP Transporters

The KUP family encodes 13 KUP/HAK/KT members in Arabidopsis that show high homology to the bacterial KUP and fungal TRK transporters (Schleyer and Bakker, 1993; Banuelos et al., 1995). Similar to *Shaker*-type channels, these KUP transporters also have diverse roles in transporting K^+ throughout different cells and tissues. In addition, KUPs are localized to the PM and different subcellular compartments, and some are capable of transporting hormones (Li et al., 2018; Lhamo et al., 2021). Among these, the PM-localized HAK5 and KUP7 mediate K^+ uptake in response to low-K^+ stress, with an

additional role of KUP7 in K^+ translocation to shoots (Gierth et al., 2005; Qi et al., 2008; Pyo et al., 2010; Han et al., 2016). Since HAK5 functions specifically under low-K^+ (10-100 µM) stress (Rubio et al., 2008; Nieves-Cordones et al., 2014), its expression was hardly detectable in young roots under the normal conditions (**Figure 10B**). However, KUP7 exhibited ubiquitous expression, suggesting its ability to transport K^+ from epidermal to stellar cells for shoot translocation during the normal growth. Although an earlier study indicated probable role of HAK5 in K^+ translocation under the sufficient-K^+ condition (Nieves-Cordones et al., 2019), our single cell profiles indicated more probable role of KUP7 than HAK5 in this process during early plant development. HAK5 may be involved in K^+ translocation during the later stages of development or specially under low-K^+ stress, requiring more detailed analysis. Like KUP7, its close homolog, KUP5, was also ubiquitously expressed (**Figure 10B**), suggesting these two KUPs may functionally overlap with AKT1 in K^+ uptake and SKOR in K^+ translocation during normal growth conditions.

In addition to K^+ transport, several KUPs function in root development. For instance, KUP4 (TRH1) located in the endomembrane promote root hair growth and gravitropic responses by modulating auxin transport in the root apex (Rigas et al., 2001, 2013; Vicente-Agullo et al., 2004). The single cell profiles indicated that KUP4 was ubiquitously expressed in young roots, suggesting it's important role in root development (**Figure 10B**). The high expression of KUP4 in trichoblast and cortex may suggest its involvement in generating auxin maxima needed for root hair elongation (Jones et al., 2009; Vissenberg et al., 2020). This is supported by genetic analyses of the KUP4 null mutants that display no root hair growth, and reduced auxin accumulation in epidermal and cortical cells compared to the wild type upon exogenous auxin treatment (Rigas et al., 2013). In addition, the expression of KUP4 in stellar cells suggests it may be involved in both acropetal and basipetal auxin distribution (Sustr et al., 2019). Its close homology, KUP3, was predominantly expressed in QC (**Figure 10B**), therefore, may fine-tune K^+ and/or hormone distribution to support meristematic activity as observed for KUP9. Recently, KUP9 is shown to mediate auxin and K^+ export from ER (Endoplasmic reticulum) to cytosol in response to low-K^+, which is essential to maintain meristematic activity and primary root growth (Zhang et al., 2020). As expected, KUP9 was expressed in QC, but also in trichoblast and cortex (**Figure 10B**). This suggests KUP9 may also contribute to polar auxin transport and K^+ distribution during plant growth. The closely-related KUP10 and KUP11 displayed broad expressions, however their single mutants did not show low-K^+ sensitive phenotype observed for *kup9* mutants (Zhang et al., 2020). Generation of higher-order mutants may dissect functional redundancies between these close-homologs, with KUP9 as the dominant player.

KUP2/6/8 are K^+ efflux transporters that are suggested to function redundantly in exporting K^+ from stellar cells to epidermis (Osakabe et al., 2013). Consistent with this study, our single cell profiling revealed overlapping expressions of these KUPs in stellar cells, with the broader expression of KUP6 in other cell types (**Figure 10B**). The coordination among KUP2/6/8 might allow efficient K^+ transport from stellar cells to root epidermis, where KUP6 along with GORK likely extrude K^+ from roots back to the soil. It remains a question whether young plants use such a mechanism to remove K^+ buildup under the sufficient-nutrient availability for osmotic balance. In addition, analysis of *kup2/6/8* triple mutants revealed the importance of these KUPs in hormone responses

that affect cell expansion and lateral root development (Osakabe et al., 2013), however the mechanism is unclear. Similar to KUP4/9, we suspect that KUP2/6/8 may be capable of transporting hormones in addition to K^+. KUP1, the founding member of the Arabidopsis KUP family, was previously shown to mediate both high- and low-affinity K^+ transport (Fu and Luan, 1998; Kim et al., 1998). However, its tissue expression and physiological role have not been examined. Our study indicated KUP1 expression in stellar cells, especially the phloem in young seedlings (**Figure 10B**). This implicated the possible role of KUP1 in phloem loading, and may work in parallel or substitute AKT2 function under the normal growth conditions. Genetic and physiological analyses are need to confirm its putative function.

CHX Antiporters

CHX is the largest family belonging to CPA superfamily, which in general plays important role in regulating pH and ion homeostasis of the subcellular organelles that affect protein processing, vesicular cargo composition, vesicle movement and protein trafficking (Bassil et al., 2012; Sze and Chanroj, 2018). The CHX family consists of 28 members in Arabidopsis, which are localized to the PM and endosomes where they perform K^+/ H^+ exchange (Chanroj et al., 2011; Sze and Chanroj, 2018). The physiological functions of many of these CHXs remain uncharacterized. Earlier studies showed that a large number of CHXs transporters are expressed in pollen, among which, some are functionally characterized to be involved in male fertility, pollen tube guidance, pollen wall construction and seed development (Sze et al., 2004; Lu et al., 2011; Chanroj et al., 2013; Sze and Chanroj, 2018). Because our single cell profiling is focused on roots of developing seedlings, the expression of many of these CHXs were detected at low levels (**Figure 11A**).

However, several CHX members are known to be expressed and function in the root system. These include the PM-localized CHX13 and CHX17 that mediate high-affinity K^+ uptake under low-K^+ (Cellier et al., 2004; Zhao et al., 2008). While expression of CHX13 was not detected in the root under the normal conditions, CHX17 was highly expressed in the epidermis and root cap (**Figure 11A**). This implies CHX17, but not CHX13, may mediate K^+ uptake in developing seedlings under the normal conditions. CHX13 is probably more responsive to low-K^+ stress, similar to HAK5. CHX20 was previously shown to mediate K^+/H^+ exchanges in ER to control the stomatal movement (Padmanaban et al., 2007), but it's role in roots have not been elucidated. We found CHX20 to be highly expressed in the root cap and atrichoblast, and may control K^+ fluxes in ER of these cells, likely to regulate protein processing and trafficking as reported (Chanroj et al., 2011). CHX7 and CHX16 were expressed at low levels in epidermis (**Figure 11A**), and may also participate in K^+ uptake or distribution depending on their membrane localizations. Between the two, CHX16 was shown to be located at the PM and ER, and implicated to function in pH homeostasis (Chanroj et al., 2011, 2013). In maintaining pH, CHX16 at the PM of root epidermis is likely to initiate K^+ acquisition in exchange.

Several CHXs have been reported to function in response to osmotic stresses. For example, the PM-localized CHX14 is suggested to mediate K^+ efflux from vascular stellar

cells under excess-K^+ condition (Zhao et al., 2015). On the other hand, the PM-localized CHX21 in root endodermis is implicated in loading Na^+ to stelar cells under salinity stress for sequestration into leaf vacuoles (Hall et al., 2006). These stress-responsive transporters, CHX14/21, were lowly detected in QC under the normal conditions, similar to CHX19/26 (**Figure 11A**). Based on their known functions, the QC-specific expression might be an artifact. It is more likely that CHX14/21 expression and function may be more pronounced in stellar cells under osmotic stresses as reported (Hall et al., 2006; Zhao et al., 2015). CHX1 was the only member in the CHX family to be highly expressed in the endodermis (**Figure 11A**), raising the interesting possibility for this CHX to play a role in long-distance transport.

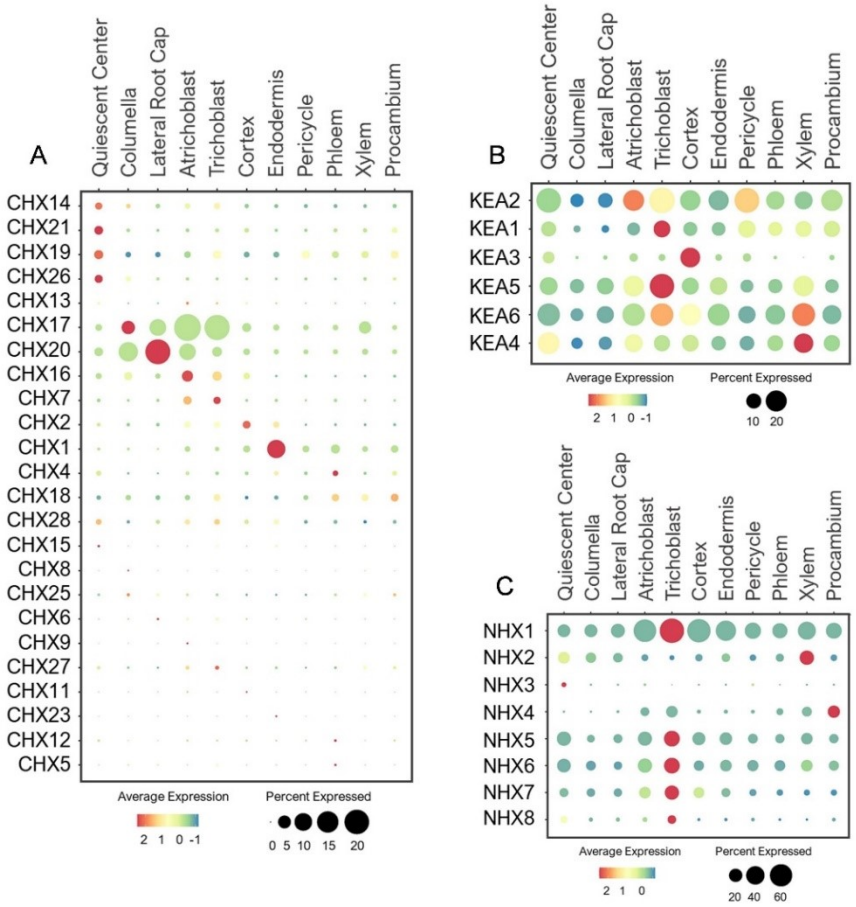

Figure 11. Expression of the (A) CHX, (B) KEA and (C) NHX antiporters in Arabidopsis root single cells. Dot plot shows proportion of cells (circle size) and mean expression (circle color).

KEA Antiporters

The KEA family is also part of the CPA superfamily, with six members that share high homology to the bacterial K^+/H^+ antiporters KefB and KefC (Mäser et al., 2001; Chanroj et al., 2012; Han et al., 2015; Sze and Chanroj, 2018). Previous studies reported

their tissue-specific expressions (Han et al., 2015; Zhu et al., 2018a), however, their expressions at a cellular level are unclear. At the subcellular level, the closely-related KEA1/2 are located at the inner envelope membrane and KEA3 at the thylakoid membrane of chloroplasts (Kunz et al., 2014). These KEAs participate in the K^+/H^+ exchange within and across chloroplasts to maintain pH and osmotic balance for chloroplast development and photosynthesis (Mäser et al., 2001; Kunz et al., 2014). Interestingly, we detected a broad expression of KEA1/2 in multiple cell-types in developing roots under the normal growth conditions (**Figure 11B**). This suggests the possible roles of KEA1/2 in K^+ and pH homeostasis of plastids in different cell-types of roots in addition to shoots. In contrast, the more distant member, KEA3, was exclusively expressed in the cortex, and may mediate K^+/H^+ exchange specifically in the cortical plastids, which require further analysis to understand its functional significance in this specific cell-type.

The closely-related KEA4/5/6 mediate K^+/H^+ exchange across trans-Golgi networks for proper vesicle trafficking that allow better adaptation to various osmotic stresses (Zhu et al., 2018b; Wang et al., 2019). These KEAs shared overlapping and ubiquitous expressions in young roots under the normal growth conditions (**Figure 11B**), emphasizing their importance in pH/ionic homeostasis to support the endomembrane function in all root cell-types. Although broadly expressed, most KEAs displayed the highest expression in root epidermis, mainly trichoblast. It would be interesting to examine if these KEAs have any specific roles in root hair development. KEAs were also widely expressed in stellar cells, with the high expression of KEA2 detected in pericycle and KEA4/5/6 in xylem. This is in agreement with the previous findings (Han et al., 2015), suggesting these KEAs may further contribute to K^+ distribution across stellar cells for shoot translocation. Currently, the physiological function of KEA members in the root system, and their possible involvement in K^+ partitioning between roots and shoots have not been elucidated. In addition, how these KEAs may allow adaptation to low-K^+ and excess-K^+ stresses at the tissue- and cell-specific levels need to be further evaluated since their higher-order mutants (e.g., *kea4/5/6)* were highly sensitive to these K^+ stresses (Zhu et al., 2018b; Wang et al., 2019).

NHX Antiporters

The NHX family is another group of the CPA superfamily. In Arabidopsis, there are eight members, originally described as Na^+/H^+ exchangers (thus NHX) (Blumwald and Poole, 1985). Later studies indicated that different NHX members can transport Na^+, K^+ or both in exchange of H^+ (Venema et al., 2002; Liu et al., 2010; Bassil et al., 2011b; Barragán et al., 2012). These transporters are found at the PM and different subcellular compartments (Bassil et al., 2012). NHX1/2 are located at the tonoplast to mediate K^+ sequestration into the vacuoles (Shi and Zhu, 2002; Bassil et al., 2011b; Barragán et al., 2012). In aerial parts, they regulate stomatal movement and flower development (Bassil et al., 2011b; Barragán et al., 2012). We found that NHX1 was ubiquitously expressed in various cell-types of roots, whereas NHX2 showed narrower expression in xylem and QC (**Figure 11C**). This is consistent with the previous reports of tissue-specific expressions (Shi and Zhu, 2002; Barragán et al., 2012). The expression dominance of NHX1

compared to NHX2 coincides with its functional dominance in maintaining turgor for cell expansion to support overall growth and development (Bassil et al., 2011b). Because NHX2 was highly expressed in xylem, it may be involved in maintaining high K^+ pools necessary for shoot translocation when plants are challenged by K^+ limitations. Their roles in the root system need to be further evaluated because the earlier studies mainly focus on aerial tissues, where NHX1/2 were also expressed. The K^+-selective microelectrode analysis revealed the maintenance of constant cytosolic K^+ concentration between different root cell-types under the sufficient-K^+ condition, and suggested the involvement of vacuolar transporters in this process (Walker et al., 1996; Tester and Leigh, 2001). The vacuolar NHXs are the prime candidates to sequester the accumulating K^+ under the nutrient sufficiency.

NHX3 and NHX4 are also localized to tonoplast, with the NHX3 expression detected in pollen grains and siliques, and NHX4 in roots and shoots (Li et al., 2009; Liu et al., 2010; Bassil et al., 2012). Consistently, NHX3 was barely detected in roots (**Figure 11C**), suggesting it may have developed a specific function in reproductive organs. In contrast, the NHX4 was highly expressed in procambium, where it may provide the K^+ supply needed for procambium differentiation into mature xylem and phloem. Similar to NHX2, NHX4 may support a long-distance K^+ transport to shoots. Its contribution to K^+ nutrition in the root system needs to be further explored. One important reason is because a previous study reported the highest K^+ concentration in vacuoles of xylem compared to all other cell types, followed by pericycle, endodermis and cortex by using the cryo-scanning microscopy with energy dispersive X-ray microanalysis (McCully, 1994). NHX1/2/4 may be the key players in maintaining the high K^+ level in xylem.

NHX5 and NHX6 are closely-related transporters with high homology to the yeast NHX1 and the human NHE6 and NHE7 (Khan et al., 2018). These NHXs maintain pH and K^+/Na^+ homeostasis in endosomes to mediate cell expansion, proliferation and vesicle trafficking by influencing auxin transport and distribution (Bassil et al., 2011a; Dragwidge et al., 2018, 2019). NHX5/6 shared broad expression in the root single cell profiles (**Figure 11C**), supporting their essential and redundant functions in the housekeeping processes. The PM-localized NHX7 (SOS1) and NHX8 are the most divergent members of the NHX family, which closely resemble the prokaryotic NhaP antiporter (Bassil et al., 2012). These NHXs maintain cellular ion homeostasis by extruding toxic Na^+ and Li^+ out of the root cells (Shi et al., 2002; An et al., 2007). Consistent with their functions, NHX7/8 were highly expressed in epidermis, especially trichoblast, to remove the toxic cations out of the cell. In return, K^+ may serve as a counter ion of Na^+ to be absorbed by NHX7 at the root surface (Wu et al., 1996).

TPK Channels

The TPK family is composed of five Tandem Pore K^+ channels (TPKs) and one K_{ir}-like K^+ channel. All of them are localized to the tonoplast except TPK4, which is found at the PM (Voelker et al., 2006, 2010; Lhamo et al., 2021). Electrophysiological studies in heterologous systems revealed TPK1/4 to be voltage-independent, K^+ selective channels, and their channel activities are regulated by calcium and pH (Becker et al., 2004; Bihler et al., 2005; Gobert et al., 2007; Tang et al., 2020). TPK4 is expressed

predominantly in pollen, where it conducts K^+ across the PM of pollen tubes (Becker et al., 2004). As reported, the expression of TPK4 was barely detected in root cells of young seedlings under the normal conditions (**Figure 12**). On the other hand, the tonoplast-localized TPK1 is ubiquitously expressed, and plays important role in low-K^+ adaptation by releasing K^+ from vacuoles (Czempinski et al., 2002; Bihler et al., 2005; Gobert et al., 2007; Tang et al., 2020). The physiological characterizations of other tonoplast-localized TPKs remain unelucidated. In root single cells, TPK1/3/5 were widely expressed in all cell-types although TPK5 was expressed at a lower level (**Figure 12**). This suggests these TPKs may share similar housekeeping functions in vacuolar K^+ remobilization throughout the root system. While their functions are likely to dominate under low-K^+ stress, TPK1/3/5 may modulate the vacuolar K^+ fluxes in coordination with NHX1/2 to maintain the constant cytosolic K^+ concentration under the normal growth conditions, similar to AKT1 and GORK at the PM. The tonoplast-localized TPK2 and KCO3 were lowly detected in trichoblast under the normal conditions. Their expressions may amplify in multiple root cell-types under low-K^+, to optimize K^+ remobilization along with TPK1/3/5. However, the presence of all vacuolar TPKs at the root epidermis might explain the previous findings that cytosolic K^+ concentration declines faster in epidermal cells than cortical cells upon continual low-K^+ treatment (Walker et al., 1996; Tester and Leigh, 2001).

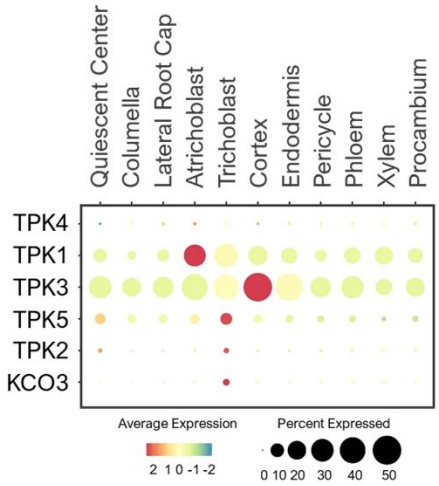

Figure 12. Expression of the TPK channels in Arabidopsis root single cells. Dot plot shows proportion of cells (circle size) and mean expression (circle color).

Potential Network of Potassium Channels and Transporters in Roots

Single cell profiling has allowed us to generate a working model of how diverse K^+ channels and transporters might coordinate to transport K^+ across different root cell-types of developing seedlings under the normal growth conditions (**Figure 13**). However, these are purely based on transcriptional profiles, which is subjected to change upon different developmental stages and K^+ status. In general, K^+ uptake and long-distance transport

are largely mediated by *Shaker*-type channels, KUP and CHX transporters. The subcellular K^+ distribution is controlled by TPK channels, KUP, KEA, NHX and CHX transporters. Although many of their membrane localizations were elucidated, the functional studies of these K^+ channels and transporters in the root system await further investigations. At the root surfaces, AKT1, HAK5, KUP7 and CHX13/17 are known to participate in K^+ uptake especially in response to low-K^+ stress (Hirsch et al., 1998; Cellier et al., 2004; Gierth et al., 2005; Qi et al., 2008; Zhao et al., 2008; Han et al., 2016), whereas KC1 channel inhibited AKT1 activity (Reintanz et al., 2002; Duby et al., 2008; Geiger et al., 2009a). Our expression analysis indicated AKT1, KUP7 and CHX17 to also mediate K^+ acquisition under the normal growth conditions in young roots. We additionally found a number of KUP, CHX, KEA, NHX transporters and TPK channels expressed at the root tip and epidermis that might be involved in K^+ influx and efflux into and out of the root cells and subcellular compartments (**Figure 13**). Among these, several showed cell-type specific expressions at low level, including KUP2/3 in QC and TPK2/KCO3 in trichoblast. Several KUP, KEA, NHX and TPK members were expressed in cortex and endodermis to distribute K^+ within and towards stellar cells (**Figure 13**). KEA3 was predominantly expressed in the cortex and CHX1 in the endodermis, suggesting their unique functions in these cell-types, which might be worth further investigations.

In stellar cells, SKOR and KUP7 were known to mediate xylem loading (Gaymard et al., 1998; Liu et al., 2006; Han et al., 2016) AKT2 in phloem loading (Marten et al., 1999; Lacombe et al., 2000), and AKT1 in xylem retrieval (Nieves-Cordones et al., 2019). Surprisingly, SKOR and AKT2 channels were lowly expressed in developing roots under the normal conditions; therefore, we anticipated the involvement of additional nutrient transporters in these processes. For instance, NPF7.3 (NRT1.5) in pericycle is shown to mediate K^+ loading to xylem under low-K^+ and the normal conditions, although its function is more pronounced under low-nutrient (K^+ or NO_3^-) stresses (Drechsler et al., 2015; Li et al., 2017b). In addition to this, we found number of KUPs, KEAs, NHXs and TPKs that might contribute to long-distance K^+ transport (**Figure 13**). Among these, NHX2/4 might be more specifically involved in xylem loading and KUP1 in phloem loading. KUP2/6/8 and GORK might coordinate with each other to extrude K^+ from stellar cells to the soil (Osakabe et al., 2013).

Overall, many KUPs were widely expressed, indicating their essential role in K^+ transport in developing roots under the sufficient-K^+ supply, and worth further studies to dissect the function of uncharacterized KUPs. Because there are large number of KUPs, functional redundancies among the KUP transporters may restrict further studies, which could be potentially resolved by generating higher-order mutants. Our analysis provides KUP genes that share overlapping expressions that may be used to generate higher-order mutants. KEAs are essential for K^+/H^+ balance in plastids and endomembranes, but their significance in K^+ transport and distribution in the root system remain unelucidated despite their broad expressions. On the other hand, the large portions of *Shaker*-type channels and CHX transporters were not much expressed in young roots, suggesting their roles in other tissues and organs or in response to specific biotic and abiotic stresses. Several NHX members were ubiquitously expressed for housekeeping functions in tonoplast and endosomes. TPK1/3/5 were broadly expressed to remobilize K^+ from vacuoles, and may function concurrently with the vacuolar influx transporter, NHX1 in maintaining cellular K^+ homoeostasis during plant development. However, TPK3/5 are not

physiologically characterized yet, and more studies of NHX members in roots are needed.

While we focus on the major families of K^+ channels and transporters, there exist other voltage-independent, nonselective cation channels such as CNGCs (cyclic nucleotide gated channels) that are capable of transporting K^+ in different heterologous systems, which include CNGC1/2/3/4/10/17 (Köhler et al., 1999; Leng et al., 1999; Balagué et al., 2003; Hua et al., 2003; Li et al., 2005; Gobert et al., 2006; Ladwig et al., 2015). This shows the complexity of K^+ transport in plants, in which various channels and transporters are able to control K^+ fluxes during different developmental stages and in response to various environmental stresses. Nevertheless, they share a similar goal of providing K^+ supply needed to support growth, which involves the coordination among different nutrient transporters and channels to maintain cellular ion homeostasis.

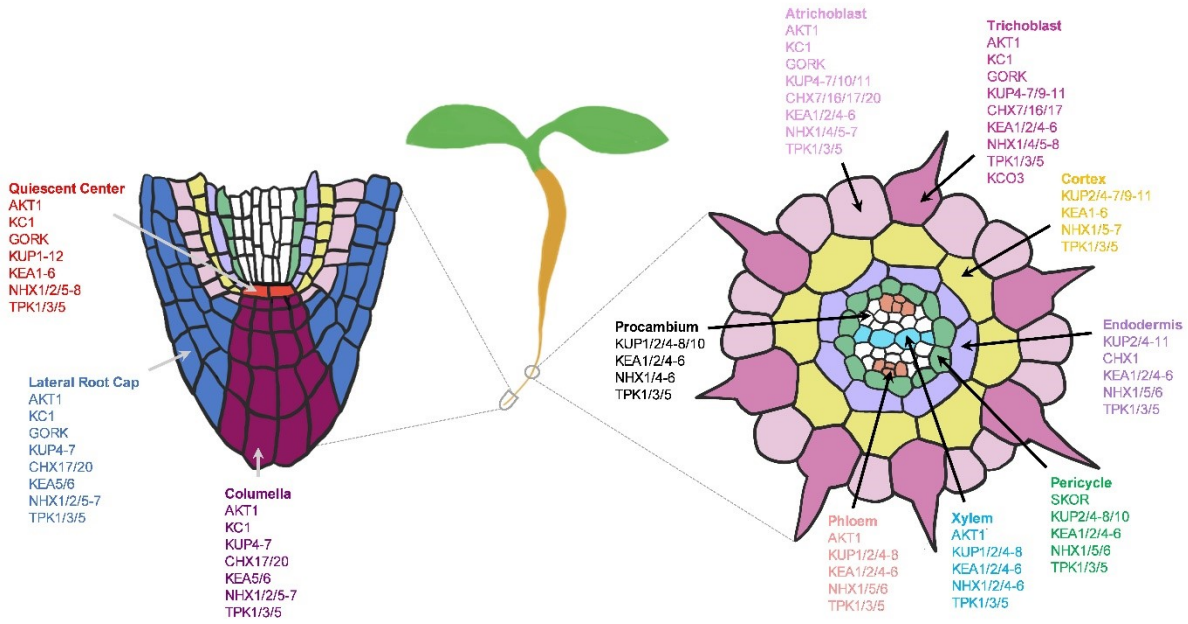

Figure 13. Potential network of potassium (K^+) channels and transporters in the root system of 5-7 days old Arabidopsis seedlings grown under the normal growth conditions. Expressed K^+ transporters involved in longitudinal transport (left) at the meristematic zone and radial transport (right) at the elongation/maturation zones were shown. K^+ transporter genes not expressed under the normal growth conditions were excluded.

CONCLUSIONS

In summary, single cell profiling has allowed us to predict the functions of many unknown NPK channels and transporters and generate their potential networks in the root system of young Arabidopsis seedlings. However, further molecular, electrophysiological and genetic studies are required to confirm their functions in the specific root cell-types, and determine functional redundancies among the closely-related transporters. In addition, generation of root atlas using a large population of cells under different developmental and low-nutrient stresses may unravel the putative channel and transporter networks that are crucial for plant development and low-nutrient adaptation.

Moreover, the spatial profiling of cytosolic and subcellular nutrient concentrations in root single cells using technologies such as microelectrodes (Zhen et al., 1991; Radcliffe et al., 2005), nuclear magnetic resonance (NMR) (Lee and Ratcliffe, 1993; Radcliffe et al., 2005; Gout et al., 2011; Liu et al., 2016) and fluorescence resonance energy transfer (FRET)-based systems (Looger et al., 2005; Mukherjee et al., 2015; Sahu et al., 2020) are much needed to dissect the complexity of nutrient transport within and between different root cell-types.

MATERIALS AND METHODS

Plant growth conditions for low-nutrient stresses

Arabidopsis seeds were sterilized in 10% bleach, rinsed with distilled water four times, and plated directly on 1/6 strength MS (Murashige and Skoog) with (NPK) and without N, P or K (0 mM) nutrients, supplemented with 1% sucrose and 1% phytoblend (Caisson labs), pH 5.7. The MS media with and without N or P were ordered from Caisson labs (North Logan, Utah). Low-K medium (1/6 MS) were prepared in-house using 0.5 mM $Ca(NO_3)_2$, 0.25 mM $MgSO_4$, 0.21 mM $NH_4H_2PO_4$, and 1/6 micronutrients (Caisson labs). Plated seeds were stratified at 4 °C for 2 days and transferred to the growth chamber in vertical position for 5 days, with a 12-hour light/12-hour dark cycle (100 µmol $m^{-2}s^{-1}$). Two sets of plates were prepared for each condition, one with plants clustered together, and the other with seedlings separated with spaces in-between for root hair phenotype, and the most representative one is presented.

Single-cell root altas data source and information

The scRNA-seq data and Seurat objects for Arabidopsis root altas were obtained from (Shahan et al., 2020) (GEO: GSE152766). Briefly, the primary roots of 5-7 days old Arabidopsis seedlings under normal growth conditions were used for root protoplast extractions. Shahan et al., (2020) generated the root atlas from 110,427 single cells, by integrating their datasets with the previously-published datasets (Denyer et al., 2019; Ryu et al., 2019). Details about the data integrations can be found in Shahan et al., (2020). The large datasets cover all the major root cell-types including QC (1834 cells), columella (11,757), LRC (14,420), atrichoblast (13,972), trichoblast (11,946), cortex (10,318), endodermis (10,541), pericycle (11,603), phloem (6758), xylem (4984) and procambium (12,294). The identity for these cell-types were assigned based on the expression of cell-type-specific marker genes that are well-studied (Shahan et al., 2020).

Data analyses and visualizations

To understand the potential networks of NPK transport systems in Arabidopsis roots, the expression patterns of all the major NPK family channels and transporters were analyzed. Extensive literature searches were performed to identify all the major NPK

transporter families in Arabidopsis. The Arabidopsis gene IDs for the NPK transporters were retrieved from The Arabidopsis Information Resource (TAIR, https://www.arabidopsis.org/). All data were analyzed in R (version 3.5.2) using the batch-corrected ('RNA' assay in Seurat object) expression value. The expression profiles of specific N, P or K transporter families were plotted using Seurat's DotPlot function. Unlike the typically-used heatmap, the dot plot visualization provides more detailed expression profiling that shows the percentage of cells expressing a specific nutrient channel or transporter gene, and the average expression of cells expressing the gene. This allows identification of the major nutrient transporters that contribute to NPK transport in specific cell type(s) under the normal growth conditions. The differentially expressed Pi transporters in response to low-Pi were extracted from root single cells analyzed by (Wendrich et al., 2020) (**Figure S1**). The artworks for potential networks of NPK channels and transporters in the root system were drawn using Adobe illustrator draw application. Nutrient channel and transporter genes expressed under normal growth conditions were included in the networks.

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SUPPLEMENTAL DATA

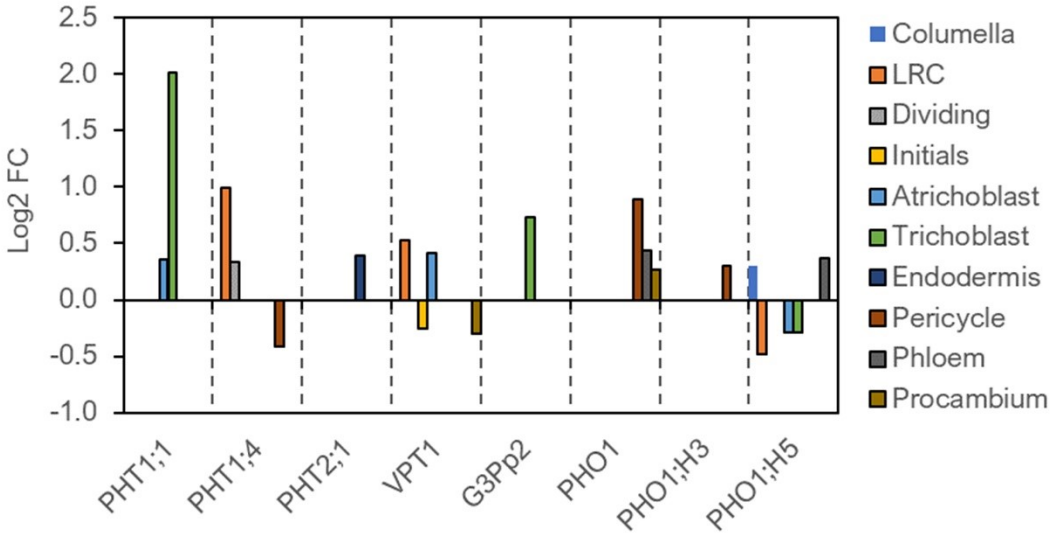

Figure S1. Differentially expressed Pi transporters in specific root cell types under Pi starvation. The data were retrieved from Wendrich et al., (2020).

Chapter 6: Concluding Remarks

Genome-wide identification and characterization of nutrient channel and transporter families are made possible with the availability of genome sequences and transcriptomic datasets. This is evident in Chapter 2 and 3 where all the major P and K channel/transporter families of *Camelina sativa* were identified and their tissue-specific expression determined. The majority of P and K channels/transporters in *Camelina* contained three copies for each Arabidopsis ortholog, resulting from genome triplication events (Kagale et al., 2014). However, several genes were further expanded beyond the ploidy norm, which was mainly caused by tandem duplication events. These genes mainly encode Pi uptake transporters and vacuolar K channels that might contribute to the better Pi acquisition and K remobilization during low-nutrient stresses, respectively.

In silico transcriptomic analysis implicated the potential roles of P and K channels and transporters in specific tissues at different developmental stages, however, their responses to various low-nutrient stresses have not been evaluated at a global transcriptomic level. Such studies in the future might provide better candidate transport genes that are involved in low-nutrient adaptive response. However, the functional studies of nutrient transport genes might be compromised by the functional redundancies among the close homoeologs. The generation of high-order mutants using CRISPR-cas9 (Morineau et al., 2017; Lyzenga et al., 2019) might provide solutions to determine the gene dosage effect. Future research on nutrient channels and transporters need to be extended to other crop species. Comparative genomic analyses may suggest the evolution of a specific nutrient channel and transporter family among different plant species, providing important clues to conserved domains that have functional significance in modulating transport activities.

At the transcriptional level, the nutrient transport genes are regulated by TFs in response to various developmental stages and stress conditions. Several TFs were found in Arabidopsis that regulate specific K transporters, and so far, the majority of them were found to regulate AtHAK5 in Arabidopsis, discussed in Chapter 3. Not many TFs that regulate K channels and transporters were identified in the crop model, rice. Our study presented in Chapter 4 identified rice SKS1 to be involved in low-K response by modulating the transcription of distinct signaling genes involved in kinase and redox activities, immune response and cell wall synthesis. In roots, SKS1 is shown to regulate a number of low-K responsive TFs that might have unique downstream targets. In shoots, low-K responsive genes encoding metabolic enzymes were differentially expressed. The expression of nutrient transporter genes and nutrient compositions especially K and P contents were also altered in the *sks1* mutants. Based on this study, SKS1 is postulated to be involved in signaling integrations between K and P, and between roots and shoots. However, further studies are required to determine the direct targets of SKS1 that are involved in low-K response. DNA affinity purification sequencing (Dap-seq) technology can be used to identify all the binding sites of SKS1 TF that allow the identification of its direct targets (Bartlett et al., 2017). In addition, how SKS1 might integrate K and P signals need to be further explored. It is anticipated that the TFs and signaling genes regulated by SKS1 might be involved in this process. For instance, N and P signaling in rice were shown to be integrated by nitrate transceptor (NRT1.1B) and phosphate signaling

repressor (SPX4), which were both under the control of NLP3 and PHR2 TFs (Hu et al., 2019).

While all the previous transcriptomics were analyzed at the tissue level, a recent bloom in single-cell RNA-seq analysis in Arabidopsis roots have shown the transcriptional dynamics in different cell types of a tissue. Recently, the generation of Arabidopsis root atlas from >110 K single cells (Shahan et al., 2020) provided the opportunity to profile the expression of nutrient channels and transporters at a single cell resolution. Through such analysis, the potential networks of NPK channels and transporters were created that show radial and longitudinal transport of nutrients, as described in Chapter 5. However, this study is currently limited to young seedlings and the normal growth conditions. In the future, profiling the expression of nutrient channels and transporters in various tissues of different developmental stages and under different nutrient conditions might decode the complex nutrient transport systems in Arabidopsis. Such approaches should also be further expanded to crop species to dissect the transcriptional programs that confer low-nutrient adaptation. Overall, this dissertation extends the nutrient studies to crop species including *Camelina* and rice, and identifies nutrient channels and transporters that may function in specific tissues and cell-types, which are under the control of TFs. Unraveling the transcriptional regulatory networks that are essential for low-nutrient stresses might aid in generating high nutrient use efficient crops. In addition, the functional characterization of candidate transport genes is needed to confirm their roles in low-nutrient adaptation.

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