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Plant Membrane Transport Research in the Post-genomic Era

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ABSTRACT

Membrane transport processes are indispensable for many aspects of plant physiology including mineral nutrition, solute storage, cell metabolism, cell signaling, osmoregulation, cell growth, and stress responses. Completion of genome sequencing in diverse plant species and the development of multiple genomic tools have marked a new era in understanding plant membrane transport at the mechanistic level. Genes coding for a galaxy of pumps, channels, and carriers that facilitate various membrane transport processes have been identified while multiple approaches are developed to dissect the physiological roles as well as to define the transport capacities of these transport systems. Furthermore, signaling networks dictating the membrane transport processes are established to fully understand the regulatory mechanisms. Here, we review recent research progress in the discovery and characterization of the components in plant membrane transport that take advantage of plant genomic resources and other experimental tools. We also provide our perspectives for future studies in the field.

Keywords: membrane transport, ion channels, transporters, signaling networks, genomics, *Arabidopsis*

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INTRODUCTION

As cell membranes serve as barriers and gatekeepers to maintain a homeostatic internal environment, they are semi-permeable to only a few small molecules such as gases while restricting the movement of the majority of solutes including charged ions and large molecules. The passage of these cellular solutes relies on specific transport proteins embedded in the membrane, which can either facilitate diffusion as channels or operate active translocation against the electrochemical gradient as pumps, transporters, and other carriers. The plant genome encodes a large number of membrane transport proteins that function broadly in a multitude of basic physiological processes including acquisition of ions, transfer of metabolites, and excretion and partitioning of waste products (Arabidopsis Genome Initiative, 2000). In the model plant *Arabidopsis thaliana*, it is estimated that about 18% of the predicted proteins appear to contain two or more transmembrane spans, among which more than half may represent transport proteins (Ward, 2001; Schwacke et al., 2003). With the development of bioinformatics tools, putative transport functions have been assigned to these proteins based on sequence homology with well-documented counterparts in bacteria, fungi, and animals, building the foundation for further functional characterization. However, as sessile organisms, plants have evolved unique transport systems to respond to various stresses in an ever-changing environment. In addition, highly compartmentalized organizations of plant cells also feature specialized organelles that bring about functional properties and mechanisms distinct from the general transport processes in bacterial and animal cells. As a result, the precise functions of many genes encoding putative plant transporter proteins remain elusive, especially in the context of plant-specific development and physiology (Barbier-Brygoo et al., 2001). The post-genomic era has witnessed extensive functional genomic studies, providing opportunities and resources to overcome challenges and advance our understanding of membrane transport in plants. The advent of high-throughput technologies such as genomics, transcriptomics, and proteomics has led to identification of thousands of gene candidates and generation of enormous datasets in a short period of time and set the stage for direct experimental efforts toward fully dissecting the functions of transport proteins.

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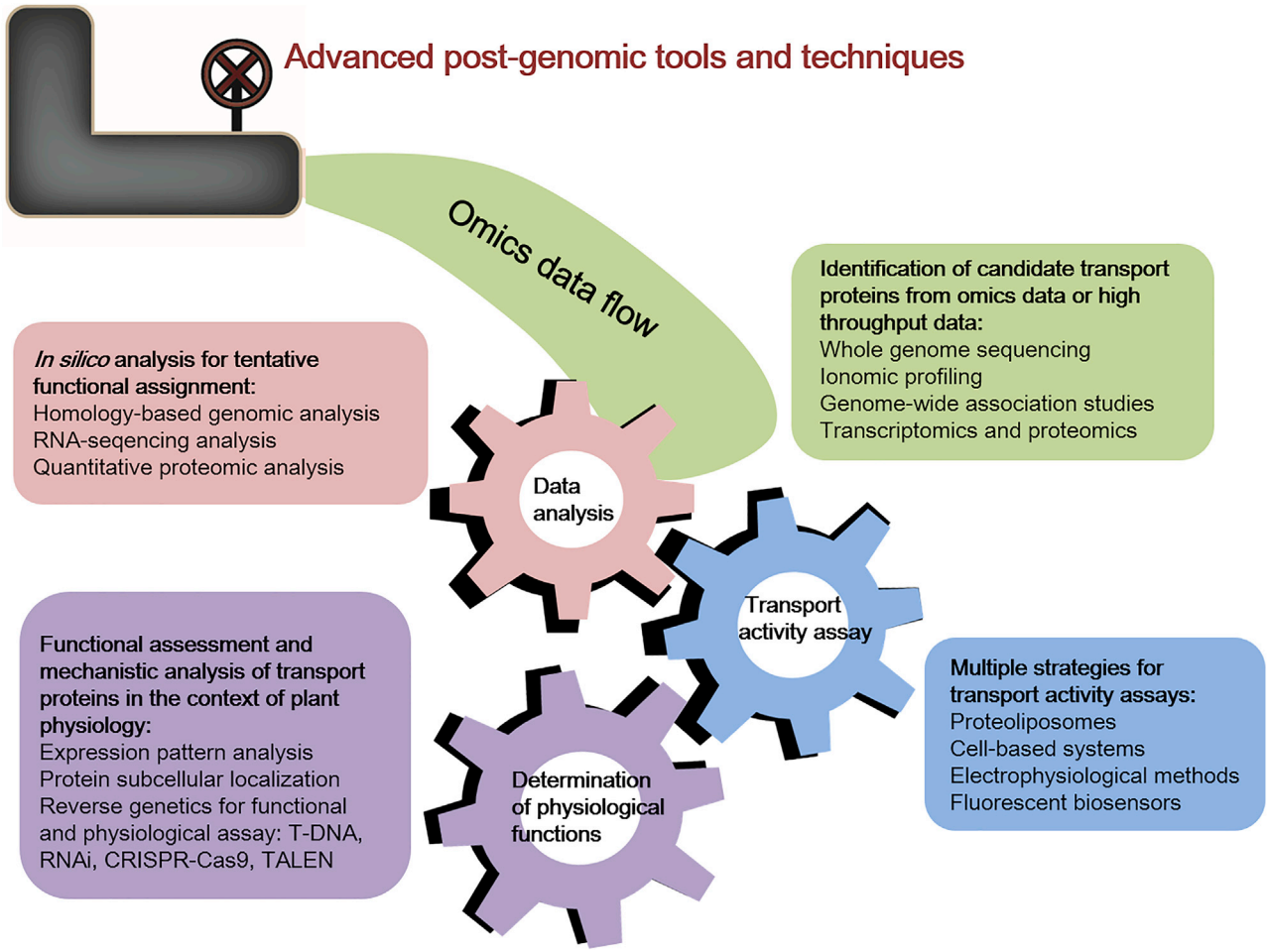

Figure 1. Overview of the Workflow in Identification and Characterization of Plant Transport Proteins in the Post-Genomic Era.
Wheel gears summarize the three major steps in defining the functions of a plant transporter protein as described in the review. The color-shaded panels list some common techniques and tools designed to address each research effort in the post-genomic era.

Moreover, within the cover of genomic tools, signaling pathways and interaction networks are established to uncover the regulatory mechanisms for plant membrane transport processes.

In this review, we highlight the impact of genome sequencing and genomic tools in the study of membrane transport proteins in plant cells. We also overview the experimental approaches widely used for analyzing the functions of transport proteins (Figure 1). We focus on ideas and examples that lead to significant advances in molecular identification and functional characterization of plant transport proteins. Regulatory networks and future perspectives are also discussed based on the studies of two decades on plant membrane transport in the post-genomic era.

SURFING THE GENOMIC WAVE: REVOLUTION IN THE RESEARCH OF PLANT MEMBRANE TRANSPORT

In the ''pre-genomic'' era, gene identification on a ''one-by-one'' basis was never an easy task. In particular, gene isolation of plant transport components appeared to be extremely challenging because the abundance of the membrane transport proteins is usually too low for biochemical purification. Therefore, although researchers observed transport activities in native membrane fractions, they found it difficult to identify causal proteins and related genes. In that era, forward genetic screening and subsequent map-based cloning, albeit rather time-consuming and labor-intensive, were occasionally successful in isolating transporter genes in model plants. Such examples include the nitrate (NO_3^-) transporter CHL1/NRT1.1 (Tsay et al., 1993), the sodium (Na^+) transporter SOS1 (Shi et al., 2000), the borate (BO_3^{3-}) transporter BOR1 (Takano et al., 2002), the auxin efflux transporters PIN1 (Galweiler et al., 1998) and PIN2 (Chen et al., 1998), as well as multidrug and toxic compound extrusion (MATE)-type transporters for plant metabolite translocation (Debeaujon et al., 2001; Nawrath et al., 2002). Forward genetics continues to be productive in the post-genomic era, especially for some specific transport processes. For instance, Lsi1 and Lsi2 were isolated from rice as two essential silicic acid (SiO_3^{2-}) transporters (Ma et al., 2006, 2007), while a unique vacuolar P-type proton (H^+)-pump predominantly expressed in petals was identified to control pH-dependent color alteration in the flowers of petunia (Verweij et al., 2008). In a more recent aequorin-based calcium (Ca^{2+})-imaging screen, OSCA1 was

identified as a previously unknown plasma membrane protein that forms a hyperosmolality-gated calcium-permeable cation channel (Yuan et al., 2014). A more convenient and straightforward strategy in identifying novel plant transport proteins is based on functional complementation screens by expressing plant cDNA libraries in bacterial or yeast mutant strains that show a clear-cut phenotype associated with a transport process. The cDNA clones that rescue mutant phenotype likely encode the proteins that participate in a particular membrane transport process. In the golden age of the 1990s, many transport genes from plants were cloned using this strategy. Some examples include plant potassium (K^+) channels (Anderson et al., 1992; Sentenac et al., 1992) and transporters (Santa-Maria et al., 1997; Fu and Luan, 1998; Kim et al., 1998), CorA-type magnesium (Mg^{2+}) transporters (Li et al., 2001), phosphate (Pi) transporters (Leggewie et al., 1997), sulfate (SO_4^{2-}) transporters (Smith et al., 1995), the wheat cation transporter LCT1 (Clemens et al., 1998), vacuolar Ca^{2+}/H^+ exchangers (Hirschi et al., 1996), the iron (Fe^{2+}) transporter IRT1 (Eide et al., 1996), ZIP-type zinc transporters (Grotz et al., 1998), the copper (Cu^{2+}) transporter COPT1 (Kampfenkel et al., 1995), SUC-type sugar transporters (Riesmeier et al., 1992), the amino acid and oligopeptide transporter (Frommer et al., 1993), and the DTX1 efflux carrier for toxic compounds (Li et al., 2002). Certainly, bacteria and yeast cannot parallel the diversity of plant transport proteins. In addition, expression of a foreign protein might result in unexpected artifacts that complicate the process to isolate the desired gene coding for a plant transport component (Lesage et al., 1994; Marini et al., 2000).

The genome sequencing project in *A. thaliana* has empowered researchers, for the first time, to read nature's complete genetic blueprint for creating a flowering plant (Arabidopsis Genome Initiative, 2000). With the help of bioinformatics programs, the majority of the open reading frames in the genome is predicted and proteins can be catalogued accordingly. The inventory suggests that among approximately 25 000 proteins in total, around 8600 possess at least one transmembrane domain and are therefore classified as "membrane proteins" (Arabidopsis Genome Initiative, 2000). Based on the sequence analysis alone, these membrane proteins and their coding genes can be divided into large groups, forming defined families. Among them, the gene families encoding putative solute transport proteins are particularly large, highlighting the complexity of plant membrane transport systems (Schwacke et al., 2003). Since then, research on membrane transport has been profoundly revamped by the genome-originated data. Functional analysis of plant transport proteins becomes much easier, faster, and more successful. For some proteins, tentative functional assignment is possible based on prior knowledge of previously characterized transporters and conserved protein domains from either plant or non-plant models. Following this idea, extensive efforts have successfully identified specific subfamilies of plant transporters, including cation transporters (Maser et al., 2001), ABC transporters (Sanchez-Fernandez et al., 2001), P-type pumps (Axelsen and Palmgren, 2001), major intrinsic proteins (Johanson et al., 2001), and major facilitator superfamily (Nino-Gonzalez et al., 2019). Moreover, searching for plant gene homologs involved in an evolutionally conserved transport process can be easily done based on the sequence information in yeast or other eukaryotes. A good example is the identification of the complete set of genes for the plant vacuolar H^+-ATPase complex consisting of multiple subunits, most of which are encoded by several gene isoforms. Although *DET3* was initially isolated as one subunit of plant V-ATPase via a forward genetic screen (Schumacher et al., 1999), many genes encoding other subunits had remained unclear until the genome sequence became available for a thorough analysis of the coding sequences that build up the whole complex (Sze et al., 2002).

In the post-genomic era, earlier functional complementation screens in bacteria or yeast have also gained more productivity. Gene families containing multiple members instead of one single gene could be readily identified because the homology-based sequence comparison enabled genome-wide analysis and cloning of all the paralogs. A typical study for this case might be the discovery of a family of at least 56 MATE-type transporters in *Arabidopsis* upon functional isolation of AtDTX1 based on the bacterial mutant strain deficient in multidrug resistance (Li et al., 2002). Forward genetic approaches also benefit from plant genome research because sequence-indexed genomic tools make the map-based gene cloning procedure more efficient than before, and help with a better-designed screening strategy. For instance, combining ionomic profiling and genomic tools, a handful of genes coding for plant transporters were isolated from *Arabidopsis* and rice as key players in plant mineral nutrition or detoxification of excessive metals (Ren et al., 2005; Rus et al., 2006; Tomatsu et al., 2007; Morrissey et al., 2009). In recent years, as genome-wide association studies become a mainstream strategy for understanding the genetic basis of phenotype variation, quite a few plant transporter genes were independently identified as the causal loci for different traits of interest (Chao et al., 2012; Huang et al., 2016; Wang et al., 2016; Hazzouri et al., 2018), thereby providing in-depth interpretation of their functional alleles in natural plant ecotypes or varieties.

With the rapid advances of genomics, other high-throughput technologies such as transcriptomics and proteomics have been established, and their applications are largely dependent on the integration of genomic database. In particular, proteomic analysis has become a powerful tool in the investigation of plant transport proteins in specific organelles. Distinct membrane fractionations of high purity coupled with proteomic experiments have proved to be effective in sorting out the transport proteins in the plasma membrane (Alexandersson et al., 2004), tonoplast (Carter et al., 2004), and endosomal membranes (Parsons et al., 2013) as well as the envelope of chloroplast (Ferro et al., 2003) or mitochondria (Heazlewood et al., 2004). Many of the transport proteins identified in these studies have homologs in other organisms based on the sequence analysis, providing initial clues on their function for further experimental verification using plant genetics and other approaches. A step further along this line is the quantitative proteomics that aims to identify post-translational modifications of membrane transport proteins as a way to explore potential regulatory mechanisms under different environmental conditions (Niittylae et al., 2007; Whiteman et al., 2008a, 2008b; Endler et al., 2009; Elmore et al., 2012; Schulze et al., 2012). The resulting datasets are valuable resources that would assist genetic and functional studies *in planta*.

The "next-generation sequencing" technology developed in the mid-2000s represents a milestone in plant genomic studies (Margulies et al., 2005). Since then, plant genome projects and

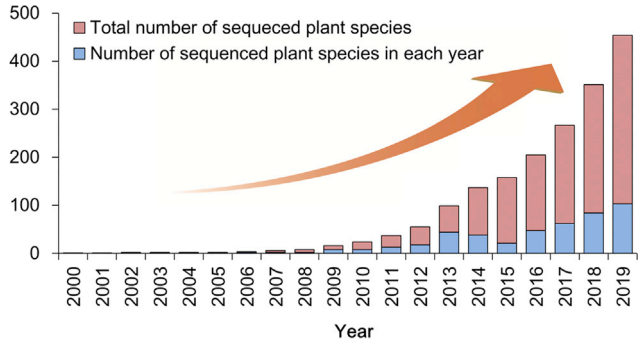

Figure 2. Increasing Number of Genome Sequencing Projects in Plant Species since 2000.
The data are extracted from the NCBI genome database updated on October 30, 2019 (https://www.ncbi.nlm.nih.gov/genome/?term=Land+Plants).

functional genomic studies have extended far beyond a small number of plant models. So far, more than 400 plant species have been sequenced at different levels of quality, enabling comprehensive understanding of membrane transport mechanisms in diverse plants at the molecular level (Figure 2). Large gene families encoding plant transport proteins can be analyzed *in silico* in any plant as long as the sequence of the genome is available. Multiple transport models and their regulatory networks can also be established using bioinformatics programs. Functional conservation and diversification of various transporters in different plant species can be analyzed, thereby providing evolutionary insights into the membrane transport processes in plants. For example, most metal transporter families can be traced back to the unicellular green plant ancestor *Chlamydomonas reinhardtii* (Hanikenne et al., 2005) and exhibit a clear presence in the charophyte algae that are basal to land plants (Nishiyama et al., 2018), highlighting conserved functions of these transporters to maintain ionic homeostasis in plant cells. On the other hand, some Ca^{2+}-conducting channels in animals such as voltage-dependent calcium channels and transient receptor potential channels are found in chlorophyte algae such as *Chlamydomonas* (Fujiu et al., 2009; Arias-Darraz et al., 2015), but become absent in the genomes of charophytes as well as all higher plants, indicating the divergent coding mechanisms for Ca^{2+} signals in response to environmental stimuli between land plants and their primitive algal ancestors (Wheeler and Brownlee, 2008). Notably, diversification of conserved transport proteins along the phylogenetic tree becomes a general theme, emphasizing more complex developmental programs and sophisticated environmental adaptation mechanisms as land plants evolve. For instance, the plasma membrane H^+-pump AHA family is expanded from two genes in the early land plant *Physcomitrella patens* to 11 genes in the flowering plant *A. thaliana*, implicating an increasing need to regulate the H^+ motive force in specialized cell types along evolution (Pedersen et al., 2012). Another example is the PUP family encoding purine uptake permease, which exists in the early vascular plant fern but is absent in a non-vascular moss, suggesting a possible role of PUP in the transport of secondary metabolites for adaptive success in vascular plants (Jelesko, 2012). The availability of genome sequence in non-model plants also provides an incredible opportunity to identify unique transporters that participate in specific transport processes, which otherwise may be difficult to study in the conventional plant models. For example, taking the advantage of RNA-sequencing analysis in the transcriptome of C3 and C4 plants of the genera *Flaveria* and *Cleome*, BASS2 was successfully identified as a plastidial sodium-dependent pyruvate transporter that exhibits a predominant transport activity in the chloroplast of C4 over that of C3 plants (Furumoto et al., 2011). In another recent study of the medicinal plant *Catharanthus roseus*, the transcriptional expression data have enabled the identification of tonoplast-localized CrNPF2.9 as a vacuolar strictosidine efflux transporter for specialized metabolism (Payne et al., 2017). Furthermore, plant genome analysis also enables comparative studies of transport proteins in plants, bacteria, and animals, which could provide insight into the conservation and uniqueness of plant membrane transport. For instance, as water and sugar are necessities to all life forms, aquaporins as water channels and SWEET-family sugar transporters appear to be structurally conserved in all organisms. However, while mammals only have a few genes encoding aquaporins, a single plant species can be equipped with more than 100 aquaporin isoforms that may be expressed in different cell types or under various environmental conditions (Bezerra-Neto et al., 2019). Genes coding for plant and animal SWEET-type sugar transporters probably originate from prokaryotic gene duplication, and as a result, the fusion of two bacterial half-sized SWEET structural units forms an asymmetrical sugar translocation path for (full) SWEET proteins in plants and animals (Jeena et al., 2019). Similar evolutionary genomic analysis suggests that plant voltage-gated K^+ channels, previously believed to derive from "Shaker-like" channels, may belong to a superfamily that comprises cyclic nucleotide-gated channels present in all lineages and thus may have derived from a prokaryotic ancestor (Jegla et al., 2018).

TRANSPORT ACTIVITY ASSAYS IN DIVERSE SYSTEMS: ALL ROADS LEAD TO FUNCTIONS

Plant membrane transport proteins possess a large set of substrates resulted from complex primary and secondary metabolism. Their transport activities need to be regulated to fit into versatile physiological programs in response to variety of environmental conditions. In the post-genomic era, although a large number of protein families with putative transport functions have been annotated, their biological functions regarding transport activity and substrate specificity must be validated experimentally. However, due to the hydrophobic property, transport proteins cannot be studied in aqueous solutions but have to be integrated into a lipid membrane system to experimentally probe the kinetics of transport activity. In addition, a vast variety of cellular solutes with disparate structures exist in the plant kingdom, serving as myriad potential substrates for distinct plant transporters. For these reasons, it is challenging to identify the substrate and measure the activity and kinetics of a plant transport protein. We discuss a list of methods and examples that have proved to be successful in assigning functional properties to plant transport proteins.

Biochemical measurement of a transporter activity requires the protein of interest to reside in a lipid bilayer-based membrane that

separates two compartments. In this regard, purified transporters can be reconstituted into liposomes of an appropriate lipid composition. The proteoliposomes can then be employed to monitor the transporter-mediated uptake of labeled substrates into the liposomes, thus measuring parameters such as transport kinetics, substrate specificity, optimal catalytic conditions, and potential activators or inhibitors (Johnson and Lee, 2015). Due to technical difficulty and complication, only a few plant transport proteins are studied using this method. These include the organelle metabolite transporters such as the mitochondrial adenine nucleotide transporter ADNT1 (Palmieri et al., 2008) and the chloroplastic ascorbate transporter PHT4;4 (Miyaji et al., 2015). For plant ion channels, electrophysiology in cell-based systems has clear advantages (see later sections), although the liposome reconstitution method may also be feasible as evidenced by the analysis of a CorA-type Mg^{2+} transporter (Ishijima et al., 2012).

A more practical approach for the transport assays is to express plant transport proteins in a cell-based system including bacterium, yeast, frog oocyte, or mammalian cell. Because these heterologous systems do not contain plant-derived gene products, the background is generally low, especially when exogenous transport proteins are overexpressed. Hundreds of plant transporter proteins were functionally analyzed in these systems. Following functional complementation of bacterial or yeast mutants by expressing plant proteins, detailed transport assays are readily carried out to study the transport activity toward a specific substrate. As a eukaryotic organism, yeast is generally preferred over the bacterial systems such as *Escherichia coli* to study plant transport proteins because many eukaryotic membrane proteins, for unknown reasons, fail to fold correctly and often cause toxicity when overexpressed in bacteria. In particular, yeast cells possess some organelles equivalent to those in plant cells, typically represented by the vacuole, which are not found in bacteria or animal cells. For instance, genes for plant vacuolar Ca^{2+}/H^+ (Hirschi et al., 1996) and monovalent cation/H^+ exchangers (Gaxiola et al., 1999; Cagnac et al., 2007) were successfully identified, and their activities were quantified in the vacuole of yeast mutant lacking the equivalent transporters. Another commonly used expression system for plant transport proteins is the *Xenopus* oocyte (Schachtman et al., 1992; Pike et al., 2019). Aside from the relative ease in manipulation, the oocyte cells are in quiescent stage, exhibiting low background transport activity but high efficiency of protein translation from foreign RNAs. In addition to electrophysiological experiments that directly monitor the ionic flows, assays in *Xenopus* oocytes to detect transport activity can be conducted by quantitative measurement of the substrates. For influx assays, quantification can be achieved through scintillation counting of incorporated radiolabeled substrates (Nour-Eldin et al., 2012), liquid chromatography–mass spectrometry (LC-MS) analyses of oocyte extracts (Jorgensen et al., 2017) or spectrophotometric detection of fluorescence-based compounds Ho and Frommer, 2014. For efflux assays, experimental setups are often more complicated owing to the pre-microinjection of the proposed substrates. The human embryonic kidney (HEK) cells feature high transfection efficiency, low levels of endogenous channels, and high compatibility to express functional plant proteins, making them an alternative host for functional studies of plant membrane transporters (Ooi et al., 2016). The HEK cell model is widely used in the current–voltage measurements of plant ion channels, but in some cases they can also assist in the assays of plant metabolite transport (Liu et al., 2001; Geisler et al., 2005). An alternative high-efficiency animal expression system is based on the insect cells, where the activities of plant K^+ channels have been successfully determined (Gaymard et al., 1996; Marten et al., 1996).

Among the plant transport proteins, channels and channel-like ion carriers are often studied by electrophysiological methods including patch clamping and two-electrode voltage clamping (Hedrich, 2012). These assays can be applied to different cell systems. For plasma membrane-localized ion channels, electrophysiological recordings are usually conducted in the *Xenopus* oocytes or mammalian cells because plant channels are likely to be targeted properly to the plasma membrane of these cell types. A plant cell system may also be feasible as long as the ectopic expression of the transporter override the endogenous background activity, as shown by the patch-clamping analysis of KAT1 activity in the tobacco mesophyll cells that lack a detectable inward K^+ current commonly found in guard cells (Bei and Luan, 1998). Following this pioneering work, transient expression of plant channels in tobacco leaves has also proved to be feasible in analyzing exogenous channel activities (Hosy et al., 2005; Latz et al., 2007). For vacuolar ion channels or transporters, the isolated vacuole from plant tissues is an ideal model (Hedrich et al., 1986; Ward and Schroeder, 1994), considering the fact that vacuolar transporters may be mistargeted in animal cells. Yeast vacuoles can also be used for patch-clamping analysis of plant tonoplast channels (Hamamoto et al., 2008). Through extensive efforts in the past 20 years, a number of ion channels and transporters predicted by genomic tools have been functionally verified by electrophysiological analyses in terms of their permeability, ionic selectivity and activation mechanism. These well-characterized channels include shaker-type K^+ channels for K^+ nutrition and homeostasis (Gaymard et al., 1998; Hirsch et al., 1998), HKT-type transporters for Na^+ tolerance (Uozumi et al., 2000; Ren et al., 2005), SLAC-type anion channels in guard cell movement (Vahisalu et al., 2008), ALMT-type channels for malate secretion and anion homeostasis (Hoekenga et al., 2006; De Angeli et al., 2013), vacuolar TPK-type K^+ channels (Gobert et al., 2007), slow vacuolar channel TPC1 (Dadacz-Narloch et al., 2011), vacuolar CLC-type NO_3^-/H^+ exchangers (De Angeli et al., 2006), CNGC- and GLR-type Ca^{2+} channels in signal transduction (Vincill et al., 2012; Gao et al., 2014; Wudick et al., 2018), and H^+-coupled sugar carriers (Carpaneto et al., 2005; Wingenter et al., 2010; Schulz et al., 2011; Jung et al., 2015), as well as many others. Recent studies reveal channel-like conductance of some ion carriers and metabolite transporters as in the cases of vacuolar Pi transporter VPT1 (Liu et al., 2015) and vacuolar MATE transporters (Zhang et al., 2017). Furthermore, some proteins facilitating transport of plant metabolites might also be applicable to electrophysiological experiments, provided that the substrate translocation by the carrier would trigger a considerable electric current. Indeed, the *Arabidopsis* sugar alcohol permease homolog AtPLT5 is found to exhibit substrate-elicited ion currents that are coupled to sugar import into the cell when expressed in *Xenopus* oocytes (Klepek et al., 2005; Reinders et al., 2005). Several plant monosaccharide transporter-like proteins are also shown to exhibit clear substrate-driven electric currents upon the uptake of different inositol epimers, either in *Xenopus* oocytes (Schneider et al., 2006) or plant vacuoles (Schneider et al., 2008).

For some plant transport proteins, an enzymatic catalysis may be tightly correlated with the transport activity, and accordingly the enzymatic assay can substitute for the direct transport measurement. A subgroup of such transport proteins includes the ATP-driven pumps that couple the hydrolysis of ATP to the transport of ionic species against their electrochemical gradient. For plant H^+-ATPases that exhibit robust activities, enzymatic measurement of ATP hydrolysis becomes particularly useful, because unidirectional ion fluxes using radioisotopes cannot be achieved for H^+. Using purified membrane vesicles, both the plasma membrane- and tonoplast-localized H^+-ATPases in plants were enzymatically analyzed with a tight kinetic correlation between proton pumping and ATP hydrolysis (Sze, 1985; Palmgren, 1990; Muller et al., 1999). Alternatively, due to the lack of defined membrane potential and solute gradients in the isolated vesicles, electrophysiology procedures can be applied to probe the H^+ pumps in both plasma membrane (Lohse and Hedrich, 1992) and vacuolar membrane (Hedrich et al., 1989; Krebs et al., 2010; Rienmüller et al., 2012) of plant cells. More recently, total internal reflection fluorescence microscopy was established to directly image a plant-derived H^+ pump in a single nanoscopic lipid vesicle, enabling a real-time analysis of the kinetics and regulation of the pump (Veshaguri et al., 2016).

Genetically encoded fluorescent biosensors have been developed to image the real-time transport activity at physiologically relevant spatial and temporal scales *in vivo* (Walia et al., 2018). As a general principle, these sensors convert a molecular event into the readout in fluorescence signal upon substrate binding to a sensing domain that undergoes a conformation change. A good example is genetically encoded calcium indicators (GECIs) designed for spatiotemporal analysis of Ca^{2+} fluxes during various physiological processes. After several generations of improvement, single fluorescence protein-based GECIs with high sensitivity and signal-to-noise ratio, represented by GCaMPs (Nakai et al., 2001; Zhao et al., 2011), have become widely used today. The intensiometric GCaMP-type probes yield fluorescence signals with a superior dynamic range as compared with the ratiometric Ca^{2+}-sensor Yellow Cameleon 3.6 (YC3.6) in plants (Keinath et al., 2015). A major drawback of the intensity-based probes such as GCaMPs is that variations in protein expression may contribute to the differences observed in the fluorescent signals. This problem can be overcome by co-expression of a reference fluorescent protein for normalization (Ast et al., 2017; Waadt et al., 2017). The GECIs have proved to be effective in research related to plant development (Ngo et al., 2014) as well as plant long-distance Ca^{2+} signaling during salt stress (Choi et al., 2014) or herbivore attack (Vincent et al., 2017; Nguyen et al., 2018; Toyota et al., 2018; Meena et al., 2019). To back up the electrophysiological recordings of plant Ca^{2+} channel activity, GECIs are also frequently used in a heterologous cell-based system for direct imaging of Ca^{2+} dynamics upon expression of a putative Ca^{2+} channel. They can offer effective measure to dissect more complicated regulatory processes at the cellular level. For example, using the GCaMP3 sensor expressed in the HEK cells, the pollen tube tripartite CNGC18–CNGC8–CAM2 complex has been reconstituted as a self-regulatory Ca^{2+} oscillator (Pan et al., 2019). Fluorescence resonance energy transfer (FRET)-based biosensors are another example that has been highly effective in measuring *in vivo* fluxes of solutes (Looger et al., 2005). The FRET sensors for ionic substrates have been developed, including a sensor for the detection of NO_3^- (Ho and Frommer, 2014), NH_4^+ (De Michele et al., 2013), Pi (Mukherjee et al., 2015), and Zn^{2+} (Lanquar et al., 2014). Other than the biosensors monitoring the ion fluxes, a growing array of FRET sensors have been developed for quantitative analyses of metabolic compounds including saccharides, amino acids, and phytohormones. One of the most successful applications resulted in identification of the SWEET family from *Arabidopsis* as evolutionarily conserved glucose and sucrose transporters (Chen et al., 2010). Co-expression of AtSWEET1 and the genetically encoded glucose sensor in HEK cells enabled analysis of the transport activity and substrate specificity of the novel sugar transporter, which was subsequently supported by yeast functional assay and the transport assays in *Xenopus* oocytes (Chen et al., 2010). Taking the advantage of both a Ca^{2+} sensor and a glutamate sensor, the amino acid glutamate is established as a wound signal that triggers long-distance, Ca^{2+}-associated plant defense signaling (Mousavi et al., 2013; Toyota et al., 2018).These studies suggest that the use of biosensors is emerging as a new powerful way to monitor metabolite transport activities and discover relevant transport proteins. In future studies, custom-designed genetically encoded biosensors ought to be generated to expand the toolkit for *in vivo* analysis of specific plant metabolites and their dynamic transport processes.

The methods described above are often used in combination to confirm activities of transport proteins. Any individual method has pros and cons depending on specific transporters in question. Each procedure may give rise to negative results in the transport assays for various reasons. For example, a plant transport protein might be incorrectly matured or targeted in a heterologous system. Alternatively, the transport system might require plant-specific accessory proteins for post-translational modifications (Li et al., 2006; Xu et al., 2006; Geiger et al., 2009b; Lee et al., 2009) or plant-derived compounds for full activation (Meyer et al., 2010; De Angeli et al., 2013) or repression (Pottosin et al., 2014). In addition, functional reconstitution of some channels appears to rely on heteromeric assembly of different subunits (Tian et al., 2019). In this context, even when a transport activity can be positively detected in a heterologous system, results should be interpreted with caution, and other lines of evidence are essential to draw conclusions on the function of the transporter in the native plant cell. To this end, noninvasive microelectrodes may be used for concurrent quantification of net fluxes of several ions with high spatial and temporal resolution (Newman, 2001; Shabala et al., 2006). This approach is particularly useful in testing the effect of environmental conditions on the dynamic ion fluxes in plant cells (Shabala and Bose, 2012). With the help of aforementioned techniques coupled with genomic tools, studies can now link the *in vitro* transport activities with physiological processes in plant cells.

REVERSE GENETICS: FROM DISCOVERY OF KEY PLAYERS TO SYSTEMATIC ANALYSIS OF GENE FAMILIES

Despite the growing body of information on protein sequences from genomic resources followed by the functional characterization in biochemical transport assays, the fundamental question concerning the physiological role needs to be addressed *in planta* for each

plant transport protein. In this context, "reverse genetics" has become a critical approach to the function of transport proteins in the post-genomic era (Alonso and Ecker, 2006). The principle of reverse genetics is to specifically modify a gene, or its expression, and characterize the phenotypic consequences of this modification. To this end, a number of genetic resources have been established not only in *Arabidopsis* but also in rice, maize, *Medicago*, and other plant species. Large collections of plant lines mutagenized by T-DNA or transposons can be screened for the lines harboring insertions within a gene of interest. Databases resulted from systematic sequencing of flanking sequences around insertion sites have been built up so that insertional mutant lines with disruption of specific genes become publicly available for functional analysis (O'Malley and Ecker, 2010). More recently, whole genome resequencing of an individual mutant has been conducted to verify all the latent mutations in the line. With the help of these mutant materials as well as other approaches to specifically modify candidate genes, many key components in plant membrane transport processes have been identified and corroborated in the genetic context, bridging the gap between physiological processes and molecular mechanisms involving particular transporters. For example, the long-sought vacuolar transporters importing Pi to the vacuolar lumen or exporting Pi from the vacuole to the cytosol were identified as two different types of transporters, respectively. Using reverse genetics, the *Arabidopsis* null mutant lacking VPT1/PHT5;1 is shown to display impaired Pi influx into isolated vacuoles and thus overaccumulate Pi in the cytoplasm, which is consistent with the role in mediating Pi sequestration into the vacuolar lumen (Liu et al., 2015, 2016). Furthermore, two rice transporters, OsVPE1 and OsVPE2, appear to export Pi from the vacuole, supported by the genetic data that double knockout of both rice genes results in retarded plant growth under Pi-deficient conditions, presumably due to defective Pi remobilization from the vacuolar store (Xu et al., 2019). An alternative procedure to disturb gene function is based on RNA interference (RNAi), which usually attenuates gene expression with wide range of inhibitory levels in a sequence-specific manner. This approach is particularly useful when T-DNA insertional knockout mutants are not available in the public stock centers. Functions of several transporter are genetically verified using RNAi including the MGT6 transporter for root Mg^{2+} acquisition (Mao et al., 2014) and the nuclear-localized CNGC channels mediating symbiotic Ca^{2+} oscillations (Charpentier et al., 2016).

In recent years, gene editing tools represented by CRISPR/Cas9 have greatly improved the feasibility and efficiency of reverse genetic studies. Generating targeted mutations in transporter genes of interest in plants has never been easier, which is especially critical for the research in crop plants because large collections of genetic mutants are not available for those species. The new technologies also hold promise for engineering agronomically desired traits in commercial crop varieties in general. Transporter genes may be targeted to for improving crop traits. For example, CRISPR/Cas9-mediated inactivation of the cesium (Cs^+)-permeable K^+ transporter OsHAK1 has been shown to reduce Cs^+ uptake in rice roots, thus restricting potential contamination of trace levels of radioactive Cs^+ in the food chain (Nieves-Cordones et al., 2017). Transcription activator-like effectors (TALEs) from some pathogen species tend to target the genes encoding SWEET transporters that release sugar into the apoplast, causing plant susceptibility to pathogens. Using another gene editing tool derived from TALEs (TALEN), OsSWEET14 promoter region was subjected to mutagenesis, which disrupted the binding site of a pathogen effector protein and ultimately resulted in disease resistance (Li et al., 2012). Similarly, a null mutation in OsSWEET13 generated by CRISPR/Cas9 enhances resistance to bacterial blight in an *indica* rice cultivar (Zhou et al., 2015).

As membrane transport represents a major housekeeping activity, disruption of a transporter function can lead to multiple defects in plants. Therefore, mutants lacking a specific transporter often exhibit pleiotropic phenotypes in the mutant plants, complicating the assessment of its physiological role in plants. To achieve a clear functional interpretation from the genetic data, other data such as gene expression pattern coupled with protein subcellular localization of a transporter should be collected to assist the analysis of the mutant phenotypes. A transport assay in an appropriate cell system with little background activity would further help with phenotypic analysis to yield a precise physiological role for the transport protein.

Genome sequencing uncovers that, like many other proteins, transporters are often encoded by multigene families in higher plants, which may have evolved from gene duplication events. The members in a transporter family can display identical substrate specificity and stand functionally redundant *in planta*. In many cases, however, the different family members are differentially expressed in specific plant cell types and thus have distinct functions. For example, the SWEET family plays a central role in sugar translocation, but different members in *Arabidopsis* specifically function in pathogen infection (Chen et al., 2015a), nectar secretion (Lin et al., 2014), phloem loading (Chen et al., 2012), pollen viability (Sun et al., 2013), and seed filling (Chen et al., 2015c). For gene members with highly redundant function, the reverse genetic approach makes it feasible to precisely delineate such redundancy by generating a higher-order mutant lacking multiple genes. For example, both the vacuolar and the endosomal K^+/H^+ exchanger families in *Arabidopsis* exhibit considerable functional redundancy, as concluded from systematic analyses using single and higher-order mutants (Zhu et al., 2018; Bassil et al., 2019; Wang et al., 2019). In some cases, transporters of different families may function synergistically in the same process, as demonstrated by genetic analyses of AKT1 and HAK5 that play a synergistic role in root K^+ uptake when external K^+ is deficient, although they belong to structurally distinct families (Pyo et al., 2010). In a more recent study, distinct K^+-permeable TPC1 and TPK-type channels in the tonoplast acted in concert to confer electrical excitability on plant vacuoles (Jaslan et al., 2019). In all these cases, the reverse genetic approach plays a crucial role in dissecting the functional diversity or redundancy of individual members in multigene families.

TO BE ACTIVE OR TO BE SILENT: REGULATION OF MEMBRANE TRANSPORT BY PLANT SIGNALING NETWORKS

Similar to many other processes in plant cells, membrane transport activity is dynamically regulated by developmental programs and environmental responses. To optimize the activity of a

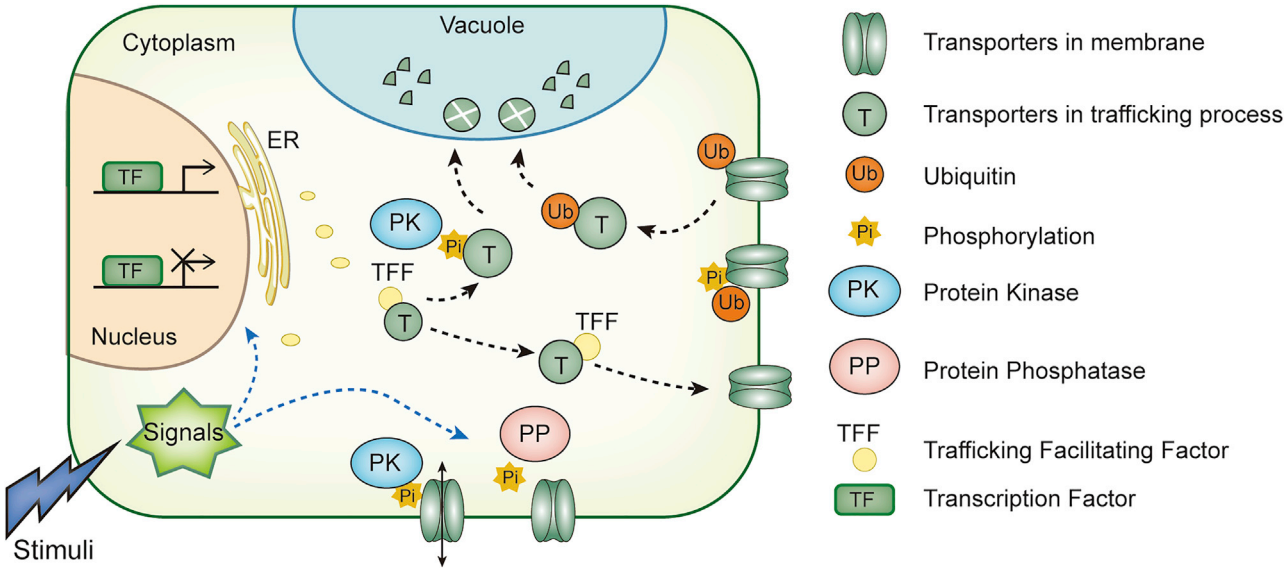

Figure 3. A Simplified Diagram of Multiple Molecular Pathways that May Regulate Plant Transport Proteins at Various Levels.
At the transcriptional level, transcription factors master the expression of genes encoding plant transport proteins in response to different developmental and environmental cues. After the proteins are synthesized, components in the secretory pathway or other trafficking mechanisms ensure the proper targeting of the transport proteins to the correct membrane fractions for physiological functions. At the post-translational level, multiple covalent modifications such as phosphorylation and ubiquitination may occur on different plant transport proteins to control their abundance, stability, targeting, or activity.

particular transport process, transport proteins are regulated by signaling pathways at multiple levels (Figure 3). For example, transcription of many transport genes is highly tissue specific and responsive to environmental cues. The transporters, once synthesized, must be targeted to the correct compartment for proper function, a process highly controlled by protein trafficking mechanisms. The transport activity can be regulated by phosphorylation and other covalent modifications of the transporters. Because genes coding for the regulatory components such as transcription factors, protein kinases and phosphatases, E3 ubiquitin ligases, and players in protein trafficking can be comprehensively identified through genomic resources, the further functional studies of the transport regulation have benefited greatly from the genome databases and genomic tools in the post-genomic era.

At the transcriptional level, a unique feature for genes encoding transport proteins is regulation by the availability of their cognate substrates, to fine-tune the transport capacity as needed in cell physiology. For example, depletion of essential mineral nutrients in the environment often enhances the expression of respective transporter genes to upregulate uptake and translocation, while high levels of the same nutrients repress gene expression (Kudoyarova et al., 2015). For metabolite transporters, however, transcript abundance may be positively correlated to the metabolic state or flux through a pathway in a specific cell type at a given time (Linka and Weber, 2010). Many plant transport-associated genes display stress-responsive transcription, suggesting that they are involved in stress-adaptation processes (Shabala et al., 2016). In the post-genomic era, reverse genetics and other functional studies have identified many key transcription factors dictating the expression of transport genes. Moreover, the *cis*-acting DNA elements can be computationally analyzed at the whole genome level, which in turn could predict potential transcriptional regulation of transport genes by transcription factors (Ibraheem et al., 2010). Co-expression analysis using transcriptomic data also serves as an effective method to identify new transporters in a metabolic pathway (Dobritzsch et al., 2016; Demurtas et al., 2019).

While it is relatively easy to monitor gene expression patterns, post-translational modifications may serve as more profound regulatory mechanisms underlying the structure, activity, and function of plant transport proteins. The modification enzymes often target plant transporters at the hydrophilic termini or loop regions on the cytoplasmic side. Protein phosphorylation is believed to act as a universal molecular switch to fine-tune the activities of transport proteins. For example, a family of plant-specific protein kinases, termed CBL-interacting protein kinases (CIPKs), preferentially regulates transport proteins by interacting with a group of calcineurin B-like (CBL) calcium sensors (Luan, 2009). Because a large majority of CBL proteins harbors lipid-modification motifs that anchor them to the membrane, CBL–CIPK complexes are also associated with cellular membranes, underpinning their central role in regulating membrane transport processes in the plasma membrane or the tonoplast of plant cells. At the plasma membrane, the CBL1/9–CIPK23 complexes target multiple plant transport proteins including the K^+ channel AKT1 (Li et al., 2006; Xu et al., 2006), the K^+ transporter HAK5 (Ragel et al., 2015; Scherzer et al., 2015), the NO_3^- transporter CHL1/NRT1.1 (Ho et al., 2009), the AMT1-type NH_4^+ transporters (Straub et al., 2017), and the anion channel SLAC1 and its homologs (Maierhofer et al., 2014a, 2014b). Interestingly, CHL1/NRT1.1 relies on CIPK23-mediated phosphorylation as a molecular switch to facilitate dual-affinity nitrate uptake as well as sensing of nitrate signals, thereby functioning as a nitrate

"transceptor" (Ho et al., 2009). Meanwhile, CBL4/SOS3–CIPK24/SOS2 stimulates the Na^+/H^+ exchanger SOS1 to confer salt tolerance (Qiu et al., 2002) and CBL1–CIPK5 regulates K^+ effluxes in the guard cell through modification of the outward K^+ channel GORK (Fotster et al., 2019). In the vacuolar membrane, CBL2/3–CIPK3/9/23/26 complexes control Mg^{2+} sequestration into the vacuole compartment by unknown transport proteins (Tang et al., 2015). In addition to CIPK-type kinases, other protein kinases can also modify transport proteins, thus linking membrane transport processes to signal transduction pathways in plant cells. For instance, the vacuolar K^+ channel TPK1 may also be phosphorylated by a Ca^{2+}-dependent kinase (CDPK) and the KIN7 receptor-like protein kinase in different physiological contexts (Latz et al., 2013; Isner et al., 2018). The SnrK2-type kinase OST1 and Ca^{2+}-dependent protein kinases (CDPKs) jointly phosphorylate the SLAC1 channel for activating anion efflux in the guard cells during abscisic acid (ABA) and CO_2 signaling (Geiger et al., 2009b, 2010; Lee et al., 2009; Brandt et al., 2012, 2015). Auxin distribution in plant requires PIN-type efflux transporters that are regulated by D6PK and PINOID protein kinase families (Zourelidou et al., 2014). In plant immunity response, the Ca^{2+} channel subunit CNGC4 is phosphorylated by the receptor-like cytoplasmic kinase BIK1 to initiate the Ca^{2+} signaling (Tian et al., 2019). To close the stomatal pores, the SLAH3 anion channel undergoes phosphorylation by PBL27, a BIK1 homolog, which is required for chitin-induced stomatal closure (Liu et al., 2019). Also, phosphorylation-dependent regulation of a sugar transporter STP13 by BAK1 could activate monosaccharide uptake for plants to compete with bacteria for extracellular sugar during antibacterial defense (Yamada et al., 2016). Together with many other examples, protein phosphorylation serves as a critical mechanism of post-translational modification for transport proteins that integrates various signaling pathways into membrane transport processes.

Ubiquitination is another common post-translational modification that often regulates transport proteins in plants. In most cases, the target proteins will be efficiently degraded by the 26S proteasome as soon as they are conjugated with ubiquitin on specific lysine residues. This mechanism allows cells to respond rapidly to the internal and external signals encountered by transport proteins. For example, the E2 ubiquitin-conjugating enzyme PHO2 and the E3 ligase NLA could work together to ubiquitinate the Pi transporter PHT1;4/PT2 for its degradation through 26S proteasome, which is thought to prevent Pi overaccumulation to toxic levels in plants (Huang et al., 2013; Lin et al., 2013; Park et al., 2014). Some other transport proteins are also shown to undergo direct ubiquitination. However, given the vast number and diversity of the transport proteins and ubiquitin E3 ligases encoded in plant genomes, transport events regulated by ubiquitination are underrepresented in current studies and await future discoveries.

Phosphorylation and ubiquitination of plant transport proteins may not simply act alone in controlling transport activity or abundance, but also work together to modify the dynamic trafficking process and stability of the transporters. For example, when heavy metals reach a toxic level in the soil, the IRT1 transporter mediating the uptake of several divalent cations will be phosphorylated by CIPK23, which in turn leads to association with IDF1 E3 ligase for endosomal sorting and protein degradation (Dubeaux et al., 2018). Phosphorylation can also work with other cellular machineries to coordinate the sorting of transport proteins. Under Pi-limiting conditions, a plant-specific SEC12-like protein, PHF1, recruits more PHT1-family transporters to the plasma membrane to boost Pi uptake (Gonzalez et al., 2005). Under Pi-replete conditions, however, interaction between PHT1s and PHF1 is hindered by phosphorylation of PHT1, which results in endoplasmic reticulum retention of PHT1s (Chen et al., 2015b). On the other hand, the localization and turnover of PHT1s are also modulated by ALIX protein, which is a subunit of the ESCRT (endosomal sorting complexes required for transport) machinery. This protein sorting machinery recognizes ubiquitinated PHT1s for endocytosis and vacuolar degradation (Cardona-Lopez et al., 2015). Interestingly, the borate exporter BOR1 is thought to be regulated by a similar mechanism. While the polar localization and constitutive endocytosis of BOR1 rely on clathrin-coated vesicles, borate-induced vacuolar sorting and degradation of BOR1 is possibly mediated by the ubiquitination-dependent ESCRT machinery (Larson, 2019; Yoshinari et al., 2019). From extensive studies on the regulation of auxin efflux carriers, a more sophisticated model has been delineated (Luschnig and Vert, 2014). Internal and external cues trigger a number of post-translational modifications of PINs, including phosphorylation (Barbosa et al., 2018) and ubiquitination (Leitner et al., 2012), which play prominent roles in the control of vesicle trafficking, polar localization, protein stabilization, and auxin transport activity, with involvement of many other components in the endosomal sorting pathway.

In addition to multiple covalent modifications of plant transport proteins, direct physical interaction between different subunits represents another mechanism of regulation, which is particularly prevalent in the gating of ion channels. One example is the heteromerization of the two shaker-like subunits AKT1 and AtKC1, which modulates the gating behavior of K^+ inward rectifier to prevent potential K^+ leakage through the uptake channels (Geiger et al., 2009a; Wang et al., 2010). On top of the channel complex, other components such as the vesicle trafficking protein SYP121 may further coordinate the channel gating process through physical interaction (Honsbein et al., 2009). In a similar scenario, the GLR3.3 channels can be activated by the CHONICHON proteins that physically interact with GLRs and play a role in the trafficking and activation of the Ca^{2+}-permeable channel (Wudick et al., 2018). In the case of S-type anion channel complex, the silent channel subunit SLAH1 interacts with SLAH3, which in turn facilitates the efflux of anions (Cubero-Font et al., 2016; Oikawa et al., 2018). Different types of ion channels can interact with each other and modulate their activities as exemplified by the interaction of guard cell-specific KAT1 and SLAC1 channels (Zhang et al., 2016). Several recent studies demonstrate that plant Ca^{2+} channel subunits tend to form oligomeric complexes for activity control. For example, the pollen tube-specific CNGC8 physically interacts with a self-active channel, CNGC18, resulting in channel inactivation (Pan et al., 2019). By contrast, CNGC2 and CNGC4 assemble into an active heterotetrameric Ca^{2+} channel and neither CNGC2 nor CNGC4 alone is active (Tian et al., 2019). Moreover, CNGC19 and CNGC20 can interact with each other as well as being self-assembled, and both the homomeric and heteromeric Ca^{2+} channel complexes appear to be functional

(Yu et al., 2019). These results strongly suggest that complex formation between different single CNGC subunits serves as a general mechanism in fine-tuning Ca^{2+} influx via CNGC channels during various physiological processes.

FUTURE PERSPECTIVES

Transport proteins are crucial for cellular function at all levels. In plants, myriad importers and exporters facilitate the translocation of various ions and metabolites intracellularly and intercellularly. Thanks to the genomic tools, genes coding for the components in a large array of transport systems from diverse plants have been identified, some of which are functionally characterized. Although the research progress of membrane transport proteins has been greatly accelerated by the new resources and tools in the post-genomic era, many questions remain unanswered.

First, many putative transport proteins identified by informatics analysis have not yet been functionally characterized in plants. It has become a common theme that one family of transport proteins could be versatile in their substrates and functions, whereas one substrate may have distinct types of transporters for translocation in different contexts. For instance, it is impressive to see that plant ABC- and MATE-type transporter families display broad substrate specificity, ranging from various secondary compounds to organic and inorganic anions (Hwang et al., 2016; Upadhyay et al., 2019). Another example is the NRT/PTR family, initially shown to transport NO_3^- or peptide, could also surprisingly facilitate the transport of plant hormones of different classes (Corratge-Faillie and Lacombe, 2017). For one substrate such as ABA, multiple types of plant transporters, including members from ABC (Kang et al., 2010; Kuromori et al., 2010), NRT/PTR (Kanno et al., 2012), and MATE (Zhang et al., 2017) families, have been identified, which may cater for diverse roles of the hormone in different developmental and physiological processes. It is therefore often difficult to predict the substrate and the function of a transporter based on the bioinformatics analysis. Experimental procedures discussed in this review are necessary to define the function of transport proteins. Notably, a high-throughput ''transportomic'' assay has recently been described on the basis of liquid chromatography–photodiode array–high-resolution mass spectrometry, which led to the identification of vacuolar transporters mediating crocin accumulation in the stigmas of *Crocus sativus* (Demurtas et al., 2019). This newly developed method may be promising to identity other transporters for important plant specialized metabolites.

Second, plant genomes encode a large number of potential transport proteins annotated with unknown function. In other words, these proteins, albeit predicted to have multiple transmembrane spans, do not have discernible similarity or structural domains to any well-documented protein in any organism. An urgent task in the field is to define their activities and characterize their functions. On one hand, reverse genetic studies to investigate their physiological roles would be helpful to speculate their potential roles in membrane transport. On the other, innovative procedures in functional screening provide alternative methods to identify and biochemically characterize new plant transporters. The recent studies in identification of SWEET family of sugar transporters and CSC/OSCA family of Ca^{2+}-permeable channels by imaging sensors set good examples (Chen et al., 2010; Hou et al., 2014; Yuan et al., 2014).

Third, even though *in vitro* transport activity is straightforward, biological functions of a transport protein *in vivo* can be complicated. During years of forward genetic studies, a number of mutants in transport genes have been unexpectedly identified based on altered developmental or physiological processes that appear to be unrelated to transport, suggesting that the loss-of-function effect of a plant transport protein can be pleiotropic. Hence, it can be challenging to link the function of a transporter to a physiological process at the mechanistic level. For instance, *dnd1* and *dnd2* (defense, no death 1 and 2) mutants are originally isolated from genetic screens as being defective in hypersensitive response but exhibiting constitutive systemic resistance and elevated levels of salicylic acid (Yu et al., 2000). Although the casual genes of the *dnd* phenotypes are two members of cyclic nucleotide-gated channel CNGC2 and CNGC4 (Clough et al., 2000; Jurkowski et al., 2004), their function *in planta* remained elusive until a recent study from an independent screen with subsequent in-depth analysis showed that they assemble into a calmodulin-gated heteromeric Ca^{2+} channel that is essential for pathogen-induced Ca^{2+} signaling in plants (Tian et al., 2019). Studies in the post-genomic era should use multiple approaches to dissect the multifunctional properties and mechanistic details of transport proteins in the context of plant physiology.

Fourth, thorough understanding of transport mechanisms and regulations heavily replies on detailed structural information of the transport proteins. Although structures of several membrane transport proteins from plants have been resolved (Liu et al., 2018; Parker and Newstead, 2014; Sun et al., 2014; Tao et al., 2015; Guo et al., 2016; Kintzer and Stroud, 2016; Zhang et al., 2018; Paulsen et al., 2019), compared with structural analysis of animal and bacterial transport proteins, membrane structure biology in the plant field is still in its infancy. While computational modeling based on the structures of homologs in animals or bacteria may be feasible and can yield helpful information for plant-derived proteins (Hua et al., 2003), a large number of plant transport proteins are unique in their structure and regulation, which presents a goldmine for future structural studies.

Last but not least, plant transport proteins operate in a network fashion, displaying functional interactions with each other and being regulated by signaling molecules. Dissecting the mechanisms underlying the coordination and regulation of multiple transport systems holds the key to fully understanding membrane transport processes. While genome projects have provided a complete list of genes and their encoded proteins, wiring the diagrams for protein–protein interaction at the genomic level becomes necessary and convenient. Using a split-ubiquitin yeast two-hybrid system, a recent study surveys global physical interactions involving membrane proteins and membrane-associated signaling machineries in *Arabidopsis*, many of which are related to membrane transport proteins and their regulators (Jones et al., 2014). This interaction database, together with other databases, serves as a valuable resource for formulating hypotheses on possible interactions between transport networks and signaling networks. In the post-genomic era, as our knowledge on plant membrane transport rapidly accumulates

at the molecular level, it should be possible soon to introduce new substrate specificities and modify specific characteristics of plant transport proteins with the goal of biotechnological applications. Meanwhile, genetic modifications of transport-associated traits in crops will aim to improve plant nutrient utilization and stress tolerance.

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