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ORIGINAL RESEARCH

Multilocation proteins in organelle communication: Based on protein–protein interactions

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Abstract

Protein–protein interaction (PPI) plays a crucial role in most biological processes, including signal transduction and cell apoptosis. Importantly, the knowledge of PPIs can be useful for identification of multimeric protein complexes and elucidation of uncharacterized protein functions. *Arabidopsis thaliana*, the best-characterized dicotyledonous plant, the steadily increasing amount of information on the levels of its proteome and signaling pathways is progressively enabling more researchers to construct models for cellular processes for the plant, which in turn encourages more experimental data to be generated. In this study, we performed an overview analysis of the 10 major organelles and their associated proteins of the dicotyledonous model plant *Arabidopsis thaliana* via PPI network, and found that PPI may play an important role in organelle communication. Further, multilocation proteins, especially phosphorylation-related multilocation proteins, can function as a "needle and thread" via PPIs and play an important role in organelle communication. Similar results were obtained in a monocotyledonous model crop, rice. Furthermore, we provide a research strategy for multilocation proteins by LOPIT technique, proteomics, and bioinformatics analysis and also describe their potential role in the field of plant science. The results provide a new view that the phosphorylation-related multilocation proteins play an important role in organelle communication and provide new insight into PPIs and novel directions for proteomic research. The research of phosphorylation-related multilocation proteins may promote the development of organelle communication and provide an important theoretical basis for plant responses to external stress.

KEYWORDS
multilocation protein, organelle separation, organelles communication, phosphorylation, protein interaction

Erhui Xiong and Di Cao contributed equally to this work.

1 | INTRODUCTION

Proteins perform the vast majority of functions in all biological domains (Subba et al., 2019). *Arabidopsis thaliana*, the best-characterized dicotyledonous plant, encodes approximately 35,000 protein-coding genes; however, the functions of the majority of these proteins remain uncharacterized, even by homology (Rhee & Mutwil, 2014). This trend is similar for *Oryza sativa*, a critical food crop and the best-characterized monocotyledonous plant (Kirkwood et al., 2013). Obviously, we still know very little about the functions of proteins in plants. Hence, it is very important to strengthen the analyses and research of plants at the proteome level.

Protein interaction networks provide new opportunities for characterizing genes and proteins (McWhite et al., 2020). Determining protein–protein interactions (PPI) is a key step in discovering both gene and protein functions, which facilitates study and manipulation of critical cellular processes (Eisenberg et al., 2000; Hartwell et al., 1999; Schwikowski et al., 2000). Systematic mapping of protein complexes in model organisms such as yeast and *Drosophila* has led to critical functional insights, and facilitated understanding of conserved and disease-related pathways (Vidal et al., 2011) and helped characterize proteins by association. With scientific and technological advancements, the study of PPIs in the model plant *Arabidopsis thaliana* has also developed rapidly. Therefore, summarizing and analyzing the PPI network in this model plant can play a guiding role for proteomics research and functional analysis of plants and crop plants.

Biological communication is ubiquitous. It exists between organic life and inorganic matter, between animals and plants, and among animals, plants, organs, cells, and organelles (Cohen et al., 2018; Frick et al., 2019; McWhite et al., 2020; Namgaladze et al., 2019; Zhang & Yang, 2018). Communication among organelles functions to regulate the size, shape, and composition of individual organelles, and coordinate transport between them (Stefan et al., 2013). The establishment of interorganellar communication, a profound consequence of the emergence of eukaryotes, enabled coordinated scaling of the biological processes necessary to meet the metabolic and energy demands of the cell (Wang & Dehesh, 2018). Recently, many studies show that the organelles of chloroplast (Chl), mitochondria (Mit), and peroxisomes (Per) actively communicate and physical interaction between peroxisomes and chloroplasts during photorespiration (Oikawa et al., 2015). Membrane contact sites (MCSs) facilitate the exchange of metabolites between organelles to support interorganellar communication (Lin et al., 2021). MCSs facilitate the exchange of metabolites between organelles to support interorganellar communication (Lin et al., 2021). Endoplasmic reticulum (ER)-plasma membrane (PM) junctions have well-established functions involved in the movement of small molecules, regulation of cell signaling, ER shape and architecture, and PM domain organization (Stefan et al., 2013). The nucleus–vacuole junctions establish physical contact between the perinuclear ER and the vacuole (Tosal-Castano et al., 2021) and the contacts between the ER and lysosomes influence organelle organization and communication (Ozkan et al., 2021). The ER-Mit encounter structure plays crucial roles in interorganelle communication, mitochondrial fission, mtDNA inheritance, lipid transfer, and autophagy (Rasul et al., 2021). A number of key molecular machinery systems participate in mediating substance exchange and signal transduction through PPI, both of which are essential processes in terms of cellular physiology and molecular biology (Lin et al., 2021), such as GRP75 interact with VDAC of mitochondrial and IP3R of ER (Kwak et al., 2020), VAPA of ER interact with KV2 of PM (Johnson et al., 2018). So, it was meaningful to study the communication between organelles based on protein interaction data.

The existence of communication between organelles raises an important question: How and by what means do organelles communicate with each other direct contact via organelle membrane contact, proteins shuttling among different organelles, or multilocation proteins interacting with other proteins to build bridges? Our previous studies showed that different pathways are involved in different organelles. In addition, we analyzed nuclear localization and its interaction proteins in *Arabidopsis* and found that multilocation proteins may play a key role among different pathways (Gong et al., 2021). It raises additional questions: Do multilocation proteins play a critical role in organelle communication? Recent studies have shown that multilocalized proteins play an important role in plant growth and development, such as WHY1 and BZR1, which plays a certain role in the communication of organelles (Lin et al., 2020; Wang et al., 2021; Zhang et al., 2020). Further, protein kinases in plants are divided mainly into serine/threonine, tyrosine, and histone kinases. The kinases are activated by phosphorylation, which in turn activates a cascade of events leading to the phosphorylation of different amino acids (Ardito et al., 2017). Phosphatases, which remove the phosphate group from phosphoproteins by hydrolyzing phosphoric acid monoesters into a phosphate group and a molecule with a free hydroxyl group, have the opposite function of kinases. In *Arabidopsis*, many kinases (such as MAPKs, CDPKs, and RPKs), phosphatases (such as PP2Cs), and PYR/PYLs (PYR1 and PYL1-PYL13) have been identified and play key roles in the signal transduction process, including hormone signaling and biotic or abiotic response (Mayer & Yu, 2018; Raghavendra et al., 2010; Tena et al., 2011). Previously study also showed that dual location of proteins mediates diverse intercellular signaling processes, for example, MAP kinase (Chan et al., 2016) and CIPK14 (Ren et al., 2017). So, do multilocation proteins or multilocation kinase bridge organelle communication to regulating plant growth and development?

In the case of the model plant *Arabidopsis*, the steadily increasing amount of information on the levels of its proteome and signaling pathways is progressively enabling more researchers to construct models for cellular processes for the plant, which in turn encourages more experimental data to be generated (Holzheu & Kummer, 2020). In this study, we conducted an overview analysis of reviewed proteins in the 10 major organelles of the model plant *Arabidopsis* and then screened and analyzed the proteins interacting with these organelle proteins. Based on the results, we performed related bioinformatics analyses, focusing on the interactions and pathways associated with these proteins. The results indicated that multilocation proteins, especially phosphorylation-related multilocation proteins, can function as

"a needle and thread" among organelles via PPIs. Similar results have been obtained in the monocotyledonous model rice. Moreover, we provide a research strategy for multilocation proteins. Our findings will guide new directions for proteomic research based on PPIs.

2 | MATERIALS AND METHODS

2.1 | Data acquisition

We searched for *Arabidopsis* and rice proteins on UniProt on May 30, 2020, and downloaded data on the reviewed proteins. We retrieved information on their interactions from the "Subunit structure" subsection of the "Interaction" section in UniProt. This subsection contains information on protein quaternary structure and interaction with other proteins and protein complexes (PPIs, host-pathogen PPIs, and protein-complex interactions, with the exception of physiological receptor-ligand interactions, which are annotated in the "Function" section). Interacting proteins from other species, such as those participating in host-pathogen PPIs, were excluded from the analysis. Only interactions associated with "Publications" were retained for further analysis.

2.2 | Protein–protein interactome network visualization

Reviewed *Arabidopsis* nuclear proteins that were classified as participating in protein interactions were used for protein interactome network construction with the network visualization program Cytoscape 3.8.1 (Shannon et al., 2003). Duplicate interactions, which would cause self-looping, were removed before network construction. The network topology was analyzed using the Network Analyzer plug-in (Assenov et al., 2008).

2.3 | Biological pathway analysis of proteins in the protein–protein interactome

Proteins are the basic components of biological pathways, and PPIs play fundamental roles in these pathways. The KEGG is a database resource for the elucidation of high-level functions and utilities of biological systems, such as the cell, organism, and ecosystem, from molecular-level information, especially large-scale molecular datasets generated via genome sequencing and other high-throughput experimental technologies (Kanehisa, 2002).

3 | RESULTS

3.1 | Overview of reviewed and their interacted proteins in *Arabidopsis thaliana*

Although the functions of the majority proteins remain uncharacterized in *Arabidopsis thaliana*, this species remains the most intensively studied plant. To data, 11,200 proteins of *Arabidopsis thaliana* located mainly in 10 organelles have been reviewed in the

TABLE 1 Details of localization and interactions of reviewed proteins in *Arabidopsis*

	Nuc	Cyt	Mem	Chl	ER	Golgi	Mit	Per	Vac	CW	Multi	Total
The reviewed proteins												
Numbers of reviewed proteins	3580	1881	2104	1534	538	540	329	144	262	288	-	11,200
Numbers of involved KEGG pathways	65	103	63	101	58	33	64	47	33	14	-	130
Top five involved KEGG pathways	PHST	MP	MP	MP	MP	MP	MP	MP	MP	MP	-	MP
	MP	BSM	PHST	BSM	BSM	EN	BSM	BSM	BSM	PGI	-	BSM
	SP	CM	PLPI	BAA	PPER	ASNSM	CM	GM	PB	SSM	-	PHST
	UMP	PLPI	EN	CM	PLPI	PGI	BAA	CM	PLPI	BSM	-	CM
	PLPI	PHST	BSM	BC	GM	PPER	OP	PE	PH	GAM	-	BAA
Protein interaction of reviewed proteins												
Numbers of reviewed proteins	1432	656	414	261	145	111	61	40	42	15	630	2416
Numbers of interactions	3170	1721	921	427	373	210	128	68	121	25	2211	4548
PPI network information of single location and multilocation proteins												
Numbers of proteins with single location	1023	189	217	204	52	45	25	16	14	1	630	2416
Number of interactions in its own location(s)	1234	46	131	149	23	35	11	2	2	0	451	3407

Abbreviations: ASNSM, amino sugar and nucleotide sugar metabolism; BAA, biosynthesis of amino acids; BC, biosynthesis of cofactors; BSM, biosynthesis of secondary metabolites; CM, carbon metabolism; EN, endocytosis; GAM, galactose metabolism; GM, glutathione metabolism; MP, metabolic pathways; OP, oxidative phosphorylation; PB, phenylpropanoid biosynthesis; PE, peroxisome; PGI, pentose and glucuronate interconversions; PH, phagosome; PHST, Plant hormone signal transduction; PLPI, plant-pathogen interaction; PPER, protein processing in endoplasmic reticulum; SP, spliceosome; SSM, starch and sucrose metabolism; UMP, ubiquitin mediated proteolysis

UniProt database (May 30, 2020) (Table S1). These 10 organelles comprise the nucleus (Nuc, 3580), PM (2104), cytoplasm (Cyt, 1181), chloroplast (Chl, 1534), ER (538), Golgi apparatus (Golgi, 540), mitochondria (Mit, 329), cell wall (CW, 288), vacuole (Vac, 262), and peroxisome (Per, 144) (Table 1). As proteins located in different organelles have different molecular functions (Huber et al., 2003), we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of proteins in the 10 major organelles of *Arabidopsis* (Table S2). The number of KEGG pathways associated with the proteins of the different organelles varied greatly, for example, 103, 101, and 65 pathways in Cyt, Chl, and Nuc, however, 33, 47, and 14 pathways in vacuole, peroxisome and CW, respectively (Table 1).

In addition, 11,200 proteins were associated with 130 KEGG pathways, the top five being the "Metabolic pathways," "Biosynthesis of secondary metabolites," "Plant hormone signal transduction," "Carbon metabolism," and "Biosynthesis of amino acids" pathways. KEGG analysis showed that 3580 reviewed proteins of the nucleus, the largest and most important eukaryotic cell structure (Janota et al., 2020), were involved in 65 pathways, mainly the "Plant hormone signal transduction," "Metabolic," "Spliceosome," "Ubiquitin mediated proteolysis," and "Plant-pathogen interaction" pathways. The reviewed cytoplasmic proteins involved in the "Metabolic," "Biosynthesis of secondary metabolites," "Carbon metabolism," "Plant-pathogen interaction" and "Plant hormone signal transduction" KEGG pathways. However, cell wall proteins were involved mainly involved in the "Metabolic," "Pentose and glucuronate interconversions," and "Starch and sucrose metabolism" KEGG pathways (Table 1). These results suggest that each organelle has unique specific functions. However, different organelle involved in some of the same pathways, and closely connected with each other.

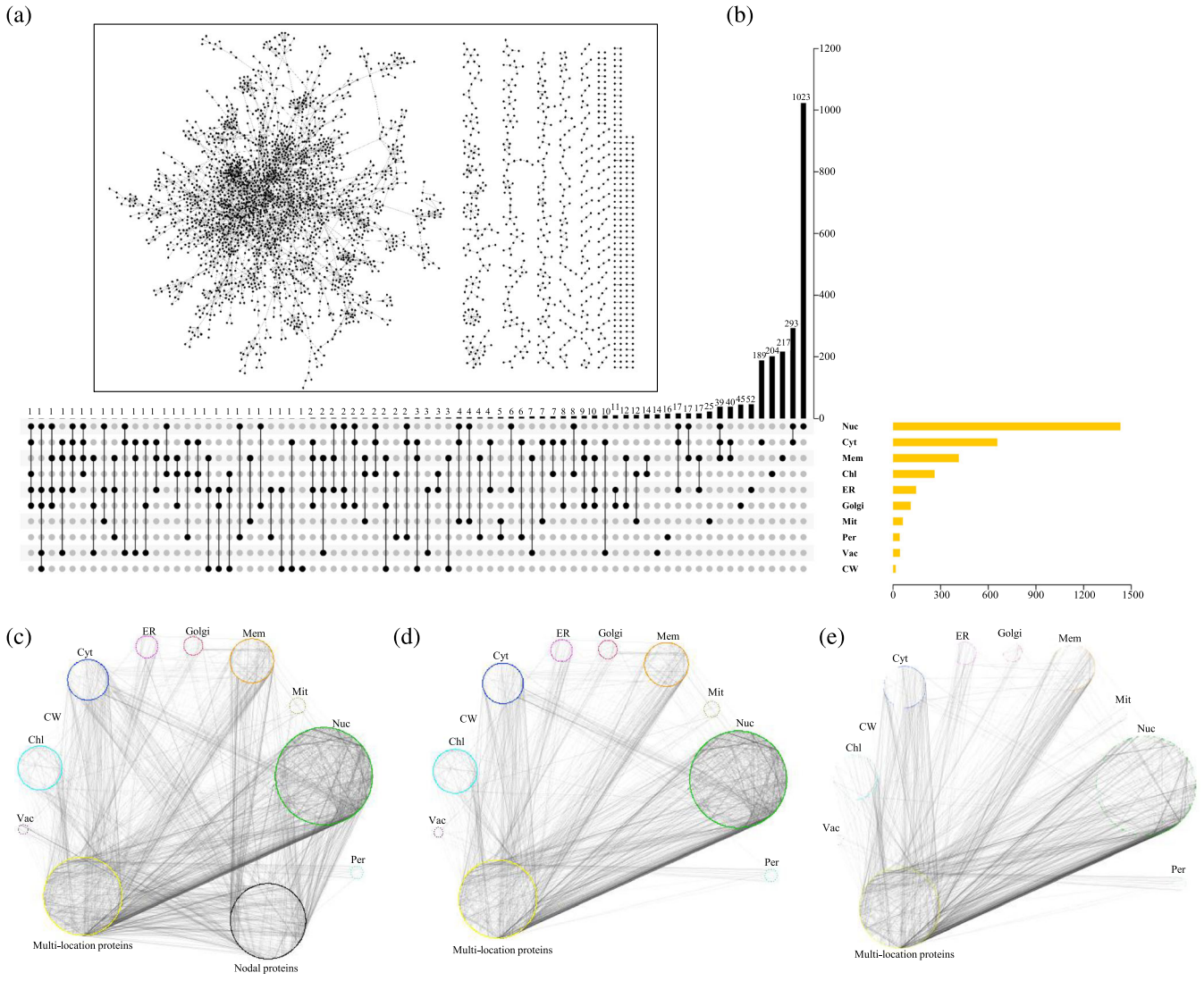

FIGURE 1 Visualization of the interactome of *Arabidopsis*. (a) The entire PPI network of the interactions among the reviewed proteins interaction. (b) Overview of single location and multilocation reviewed proteins in *Arabidopsis*. (c) Subnetwork organized by multilocation and nodal proteins. (d) Subnetwork organized by single and multilocation proteins. (e) Subnetwork of interactions between multilocation and signal-location proteins in different organelles

PPI networks provide valuable information for understanding plant biological processes (Xiong, Dong, et al., 2021; Xiong, Li, et al., 2021). A PPI network provides crucial information about how biological pathways are structured and coordinated based on the functions of individual proteins. At present, many predictional analyses of PPIs have been conducted in some model plants (Ding & Kihara, 2019). To obtain the most realistic and current state of PPI research progress, we screened the interacting proteins among the 11,200 reviewed proteins of *Arabidopsis*. In the UniProt database, Only interactions associated with "Publications" were retained for further analysis. The results of the statistical analysis showed that 4548 pairs interacting proteins were screened (Table S3), which are involved in 3004 proteins, including 2416 reviewed proteins and 589 non-reviewed interacting proteins. The number of proteins from different organelles and the logarithm of their participation varied greatly (Table S4). There were 1432 Nuc and 656 Cyt proteins involved in 3170 and 1721 interaction pairs, respectively, whereas only 42 Vac and 15 CW proteins were involved in 121 and 25 interaction pairs, respectively. The different organelles were ranked in terms of breadth and depth of research. There is such a sequence of different organelles at the protein interaction level as follows: Nuc > Cyt > PM > Chl > ER > Golgi > Mit > Per > Vac > CW (Table 1). Here, the research conducted on the nucleus, the central organelle of the cell, is nearly equal to the sum of the research conducted on all the other organelles, which is still the central guiding role among organelles in the cell.

3.2 | Multilocation proteins play important roles in *Arabidopsis* PPI networks

To further understand how these proteins relate to each other, and what are the regulatory networks among them, 4548 pairs of interacting proteins were used for protein interactome network construction using the network visualization program Cytoscape 3.8.1 (Shannon et al., 2003) (Table S5). On the whole, the current research on the *Arabidopsis* PPIs is relatively close, to being complete, with approximately 75% of proteins (2247) participating in a large network and 25% (754) interacting in a discrete distribution (Figure 1a). However, the functions of the majority of *Arabidopsis* proteins remain uncharacterized, even by homology (Rhee & Mutwil, 2014). We speculate that the interactions will reveal a relatively complete protein network representing the coordinated participation of proteins in biological processes. Therefore, we believe that great potential remains for PPIs studies in *Arabidopsis*, which can complement and complete this network, facilitate understanding of more detailed relationships among proteins, and provide a theoretical basis for the study of crops. Further analysis revealed 630 multilocation proteins among 2416 reviewed proteins, of which 509, 113, 6, and 2 localized in two, three, four, and five locations, respectively (Table 1, Figure 1b). Based on the thickness of the lines in Figure 1c, we found that the ten organelles were closely connected via multilocation or interacting proteins (no location information). By comparison, the organelles were less closely related to each other directly (Figure 1c). Hence, whether these multilocation proteins or interacting proteins play important roles in communication among organelles is an intriguing question. Because the location information of these interacting proteins is not completely clear, there may be important interactions between multilocation or single-location proteins and single-location organelles. We focused mainly on the multilocation proteins in the PPI analysis and their relationships among the various organelles. For better observation and analysis, single- and multilocation proteins were selected to build a sub-network (Figure 1d). From the network, it seems that almost all organelles interact with multilocation proteins. In addition, Nuc, Cyt and ER are also closely related (Figure 1d). To confirm further the role of the multilocation proteins in the various organelles, we constructed a sub-interaction network of multilocation proteins interacting with signal-locating proteins in different organelles (Figure 1e). The network clearly shows that the multilocation proteins are in the centre of a vortex that acts as a "bridge" among different organelles. Therefore, we believe that multilocation proteins may function mainly as bridges in the PPI network.

To prove that multilocation proteins play an important role in organelle communication, we further analyzed the PPI network between single (1786) and multilocation proteins (630), which involved in 3407 pairs interaction. Among these, 1786 single localized proteins were involved in 1782 interactions, and total 1633 pairs (about 91.7%) were interacting proteins within an organelle. Therefore, there were only about 8.3% (149/1782) interaction pairs among different organelles. In addition, 630 multilocation proteins were involved in 451 interaction pairs. Thus, 1174 protein interaction pairs were involved in communication between the multilocation proteins and the various organelles, eight times (1174/149) as many single-location proteins (Table 1, Figure 1d). This confirms that multilocation proteins may play a crucial role in organelle communication, which is consistent with our above results.

Thus, we evaluated how multilocation proteins function in organelles and which pathways are associated with these proteins? KEGG pathway analysis showed that "Plant hormone signal transduction" and "MAPK signaling pathway" were the top two KEGG pathways associated with the multilocation proteins. MAPK modules play key roles in the transduction of environmental and developmental signals via phosphorylation of downstream signaling targets (Jagodzik et al., 2018), and hormones such as auxin, abscisic acid, jasmonic acid, salicylic acid (SA), ethylene, brassinosteroids, and gibberellins influence signaling via MAPK cascades (Hettenhausen et al., 2015; Lu et al., 2015; Mishra et al., 2006; Rodriguez et al., 2010; Smekalova et al., 2014). To assess whether "Plant hormone signal transduction" and "MAPK signaling pathway" are important for organelle communication, we additionally performed a KEGG pathway analysis of 3004 currently reviewed proteins and their interactions, and also determined which are involved mainly in "Plant hormone signal transduction" and "MAPK signaling pathway." The results showed that the major pathway of associated with the multilocation proteins was the same as that associated with the total proteins by KEGG analysis, indicating that multilocation proteins play an important role in biological activities.

3.3 | Multilocation kinases or phosphatases in organelle communication

This raises several important questions. Which organelles are bridged by multilocation proteins, and how are they bridged? Which proteins participate in the bridging? How do they communicate with organelles and regulate biological activities? Unlike most animals, plants are immobile and cannot actively escape the effects of harsh environmental factors. Plants tend to respond to stress in the most rapid way to minimize the damage that could occur under the stress (Ashapkin et al., 2020). To address the above questions, we screened for the shortest path interaction circuits bridging multiple organelles from the network diagram of 75% of proteins (2247) participating in a large network (Figure 2a). There is only one interaction circuit connecting the 10 organelles in this network (Figure 2b), and the KEGG pathway analysis showed that the multilocation proteins connecting these 10 organelles are involved mainly in the MAPK signaling pathway, such as kinases MKK4, MPK6, MPK3, MPK4, and NDPK1. The second largest circuit involved seven organelles (Figure 2c), and KEGG analysis showed that the multilocation proteins of these organelles are mainly histidine-related kinase such as AHP2, AHP3, AHP5, ARR9, HK2, HK3, and WOL, which are involved in "Plant hormone signal transduction." The third interaction circuit involved six organelles in the network (Figure 2d), and KEGG analysis showed that the multilocation proteins of these organelles are mainly kinase- and phosphatase-related proteins such as CPK6, CPK33, ABI1, CIPK24, KIN10, and RCAR3, which are involved in "Plant-pathogen interaction" and "Plant hormone signal transduction." The analysis revealed that the multilocation proteins connecting the various organelles are mainly kinases or phosphatases, suggesting that phosphorylation and dephosphorylation are key processes in organelle communication.

These directly interacting proteins elegantly link various organelles and form a path for organelle communication. On this basis, the proteins in each organelle interact with each other, and finally interact to build a PPI network that participates in the regulation of biological activities. We hypothesize that additional multilocation proteins involved in organelle communication have yet to be discovered, beyond those of the current single pathway connecting the 10 organelles. Therefore, screening of multilocation proteins has great value for understanding the growth and development of plants.

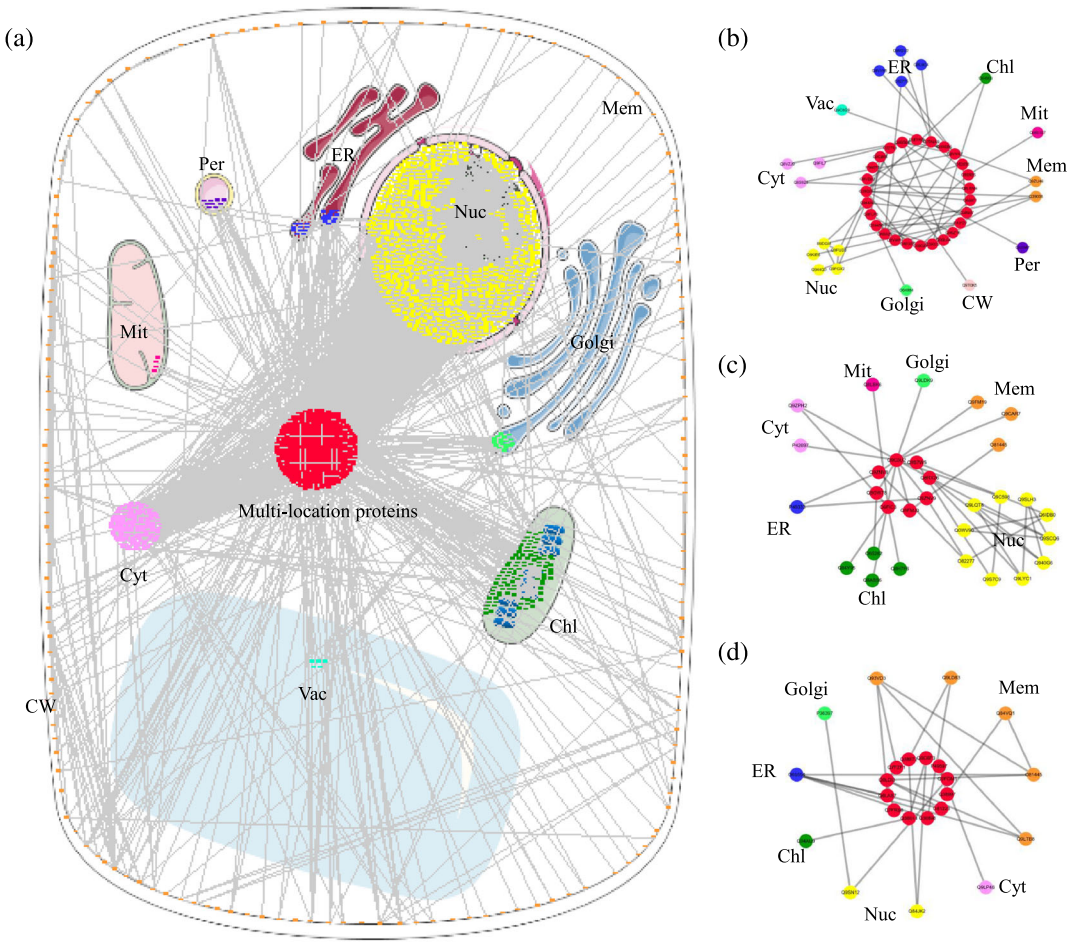

FIGURE 2 Shortest path interaction circuits bridging multiple organelles from the network diagram of 75% of proteins participating in a large network. (a) Main network. (b) Circuit of connecting the 10 organelles. (c) Circuit of connecting the seven organelles. (d) Circuit of connecting the six organelles

The post-translational modification phosphorylation can alter the properties of proteins such as enzyme activity and intracellular localization. Thus, it is important to consider the localization of kinases or phosphatase, and whether they are multilocation proteins. In 630 multilocation proteins, 90 proteins (about 14.3%) were kinases or phosphatase. Moreover, these proteins involved in 529 interaction pairs (about 32.7%) of multilocation proteins. Especially pentuple location protein SNF1-related protein kinase catalytic subunit alpha KIN10 (Q38997), involved in 51 interaction pairs. The results suggested that multilocation kinases or phosphatases may have significant roles in PPI network. Further, a total of 705 kinases- and 143 phosphatase-related proteins were screened using the subcellular localization details of 11,200 *Arabidopsis* reviewed proteins, which were concentrated mainly in the Nuc, Cyt, PM and Chl, and among these, 129 were multilocation proteins. In addition, some widely studied kinase families, such as MAPKs and CDPKs, were selected from 11,200 reviewed proteins for further analysis. A total of 19 MAPKs, 25 CDPKs, and 212 serine/threonine-protein related-kinases with locational information were screened. Among the19 MAPKs, 14 (73.6%) are multilocation proteins; among the 25 CDPKs, nine (36%) are multilocation protein and 16 are membrane-located proteins. In total, about 53% MAPKs and CDPKs were multilocation protein. The results of the analysis suggest that phosphorylation-related proteins are not all multilocation proteins. However, the phosphorylation-related proteins with multiple locations plays an important role in organelle communication. Compared to the results of multilocation kinases or phosphatases in PPI network, there is still a large research gap for multilocation kinases or phosphatases in 11,200 *Arabidopsis* reviewed proteins, and with further research, it may be found that more phosphorylation-related proteins are multilocalized proteins. Hence, we hypothesized that although phosphorylation-related proteins play an important role in signal transduction, not all phosphorylation-related proteins are equally important in the plant. Some key phosphorylation-related proteins, such as multilocation kinase proteins, may play an important role in organelle communication. Therefore, screening and obtaining more kinases or phosphatases with multilocation information may be critical for understanding cell-to-cell and organelle-to-organelle communication.

3.4 | PPI network and multilocation proteins in the monocotyledonous model crop *O. sativa* L.

Rice is a staple food for most of the world's population. However, the PPI networks of rice have not been identified by large-scale experiments. Here, we also summarize the recent achievements toward PPIs in the monocotyledonous model crop rice. Using the UniPort database, we reviewed 2746 proteins of rice, of which 291 were involved in 367 interaction pairs (Table 2). Similar to *Arabidopsis*, proteins here in Nuc and Cyt have more logarithmic interaction, with 183 proteins in Nuc involved in 249 interaction pairs and 74 proteins in Cyt involved in 147 interaction pairs. However, proteomic studies in rice are only 10%–20% of that in *Arabidopsis*. In addition, the PPI network with high dispersion was not a large network (Figure 3a, Table S6). Among these proteins, 63 are multilocation proteins (59 located in two and 4 in three locations), which is a lower proportion than that in *Arabidopsis* (Figure 3b). Although the rice genome is much larger than that of *Arabidopsis*, the scope and depth of research on rice are much lower, as can be seen by comparing Table 1 and Table 2. Rice protein level research is lagging, and a large gap still exists between the 40,000 annotated rice proteins and fewer than 1000 proteins that have been characterized, even with limited in vivo analyses (Rahiminejad et al., 2019). It is likely that potential interactions can be screened via comparative analyses with *Arabidopsis*, and made available for future reference.

Recently, a genome-wide protein labeling program for rice (RPTP) was proposed (Lu, Ronald, et al., 2020). In situ labeling of rice proteins will contribute to functional analysis of the rice proteome (Lu, Tian, et al., 2020). In this program, many proteins of interest need to be selected for an initial labeling effort, and we believe that the criterion of multilocation is important selection indicator. Here, we also conducted KEGG analysis of the multilocation proteins of rice, and "Plant hormone signal transduction" and "MAPK signaling pathway" as the top two KEGG pathways. Like *Arabidopsis*, the analysis showed that the multilocation proteins connecting various organelles are mainly phosphorylation related proteins, such as PP2C06, PYL8, SAPK10, SAPK8, GSK2, PYL5, PP2C53, PYL2, SAPK2, PYR1, and SAPK9. These results also suggest that multilocation proteins and phosphorylation play important roles in organelle communication in

TABLE 2 Details of localization and interactions of reviewed proteins in rice

	Nuc	Cyt	Mem	Chl	ER	Golgi	Mit	Per	Vac	CW	Multi	Total
The reviewed proteins												
Numbers of reviewed proteins	951	463	398	459	133	114	37	30	69	62	-	2746
Protein interaction of reviewed proteins												
Numbers of reviewed proteins	183	74	53	126	7	4	1	4	5	1	63	291
Numbers of interactions	249	147	68	26	10	5	1	7	6	1	142	367
PPI network information of single location and multilocation proteins												
Numbers of proteins with single location	131	18	41	26	3	2	1	2	3	1	63	228
Number of interactions in its own location(s)	59	2	25	12	0	1	0	0	0	0	27	99

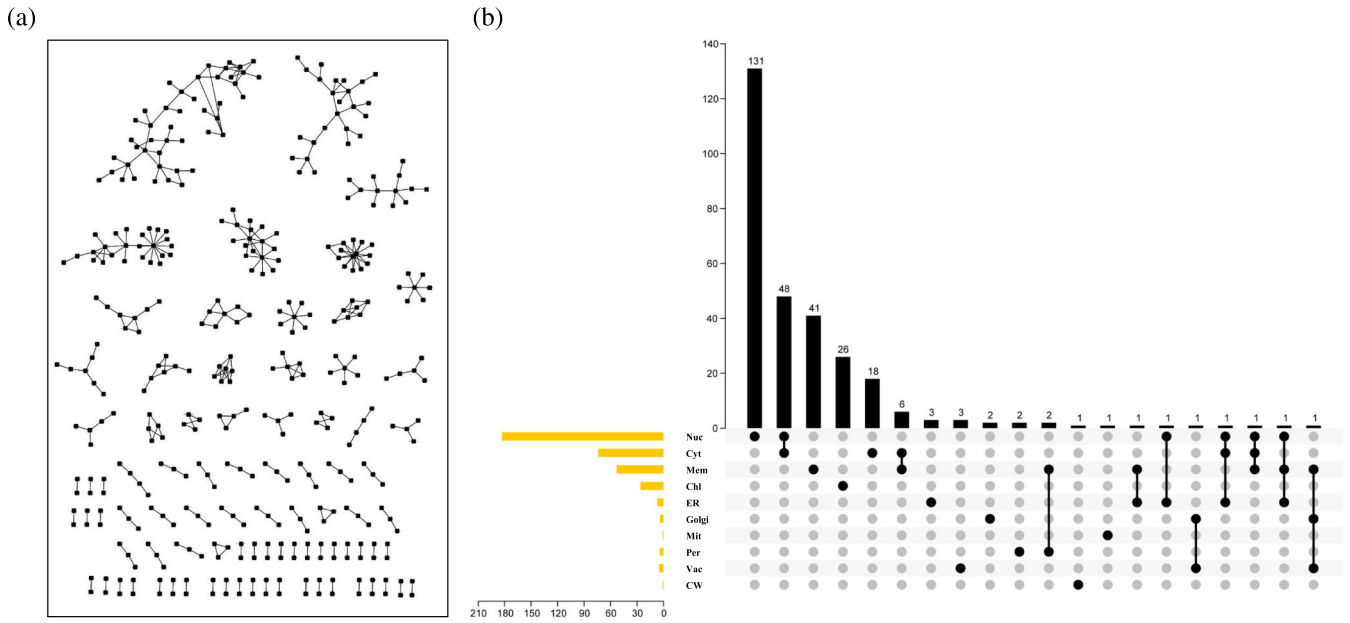

FIGURE 3 Visualization of the interactome of rice. (a) The entire PPI network of the interactions among the reviewed proteins interaction. (b) Overview of single location and multilocation reviewed proteins in rice

rice. Compared with *Arabidopsis*, there are few protein-level interaction networks; thus, multilocation protein analysis may be a relatively rapid method to elucidate rice PPIs. We believe that this research direction will also be fruitful in the study of other plants and even animals.

4 | DISCUSSION

4.1 | Multilocation proteins function in PPI network and organelle communication

PPIs play a crucial role in cellular functions and biological processes in all organisms. Crosstalk among organelles can involve direct physical contact (Lin et al., 2021), especially through PPI (Johnson et al., 2018; Kwak et al., 2020). Direct physical contacts between mitochondria and the endolysosomal compartment have been reported as a rapid means of interorganelle communication, mediating lipid or other metabolite exchange (Soto-Heredero et al., 2017). In addition, MCSs are also defined as regions where two organelles are closely apposed, and most MCSs associated with each other via protein–protein (Lin et al., 2021). For example, ER is the largest reticulum network within the cell and has extensive communication with other cellular organelles, including the PM, mitochondria, Golgi, endosomes and lipid droplets (Lin et al., 2021). GRP75 located in Mit interacts with VDAC located in mitochondrial and IP3R located in ER (Kwak et al., 2020), and VAPA located in ER interacts with KV2 located in PM (Johnson et al., 2018). In addition, proteins travel through different organelles and interact with proteins in organelles under different conditions to regulate growth and development (Isemer et al., 2012; Lin et al., 2020; Wang et al., 2021). So, PPI can be acting as a means of analyzing the degree to which organelles are linked. Here, we performed a detailed analysis using PPI data of reviewed *Arabidopsis* proteins, and found that 1786 single localized proteins were involved in 1782 interactions. Compared to single localized proteins, multilocation proteins are prominent in PPI networks. Among them, 630 multilocation proteins were involved in 1621 interaction. The results suggested that these multilocation proteins may play a greater role in PPI networks. So far, although many PPI networks have been analyzed, the role of multilocation proteins in PPI networks remains to be further studied. The acquisition of multilocation proteins may be more beneficial to the construction of protein interaction network.

PPIs have recently come to the fore of our understanding of organelle communications (Falz & Muller-Schussele, 2019; Lin et al., 2021). Since multilocation proteins play an important role in PPI networks, are they equally important in organelle communication? Further analysis showed that there were only 149 interaction pairs (about 8.3%) among different organelles of signal location protein, and most interactions (about 91.7%) occur within the own organelles. If the degree of PPI connection between organelles was assumed to be an indicator of organelle information exchange. Thus, single localized proteins may play a limited role in organelle communication. Of course, it could not rule out that these few interactions may also play a decisive role in organelle communication and growth and development. Compared with single localized proteins, in the 1621 interaction pairs of 630 multilocation proteins, 1170 interaction pairs (about 72.1%) were involved in communication between the multilocation proteins and the various organelles, which is about eight times (1170/149) as many as single-location proteins (Table 1, Figure 1d). From this point, multilocalized proteins may be more important in

organelle communication than single-localized proteins. Further, from the depth and breadth of some gene studies, some multilocalization proteins may indeed play a more important role in plant growth and development (Lin et al., 2019, 2020; Nevarez et al., 2017; Rasul et al., 2021; Swida-Barteczka et al., 2018; Wang et al., 2016, 2021; Wang & Dehesh, 2018; Zhang et al., 2020).

Proteins will be effective only when they are in the right subcellular structure. The same protein may play different roles in different organelles and regions. Proteins with dual subcellular localization can affect transcription and display various functions in intracellular signaling. Recently, there are more and more studies on the involvement of multilocation proteins in the communication of different organelles in the regulation of plant growth and development (Li et al., 2017; Lin et al., 2020; Wang et al., 2021; Zhang et al., 2020; Zhuang et al., 2019). For example, WHY1, which is a plant-specific DNA- and RNA-binding protein that locates both in chloroplasts and nucleus (Krause et al., 2005; Prikryl et al., 2008). Due to dual location and function in the nucleus and plastids (Krause & Krupinska, 2009), it is assumed that WHY1 can move from plastids to the nucleus (Isemer et al., 2012). The plastid isoform of WHIRLY1 affects miRNA biogenesis in the nucleus (Swida-Barteczka et al., 2018). SA accumulation could be prevented by ectopic expression of the nuclear WHY1 isoform. However, expressing the plastid WHY1 isoform greatly enhanced cellular SA levels. Altered localization of *Arabidopsis* WHY1, in the nucleus or chloroplast causes a perturbation in SA homeostasis, resulting in adverse plant senescence phenotypes (Lin et al., 2020). *Arabidopsis why1* mutants are insensitive to ABA, it was found that WHY1 located in chloroplast enhanced the sensitivity of plants to ABA after knocking out the chloroplast WHY1 signal peptide, while WHY1 in nucleus was still not sensitive to ABA (Isemer et al., 2012). These studies also suggested that multilocalized proteins may play an important role in cell or organelle communication and participate in plant growth and development.

4.2 | Multilocation kinases or phosphatases may have significant roles in organelle communication

Multilocation proteins, such as the phosphorylation-related proteins MKK4, MPK6, MPK3, MPK4, and NDPK1, were found to be bridged with Nuc (WNK8 and BZIP1), CW (LRX3), Golgi (OFUT20), Mit (ATG11), Per (CAT3), Vac (ATG8B and ATG8D), ER (KCR1 and ATI2), Chl (NDPK2), Cyt (CRCK1 and CRCK2), and Mem (MEKK1 and ZAR1) (Figure 2a) to regulate autophagy (Li et al., 2014; Michaeli et al., 2014), phosphorylation (Hong-Hermesdorf et al., 2006), photoperiod pathway (Wang et al., 2008), amino acid metabolism (Dietrich et al., 2011), sugar signaling (Kang et al., 2010), cell morphogenesis (Velasquez et al., 2011), reactive oxygen species (Li et al., 2015), fatty acids (Beaudoin et al., 2009), cellular redox state (Moon et al., 2003), innate immunity (Asai et al., 2002; Gao et al., 2008), and zygote development (Yu et al., 2016). In eukaryotes, cell–cell and cell–environment communication often involve cell surface receptors (Xu & Zhang, 2015). MAPK related proteins are ubiquitous signaling modules in eukaryotes. The functions of MAPK-related proteins include immunity and stress responses. They also play essential roles in plant growth and development downstream of receptor-like protein kinases (Wang et al., 2007; Xu & Zhang, 2015). Multiple functional pathways initiated from different receptors often involve the same MAPK components or even a complete MAPK cascade. Considering our results, in addition to their important role in cell–cell or cell-environment communication, MAPK-related proteins may also play an important role in organelle communication via PPIs.

Histone kinases or phosphatases are also important proteins involved in phosphorylation processes, transmitting the stress signal to a downstream MAPK cascade. Here, in the second interaction circuit, multilocation proteins such as phosphorylation-related histidine-containing phosphotransfer protein 1 (AHP1), AHP2, histone kinase 2 (AHK2), and PI4KB1, interact with GID1C, AHL27 and TCP10 of Nuc, GRXS15 of Mit, ETR1 of ER, DRP1A of Cyt, BETAA-AD of Golgi, HIR1 of PM, and PNSL5 of Chl (Figure 2b). In plants, histone kinases are involved in a variety of stress responses (cold, drought, salt) by regulating hormonal signaling such as abscisic acid or cytokinin signaling, and also regulate many developmental processes including seed germination, cell division, seed size, chlorophyll retention during leaf senescence, root repression and shoot promotion (Miwa et al., 2007; Riefler et al., 2006; Tran et al., 2007). In addition, AHP has histidine phosphotransfer kinase activity, functioning as a two-component phosphorelay mediator between cytokinin sensor histidine kinases and response regulators.

Calcium is a ubiquitous second messenger that mediates plant responses to developmental and environmental cues. In the third interaction circuit, CDPK6, CDPK33, ABI1, CIPK24, KIN10 and RCAR3 interact with MYB77 of Nuc, RBCX1 of Chl, ARF1 of Golgi, SCAR3 of Cyt, CIPK6 of ER, and CBL1 of Mem (Figure 2c). CDPKs are key factors in plant signaling that convey Ca^{2+} signals into physiological responses by phosphorylating various substrates, including ion channels, transcription factors and metabolic enzymes (Yip Delormel & Boudsocq, 2019). The stress-induced Ca^{2+} signaling was dependent on OsPLC1, and the PLC-mediated Ca^{2+} signaling was essential for controlling Na + accumulation in leaf blades. Salt stress induced the recruitment of OsPLC1 from cytoplasm to plasma membrane, where it hydrolyzed PtdIns4P, thus establishing whole plant salt tolerance (Li et al., 2017). Because of this large diversity of targets, CDPKs play pivotal roles in shoot and root development, pollen tube growth, stomatal movements, hormonal signaling, transcriptional reprogramming, and stress tolerance (Almadanim et al., 2018; Baba et al., 2018; Boudsocq & Sheen, 2013; Mori et al., 2006).

In this study, we found that multilocalized proteins may play an important role in PPI network and organelle communication both of *Arabidopsis* and rice, especially in multilocation kinases or phosphatases. Protein phosphorylation is an important cellular regulatory mechanism as many enzymes and receptors are activated/deactivated by phosphorylation and dephosphorylation via kinases and phosphatases (Tena et al., 2011). Signal transduction systems based on phosphorylation are central to cell–cell communication in multicellular

organisms (Mayer & Yu, 2018) and reversible phosphorylation of a protein often serves as a signal modulation mechanism to regulate cellular activities (Hoang et al., 2019). In addition, phosphorylation can also affect the subcellular localization of proteins, such as BZR1. The subcellular localization and transcriptional activity are tightly regulated by reversible protein phosphorylation (Wang et al., 2021). Our study provides a new view that the phosphorylation-related multilocation proteins plays an important role in organelle communication. The research of phosphorylation-related multilocation proteins may promote the development of organelle communication and provide an important theoretical basis for plant responses to external stress.

4.3 | Techniques, technical difficulties, and prospects of multilocation proteins

The smallest unit of life is the cell, which contains numerous protein molecules. Most of the functions critical to cell survival are performed by these proteins, which are located in different organelles, usually called "subcellular locations" (Chou, 2019). As a result of the above analyses, it speculated that multilocation proteins play key roles in the communication among organelles, which can also be used as an indicator of the closeness organelles. Thus, the method used to screen multilocation proteins is important. At present, research pertaining to subcellular localization is divided mainly into experimental evidence and software predictions. The experimental methods include immunofluorescence (Stadler et al., 2013) and expression of green fluorescent protein fusion proteins (Cui et al., 2016), and software performed predictions are based mainly on bioinformatics (Bouziane & Chouarfia, 2020; Chou, 2019; Chou et al., 2019a). The application of these experimental methods can provide more accurate information on protein subcellular localization, and the present research is based mainly on this type of method. Unfortunately, determining the subcellular locations of proteins based solely on experiments is time-consuming and costly. In addition, external factors such as light signals induce changes in the subcellular localization of some proteins (Oikawa et al., 2008; Sakamoto & Briggs, 2002; Wan et al., 2008). Thus, inaccurate subcellular locations can be obtained by confocal microscope. In this case, many subcellular localization prediction software programs, including multilocation prediction software, have been developed (Cheng et al., 2017, 2018, 2019; Chou et al., 2011, 2019a, 2019b; Chou & Shen, 2008, 2010; Jiang et al., 2019; Sahu et al., 2019; Wu et al., 2011; Yu et al., 2014). Through comparisons with relevant proteins verified by experimental evidence, several software location information predictors, such as iLoc-Plant and Plant-mPLoc, have high confidence in predicting plant nuclear localization (Xiong et al., 2016). However, the prediction software results still need to be supported by experimental evidence, and a method that can ensure a certain accuracy and high-throughput is needed.

In protein studies, one of the fundamental goals is determining the subcellular locations of proteins within an entire cell (Chou, 2019). Some proteomic techniques, such as iTRAQ, 2-DE, and Lable-free, are mature and can be used to analyze organelle proteins (Lv et al., 2019; Ning et al., 2016; Paik et al., 2019). If organelles are isolated effectively, the proteins of each organelle can be identified using proteomic technologies, and multilocation proteins can be obtained. However, separation and acquisition of high purity organelles are difficult. Organelle separation is usually achieved by differential centrifugation (Liao et al., 2020), but the separation efficiency and purity of this method do not meet the standards of organelle proteomic analyses. Recently, dynamic endomembrane organelle profiling has provided a novel approach, localization of organelle proteins by isotope tagging (LOPIT), for isolating and detecting protein components from multiple organelles simultaneously (Chen & Heazlewood, 2020; Geladaki et al., 2019; Mulvey et al., 2017). In addition, this method can detect and locate live proteins moving between different sub-organelles and membrane systems, which overcomes the technical challenge of routine laboratory separation of sub-organelles. Furthermore, dynamic endomembrane organelle profiling can be used for large-scale qualitative and quantitative analyses of different sub-organelle proteins by valuating localization of organelle proteins by LOPIT, free-flow electrophoresis (FFE) and mass spectrometry. In addition,

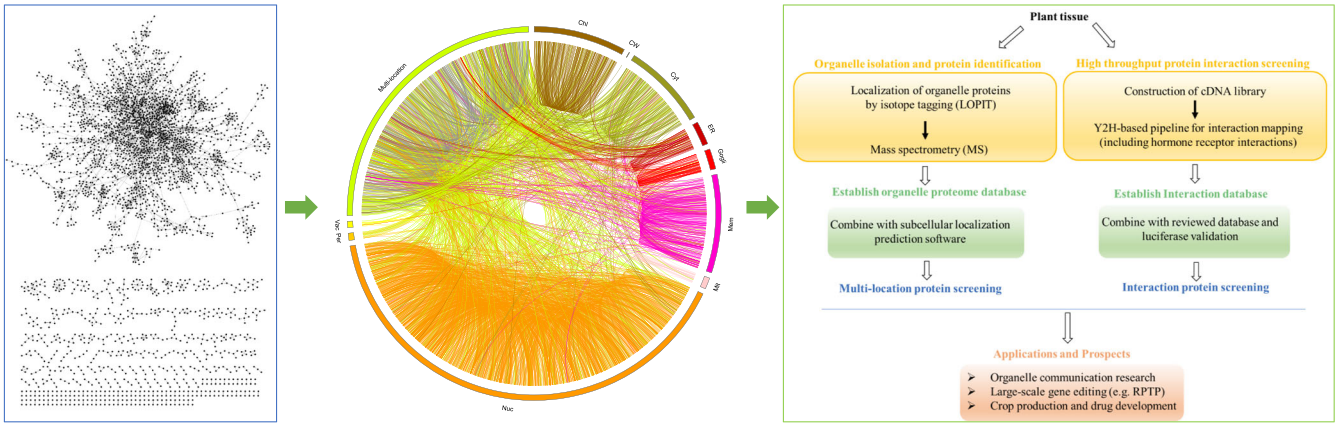

FIGURE 4 Schematic overview of multilocation protein analysis in plant tissue

protein from different components of the Golgi, such as the cis-, Medial-, and trans-Golgi proteins, can be isolated accurately (Chen & Heazlewood, 2020). By this method, it is possible to isolate the proteins of each organelle and then screen them to obtain multilocation proteins. By combining the protein location information obtained by experimental evidence, homology alignment analysis, and software prediction, more accurate location information should be obtainable. The multilocation proteins can be used for further research, such as the RPTP for rice or large-scale gene editing, and applied to crop production, drug development, and other applications. In addition, this method (Figure 4) can be used to build databases of multilocation proteins from different species on a large scale. The databases will be important, new and comprehensive protein research resources for many researchers, allowing research to be widely extended to the protein level, which plays an important role in the regulation of biological processes. It is also expected that many new discoveries advancing animal and plant sciences will be made.

4.4 | Conclusion and perspectives

PPI networks often suggest protein functions and open new avenues for characterizing genes and proteins (McWhite et al., 2020). Although research on PPIs has advanced rapidly in recent years, it remains poorly developed, especially in terms of communication among organelles at the proteomic level. By summarizing and analyzing the model organisms that have been investigated thoroughly, we can find new clues and identify new research directions, which will have important impacts on research of other crops and animals. In this study, through the analysis of PPIs in the model plant *Arabidopsis thaliana*, we found that multilocation proteins, especially those related to phosphorylation, play an important role in organelle communication. In addition, we analyzed the research progress on PPIs and the potential role of multilocation proteins in the rice RPTP program. Considering the current research progress, we provided a research strategy and direction for multilocation proteins, which provides a theoretical basis for research pertaining to organelle communication.

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

XEH and GFP conceived the idea of this research. XEH, CD, GFP, ZPF, WZK, and QCX processed and analyzed the data. GFP and XEH wrote the original manuscript. YDM and ZQZ revised the manuscript. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the plots within this paper and other finding of this study are available from the corresponding author upon reasonable request.

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