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Selective chemical probes can untangle the complexity of the plant cell endomembrane system

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Abstract

The endomembrane system is critical for plant growth and development and understanding its function and regulation is of great interest for plant biology research. Small-molecule targeting distinctive endomembrane components have proven powerful tools to dissect membrane trafficking in plant cells. However, unambiguous elucidation of the complex and dynamic trafficking processes requires chemical probes with enhanced precision. Determination of the mechanism of action of a compound, which is facilitated by various chemoproteomic approaches, opens new avenues for the improvement of its specificity. Moreover, rational molecule design and reverse chemical genetics with the aid of virtual screening and artificial intelligence will enable us to discover highly precise chemical probes more efficiently. The next decade will witness the emergence of more such accurate tools, which together with advanced live quantitative imaging techniques of subcellular phenotypes, will deepen our insights into the plant endomembrane system.

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Keywords

Endomembrane trafficking, Chemical genetics, Small molecule, Endosidins, Mechanism of action, Target identification, Chemoproteomics, Virtual screening, Rational design, Artificial intelligence.

Introduction

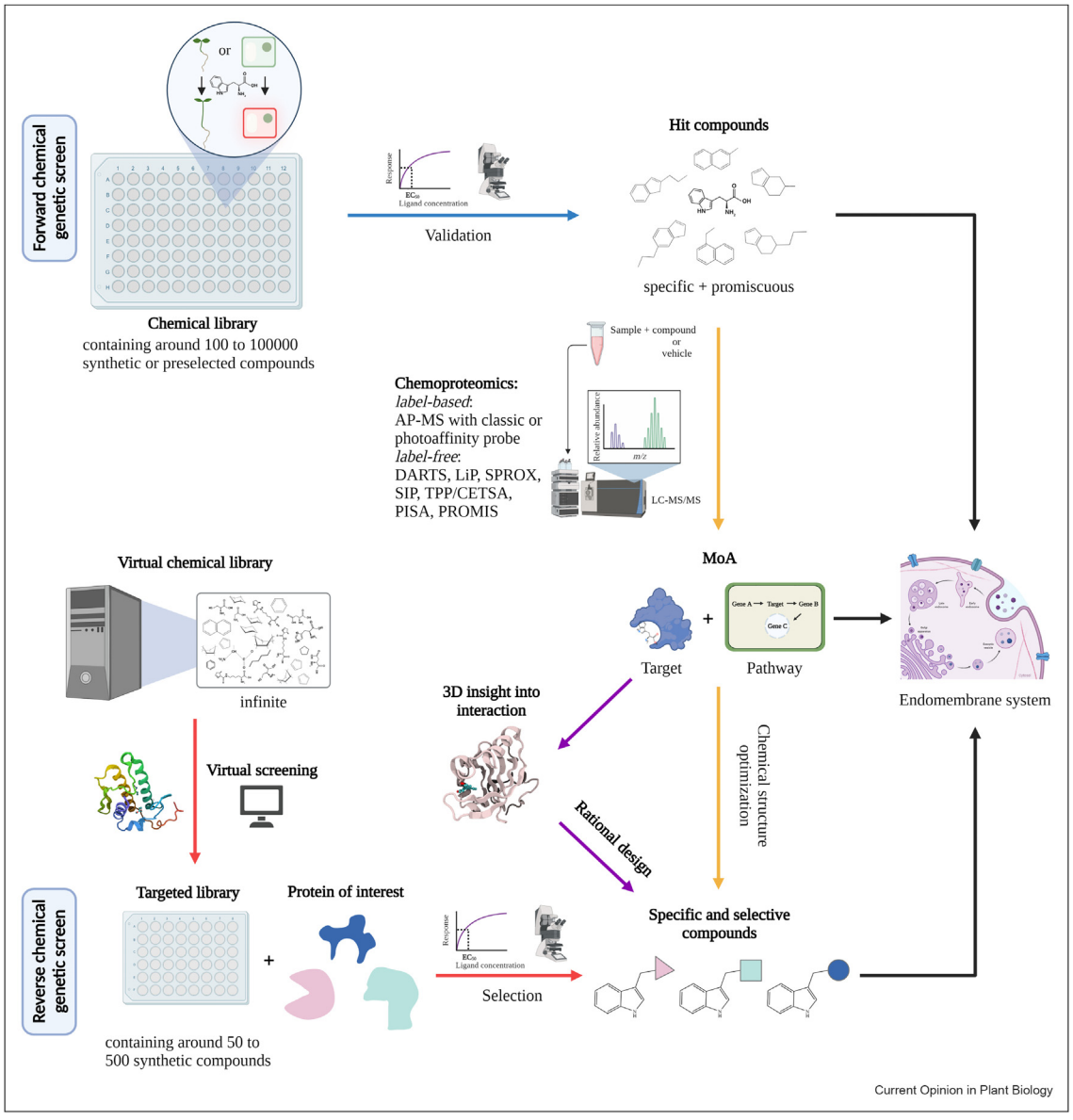
Despite its rather recent establishment, chemical genetics has already proven valuable for plant biology research [1]. The use of small molecules can overcome issues of gene redundancy, lethality, and pleiotropy in classical genetics and they can be applied in a reversible, temporal, and dose-dependent manner, enabling cell and tissue specificity. Small molecules are powerful tools to decipher highly dynamic and essential cellular processes, such as endomembrane trafficking. Although the last two decades of plant chemical genetics have delivered numerous endomembrane trafficking modifiers [2,3], major bottlenecks preventing their wide application are the limited knowledge about their direct targets, mechanism of action (MoA), and their low specificity.

In this review, we provide an overview of the usefulness of the available chemical tools for endomembrane trafficking studies in plants and we discuss recent advances in MoA and structure-based design strategies that can help improving the specificity and selectivity of the small molecules.

Historical enrichment of the small-molecule collection targeting the plant endomembrane system

In the mid-2000, the most popular chemical probe used to investigate Golgi-mediated trafficking in plants was Brefeldin A (BFA) that has been instrumental in dissecting the function of the plant ADP-ribosylation factor–guanine–nucleotide exchange factors (ARF-GEFs) [4]. The broad BFA spectrum prompted the need for more specific probes that could assess complex pathways. A comprehensive forward chemical screen for pollen germination inhibition of nearly 50,000 compounds established a small collection of 360 inhibitors and a toolbox of 123 endosidin (ES) compounds selected through secondary and tertiary microscopy-based screens with fluorescently-labeled endomembrane markers [5]. Later on, more specialized screens of this chemical collection delivered inhibitors of endocytosis [6], vacuolar trafficking [7,8], and autophagy [9]. Conversely, only a few plant growth phenotype-based screens identified endomembrane trafficking modifiers, as for instance Secdin [10].

Figure 1



Routes to develop precise chemical probes to dissect membrane trafficking. In plants, a predominant route (blue line) usually starts with a forward chemical genetic screen based on a phenotype or a molecular reporter closely linked to a cellular process under study as the readout. A variety of chemical libraries containing comprehensive synthetic small molecules, natural products or preselected bioactive compounds can be used in the screen. After validation of the efficacy of hit compounds, typically through multitier phases, they can be used as tools to study the endomembrane system. However, in most cases, these compounds exhibit pleiotropic effects, possibly due to their promiscuous binding behavior. Hence, results obtained by their use require careful interpretation. Elucidation of their MoA, especially pinpointing the cognate targets, is crucial to improve their specificity and selectivity (orange line), through, for instance, chemical structure optimization. In recent years, a growing number of key components in diverse membrane trafficking pathways have been uncovered in plants. Taking advantage of this advancement, an alternative route (red line) implements a reverse chemical genetic screen assisted by virtual screening (VS) to discover bioactive compounds that bind specifically to a particular protein and selectively affect its function. When the structural knowledge of an interaction between a bioactive compound and the binding site of its target is available, rational design can be utilized following the third route (purple line) as a targeted chemical engineering method, operating at the atomic level, to create new compounds with ultra-high affinity, specificity, and even new desirable properties. The precise chemical tools developed by the above-mentioned ways can facilitate mechanistic understanding of the function and regulation of membrane trafficking pathways in plants in an unambiguous manner. Abbreviation: AP-MS, affinity purification coupled with mass spectrometry; CETSA, cellular thermal shift assay; DARTS, drug affinity-responsive target stability; LiP, limited proteolysis; MoA, mechanism of action; PISA, proteome integral solubility alteration; PROMIS, protein–metabolite interactions by means of size separation; SIP, solvent-induced protein precipitation; SPROX, stability of proteins from rates of oxidation; TPP, thermal proteome profiling. Created with BioRender.com.

The available ES compounds affect various aspects of endomembrane trafficking in plants [5], but the lack of information on their direct target(s) and the fact that some ES compounds are promiscuous result in ambiguous interpretations. Below, we give examples of chemical probes that have helped addressing cell biology questions in plants. The actin filament stabilizer ES1 [11,12] and ES16 that target the RabA GTPase subfamily [13] have contributed to the understanding of endosomal recycling of plasma membrane proteins and Rab GTPase-mediated trafficking routes in plants [11,14]. The inhibitor of the cytokinesis-specific callose deposition ES7 [15] has allowed the examination of the spatiotemporal regulations of secretory and endosomal vesicles in cell plate maturation [15,16]. Furthermore, ES7 application has validated the role of polysaccharides in cell plate expansion, as predicted by biophysical modeling, and the evolutionary conservation of its role [17*,18]. ES8, ES4, and Secdin affect a number of ARF-GEF-regulated pathways, but the direct targets of only ES4 and Secdin were identified as the ARF-GEFs [10,19,20]. The successful target identification revealed that ES9 and ES9-17 bind the plant and mammalian clathrin heavy chain (CHC), thus, providing a much-needed probe to study plant endocytosis [21]. Moreover, ES2 that targets the EXOCYST COMPONENT OF 70 KDa A1 (EXO70A1) subunit of the exocyst complex [22] has become a powerful tool to investigate exocytosis in both plant and mammalian cells [23,24]. The recent application of ES16, ES2, and BFA also helped assess the polarization mechanisms of two receptor kinases that control root cell division and cell patterning [25*].

In summary, despite some successful examples, the number of cell biology studies benefiting from the available small molecules is still limited. In the following sections, we explore several strategies (Figure 1) that can help develop highly precise chemicals with the aim to enhance their application potential in plant cell biology.

Direct protein target identification of bioactive small molecules applicable in plant cell biology research by chemoproteomics

Identification of the cognate target(s) of the small molecules is a critical step in unraveling their MoA and is essential for the further improvement of their selectivity and specificity. At present, the knowledge about the direct targets of the chemical tools discovered via chemical genetics is limited [3,26,27]. The two main reasons are: first, the traditional ethyl methanesulfonate (EMS) mutagenesis-based forward genetic screens used for identifying small molecule-responsive mutants often do not deliver direct targets or they might be missed, when the target is an essential gene; and second, the small molecules identified thus far are either not optimized in terms of bioactivity or regulate the target protein function via transient, low-affinity, non-covalent interactions. Capturing such dynamic and weak interactions is a formidable challenge for target identification. Nevertheless, thanks to important technological advancement in the past decades, chemoproteomics has emerged as an attractive strategy for proteome-based discovery of small-molecule target(s) [28]. As this review is not intended to be exhaustive on the established techniques for small-molecule target identification, we will introduce only the most representative chemoproteomic techniques (Figure 1 and Table 1). We classify them into two general groups, label-based or label-free approaches, depending on use of bioactive small molecules either chemically conjugated or intact, respectively (Table 1).

Label-based approaches

The classical affinity purification (AP) coupled with quantitative mass spectrometry (MS) is still the most widely applied target identification method in plant chemical genetics [3,29]. This approach uses a bipartite small-molecule affinity probe that is generated through chemical conjugation of the small molecule (the ligand) with a functional group (usually a biotin tag) via a linker at a position that based on the structure—activity relationship (SAR) is not essential for its binding capability. After incubation of the affinity probe with cell lysates, the target is recognized by the ligand moiety, pulled-down, enriched by streptavidin-coated beads and characterized by MS. For example, AP-MS experiments using biotin-labeled compounds revealed that EXO70A1 [22], the ARF-GEFs [10] and CHC [21] are the protein targets of ES2, Secdin and ES9, respectively, in *Arabidopsis thaliana*.

Major challenges faced by the affinity-based proteomics are: (*i*) the time-consuming development of a small-molecule affinity probe might affect its activity or function [29]; (*ii*) the pull-downs are often performed out of the endogenous cellular context in cell lysates; and (*iii*) the weak non-covalent interactions between small molecules and proteins can be affected by the purification conditions. Some of the drawbacks can be resolved by generating and using bioorthogonal photoaffinity probes, also called trifunctional probes, which are designed to capture the non-covalent protein—compound interactions in living cells [30,31]. These probes harbor a smaller bioorthogonal tag (alkyne, azide, or others) to enable *in situ* labeling with a functional group, such as biotin, via a "click" reaction and a photoreactive group that is coupled with the linker to covalently cross-link the compound to its target protein. As a result, the reversible non-covalent binding is transformed into a stable covalent interaction (Figure 2). Thus far, this

Table 1

Features of different chemoproteomics approaches applicable for target identification.

Approach	Sample source	Target validation combined with WB	Detection level	Binding site information	Binding affinity estimation	Ligand(s) in one run	Publication
Label-based approach (requires chemical modification of small molecule)							
Classic AP-MS	Cell lysate	Yes	Protein level	No	No	Single	[10,21,22]
AP-MS using trifunctional photoaffinity probes	Cell lysate and living cell	Yes	Protein level	No	No	Single	[32,33]
Label-free approach (does not require chemical modification of small molecules)							
DARTS	Cell lysate	Yes	Protein level	No	No	Single	[34,35]
LiP	Cell lysate and living cell	No	Peptide level	Yes	Yes	Single	[36,37]
SPROX	Cell lysate	No	Peptide level (Met-containing peptides)	No	Yes	Single	[38]
SIP	Cell lysate	Yes	Protein level	No	No	Single	[39]
TPP/CETSA	Cell lysate and living cell	Yes	Protein level	No	No	Single	[42–44]
PISA	Cell lysate and living cell	No	Protein level	No	No	Single	[45]
PROMIS	Cell lysate	No	Protein level	No	No	Multiple	[48,49]

type of sophisticated bioorthogonal photoaffinity probes are mostly designed and applied in mammalian research [32], although a few applications in plant chemical genetics are emerging [33*].

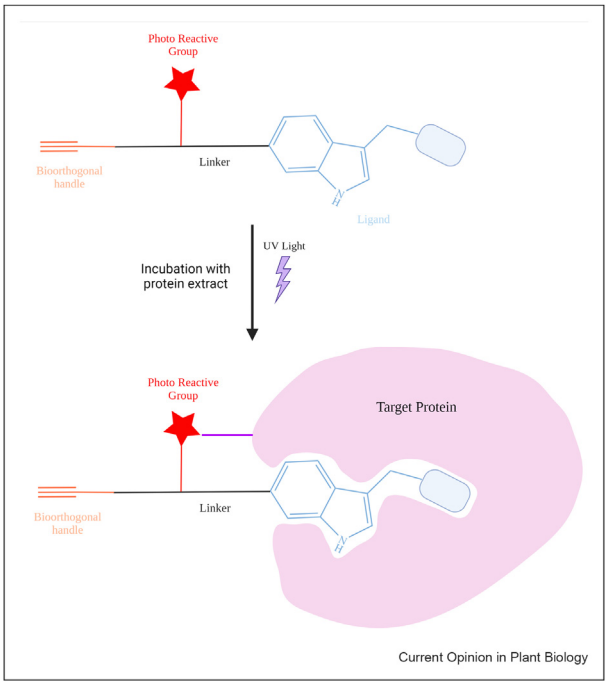
Label-free approaches

The recently introduced label-free strategies mainly into non-plant systems can nicely complement the traditional affinity methods and overcome some of their limitations in plants (Table 1). Despite their diversified technical details, the label-free approaches rely on the principle that the binding of a ligand to a protein triggers a change in the protein's biochemical and/or biophysical properties often manifested as altered stability, which can be detected on a proteome-wide scale by the modern MS technologies in a sensitive and quantitative manner. Below, we highlight several recent and representative label-free approaches.

Drug affinity-responsive target stability (DARTS) and limited proteolysis-coupled mass spectrometry (LiP-MS) are both based on the differential susceptibility to a partial proteolysis incurred by a promiscuous protease upon a small molecule or vehicle treatment [34–36]. DARTS detects the abundance change at the protein level, whereas LiP-MS does this at the peptide level, making it uniquely able to predict ligand-binding sites [36]. More recently, an improved LiP-MS method, called LiP-Quant, has been developed via machine learning to effectively prioritize true small-molecule target identification and pinpoint ligand-binding regions in complex eukaryotic proteomes [37**]. Other established approaches include stability of proteins from rates of oxidation (SPROX) and solvent-induced protein precipitation (SIP), which rely on measurements of altered susceptibility to oxidation and of ligand-binding–induced organic solvent denaturation, respectively [38,39]. Application of these approaches is still in their infancy for plant research, but unlike LiP and SPROX that operate solely via quantitative MS [36,38], DARTS and SIP can be also coupled with western blot (WB) to validate small molecule–candidate target protein interactions [34,39]. For instance, the binding of ES2, ES2-14, ES9-17, ES16, ES4, Secdin, and ES20 to their corresponding targets had been verified by DARTS-WB [10,13,20–22,40,41].

Another rapidly evolving chemoproteomic approach is the thermal proteome profiling (TPP) that allows the study of ligand binding to proteins in living cells or even tissues, through a proteome-wide cellular thermal shift assay (CETSA) that monitors the melting temperature shift of a protein in the presence or absence of a small molecule [42,43]. Soon hereafter, the sensitivity and specificity of the approach is increased by two-dimensional TPP (2D-TPP) [44], whereas a multidimensional or high-resolution format, called proteome integral solubility alteration (PISA), further augments the throughput and reduces the experimental cost dramatically [45]. Similar to DARTS, CETSA coupled to WB was used to prove the interactions between ES9 and ES9-17 and their plant target [21]. To broadly adapt CETSA to plant chemical genetics, a recent proof-of-concept study applied CETSA-MS in intact *Arabidopsis*

Figure 2



Bioorthogonal photoaffinity probe principle. In plants, a predominant route (blue line, Figure 1) usually starts with a forward chemical genetic screen based on a phenotype or a molecular reporter. Based on SAR, the small molecule of interest (ligand in light blue) is modified to incorporate a linker (black line), which harbors a photoreactive group (red line and star) and a bioorthogonal handle (in orange). The probe is incubated with protein extracts to allow binding to the potential target protein (in pink) in the binding pocket. A UV illumination triggers the covalent crosslinking (purple line) between the probe and the target protein. The small molecule–protein complex can be isolated with an affinity tag (not shown here) added to the probe via the bioorthogonal handle by the "click" reaction. Created with BioRender.com.

cells to map the interacting proteins of bikinin [46**]. Bikinin is a potent and well-characterized small-molecule inhibitor targeting the plant-specific glycogen synthase kinase 3 (GSK3), the core negative regulator of brassinosteroid (BR) signaling [47]. CETSA-WB validated a subset of the GSK3s as direct bikinin targets. Notably, CETSA-MS also identified the auxin carrier PIN-FORMED1 (PIN1) as an indirect target of bikinin [46**]. This study not only benchmarks the application of CETSA-MS for target identification in plants, but also illustrates the usefulness of this approach for the discovery of novel signaling components downstream of the small molecule's direct target.

In contrast to the approaches described above that originate from non-plant systems, the protein–metabolite interactions using size separation (PROMIS) method was devised for the systemic detection of endogenous protein–metabolite interactions in plants [48,49]. PROMIS relies on the co-elution behavior of proteins and small molecules during size-exclusion chromatography (SEC), which is determined by quantitative metabolomics and proteomics. In addition, PROMIS can be used as a tool to identify interactions between proteins and synthetic small molecules across all biological systems. As a proof-of-concept, this approach reliably separated the known targets of several *Arabidopsis* protease inhibitors in total protein lysates [48]. However, rather than pinpointing the target, PROMIS narrows down the target range and requires independent techniques to find the target [48].

Searching for novel bioactive compounds targeting key cellular players by reverse chemical genetics

In plants, the predominant approach to identify small molecules that can perturb specific processes has been the phenotype-based forward chemical genetics [26] (Figure 1). According to our current knowledge, a trend has become apparent that some proteins, such as the ARF-GEFs, are chemical-prone targets in forward chemical screens for trafficking modifiers [10,19,20]. The *ARF-GEF* genes are essential and play central regulatory roles [4], possibly the reason for the high frequency of small-molecule "hits" that affect this family of proteins. Hence, a screening pipeline, including additional validation steps based on quantitative analyses or specific phenotypic readouts, can broaden the spectrum of chemical modulators targeting the pathway of interest. For instance, a recently established autophagy multitier-based screen attempted to improve the specificity and selectivity of screened chemicals and eliminate general toxicity enhancers [9], even though the corresponding targets and MoAs remained to be identified.

Although forward chemical screens can be fine-tuned for specificity, more precise chemical probes can be identified via target-based reverse chemical screens that mostly generate target protein-specific compounds, thereby largely avoiding the off-target effects. In the past, the number of ligandable proteins for cellular processes of interest and the mechanistic knowledge regarding their functions were limited, among others, impeding the application of target activity-based reverse chemical genetics. The increasing knowledge about proteins involved in diverse cellular processes provides a new avenue for employing the reverse chemical genetics method in plants [1]. Nonetheless, similar to the forward approach, the reverse chemical screens are laborious and costly, because they require screening of a large number of compounds, hence restricting the types and scales of phenotypes or protein bioactivities that can be examined.

Instead, virtual screening (VS), originally developed as a promising computational chemistry approach to increase the efficiency of drug development for proteins with known or predicted structures, is a robust *in silico* technique that can markedly decrease the infinite virtual

space of chemical compounds to a manageable scale for further reverse chemical screening [50,51]. With the aid of VS, the number of compounds to be tested is dramatically reduced (Figure 1). Therefore, the potency of the predicted active compounds can be examined by means of the informative readout that directly reports the target-specific bioactivity. Particularly beneficial for plant cell biology studies, the "wet-lab" screen can be conducted under confocal microscopy in a sensitive and quantitative manner. As a consequence, the chance to isolate selective and specific compounds is increased, while time and cost are reduced. Once a target is identified, resolving the structure of the small molecule bound to the target protein elucidates the mechanism of binding and activity [21,52].

Rational design of chemical scalpels for applications in plant cell biology

Thanks to chemical genetics combined with structural biology, insights into the three-dimensional (3D) interactions between small molecules and their protein targets are proliferating, enabling rational compound design with increased affinity and specificity and/or even new functionality, based on the structure—function relationship [53,54]. This strategy has been widely used for drug development and gains more and more attention in plant chemical biology in general, but is lacking in plant cell biology. For instance, an orthogonal pair, in which the synthetic small molecule convex indole-3-acetic acid (cvxIAA) binds only to an engineered concave TRANSPORT INHIBITOR RESPONSE1 (ccvTIR1) receptor has been designed based on structural information [55]. This system provides a unique way to manipulate auxin-mediated processes in a controllable manner and to bypass genetic redundancy and feedback regulations. Among other prominent examples are the specific auxin antagonist auxinole [56] and the highly potent and specific abscisic acid (ABA) agonist opabactin [52] and antagonist antabactin [57*]. These powerful chemical tools demonstrate how rational design based on the structural knowledge of ligand—target interactions could facilitate the development of potent and precise chemical probes for plant cell biology.

The availability of 3D structures of proteins in complex with ligands is indispensable for rational design application. This type of information is usually obtained by X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy or cryogenic electron microscopy (cryo-EM). Structural knowledge in plants is far behind that in mammals. Nonetheless, the ample structural information in non-plant systems can be extensively exploited via computational protein structure prediction approaches, such as homology modeling. Excitingly, artificial intelligence (AI) deep learning-based approaches, such as AlphaFold [58**] and RoseTTAFold [59], unprecedentedly increase the atomic accuracy of protein structure prediction and can theoretically provide deep structural coverage for any plant species of which the proteome is available. Once the protein structure is known, its interaction with the ligand can be obtained by molecular docking. In this manner, rational design can be performed for any protein that is interesting for plant cell biology study, but still lacks the structural information.

Conclusions

A decade ago, Hicks and Raikhel [2] published a comprehensive review that systematically delineated plant chemical biology and deciphered how it would overcome the constraints of the conventional strategies and assist in unraveling the mechanisms of plant endomembrane trafficking. Recent advances in this field highlight the substantial contribution made by chemical genetics to membrane trafficking research [3,27], providing an enriched chemical toolbox for investigation, despite caveats for the use of these chemical probes. An evident concern lies in the pleiotropic effects of some compounds, confounding the result interpretation [6], but could be resolved by developing and using compounds with an increased specificity (Figure 1). We envision that precise chemical tools coupled with advanced live quantitative imaging techniques at a subcellular level, followed by modeling, will provide unparalleled opportunities to obtain deeper insights into membrane trafficking.

In the last decade, technological and computational developments in proteomics propelled the generation of various powerful chemoproteomic tools for mapping small molecule—protein interactions (Table 1). They can be used in a direct manner or in a competitive format to distinguish the non-specific binding [29,31]. Moreover, these chemoproteomic approaches are complementary to each other, because they utilize distinctive protein properties that change upon binding to ligands, and they can be combined to help reducing the false positive proteins during target identification.

Application of AI in biological research is transforming our way of studying protein—small molecule interactions [60]. AI-based algorithms can enhance the robustness and efficiency of molecular docking generating structural models of how a ligand binds to the potential binding site at the atomic level, but also provide a feasible manner to probe the vast chemical space [51]. Conversely, both VS and rational design rely on protein structural knowledge and AI seems one of the most promising technologies to tackle this bottleneck [61]. The eminent AI tool AlphaFold brings the accuracy of protein structure prediction to an exceptionally near-experimental level [58,61] and is expected to expand to 130 million—nearly half of all known proteins—by

the end of 2022. This progress in the knowledge of protein structures as well as the efficacy in exploiting the enormous chemical space could revolutionize chemical genetics.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

Box 1. Glossary

Chemical genetics: The use of bioactive small molecules that are able to cause phenotypic perturbations as tools to dissect biological systems through the identification of target biomolecules, most often proteins, and downstream effectors and signaling pathways.

Chemoproteomics: Also known as chemical proteomics refers to studies involving a plethora of mass spectrometry-based techniques used to identify and assess protein–small molecule interactions on a proteome-wide scale.

Ligandable: Describes a biomolecule that is capable of binding to a ligand or small molecule and of which the activity can be modulated by the small molecule.

Mechanism of action: Defines how a compound exerts its physiological effect at the molecular level; it usually includes the characterization of the pathway affected by the compound and the identification of the specific molecular target to which the compound binds.

Rational design: The design of a small molecule that is able to bind to its biomolecular target, in most cases a protein, based on the rationale that originates from the detailed knowledge about a known protein–small molecule interaction.

Virtual screening: A computational approach that is usually used in tandem with reverse chemical genetic screens to search virtual libraries of small molecules to detect chemical compounds that are likely to bind to the target protein of interest. This type of computation is analogous to biochemical high-throughput screening performed *in silico*.

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References

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest

1. Halder V, Russinova E: **Understanding the language of drugged plants**. *Nat Chem Biol* 2019, **15**:1025–1028.

2. Hicks GR, Raikhel NV: **Small molecules present large opportunities in plant biology**. *Annu Rev Plant Biol* 2012, **63**:261–282.

3. Huang L, Li X, Zhang C: **Progress in using chemical biology as a tool to uncover novel regulators of plant endomembrane trafficking**. *Curr Opin Plant Biol* 2019, **52**:106–113.

4. Singh MK, Jürgens G: **Specificity of plant membrane trafficking - ARFs, regulators and coat proteins**. *Semin Cell Dev Biol* 2018, **80**:85–93.

5. Drakakaki G, Robert S, Szatmari A-M, Brown MQ, Nagawa S, Van Damme D, Leonard M, Yang Z, Girke T, Schmid SL, *et al.*: **Clusters of bioactive compounds target dynamic endomembrane networks in vivo**. *Proc Natl Acad Sci USA* 2011, **108**:17850–17855.

6. Dejonghe W, Kuenen S, Mylle E, Vasileva M, Keech O, Viotti C, Swerts J, Fendrych M, Ortiz-Morea FA, Mishev K, *et al.*: **Mitochondrial uncouplers inhibit clathrin-mediated endocytosis largely through cytoplasmic acidification**. *Nat Commun* 2016, **7**:11710.

7. Rivera-Serrano EE, Rodriguez-Welsh MF, Hicks GR, Rojas-Pierce M: **A small molecule inhibitor partitions two distinct pathways for trafficking of tonoplast intrinsic proteins in Arabidopsis**. *PLoS One* 2012, **7**, e44735.

8. Dünser K, Schöller M, Löfke C, Xiao N, Pařízková B, Melnik S, Stöger E, Novák O, Kleine-Vehn J: **Endocytic trafficking promotes vacuolar enlargements for fast cell expansion rates in plants**. *bioRxiv* 2021, **2021**.2021.2011.2029.470358.

9. Dauphinee AN, Cardoso C, Dalman K, Ohlsson JA, Fick SB, Robert S, Hicks GR, Bozhkov PV, Minina EA: **Chemical screening pipeline for identification of specific plant autophagy modulators**. *Plant Physiol* 2019, **181**:855–866.

10. Mishev K, Lu Q, Denoo B, Peurois F, Dejonghe W, Hullaert J, De Rycke R, Boeren S, Bretou M, De Munck S, *et al.*: **Nonselective chemical inhibition of Sec7 domain-containing ARF GTPase exchange factors**. *Plant Cell* 2018, **30**:2573–2593.

11. Robert S, Chary SN, Drakakaki G, Li S, Yang Z, Raikhel NV, Hicks GR: **Endosidin1 defines a compartment involved in endocytosis of the brassinosteroid receptor BRI1 and the auxin transporters PIN2 and AUX1**. *Proc Natl Acad Sci USA* 2008, **105**:8464–8469.

12. Tóth R, Gerding-Reimers C, Deeks MJ, Menninger S, Gallegos RM, Tonaco IAN, Hübel K, Hussey PJ, Waldmann H, Coupland G: **Prieurianin/endosidi 1 is an actin-stabilizing small molecule identified from a chemical genetic screen for circadian clock effectors in *Arabidopsis thaliana***. *Plant J* 2012, **71**:338–352.

13. Li R, Rodriguez-Furlan C, Wang J, van de Ven W, Gao T, Raikhel NV, Hicks GR: **Different endomembrane trafficking pathways establish apical and basal polarities**. *Plant Cell* 2017, **29**:90–108.

14. Qi X, Zheng H: **Rab-A1c GTPase defines a population of the *trans*-Golgi network that is sensitive to endosidin1 during cytokinesis in *Arabidopsis***. *Mol Plant* 2013, **6**:847–859.

15. Park E, Díaz-Moreno SM, Davis DJ, Wilkop TE, Bulone V, Drakakaki G: **Endosidin 7 specifically arrests late cytokinesis and inhibits callose biosynthesis, revealing distinct trafficking events during cell plate maturation**. *Plant Physiol* 2014, **165**:1019–1034.

16. Davis DJ, McDowell SC, Park E, Hicks G, Wilkop TE, Drakakaki G: **The RAB GTPase RABA1e localizes to the cell plate and shows distinct subcellular behavior from RABA2a under Endosidin 7 treatment**. *Plant Signal Behav* 2016, **11**, e984520.

17. * Jawaid MZ, Sinclair R, Bulone V, Cox DL, Drakakaki G: **A biophysical model for plant cell plate maturation based on the contribution of a spreading force**. *Plant Physiol* 2022, **188**:795–806.
Application of ES7 validates the contribution of callose in membrane network transition and cell plate expansion, as predicted by biophysical modeling.

18. Davis DJ, Wang M, Sørensen I, Rose JKC, Domozych DS, Drakakaki G: **Callose deposition is essential for the completion of cytokinesis in the unicellular alga *Penium margaritaceum***. *J Cell Sci* 2020, **133**:jcs249599.

19. Doyle SM, Haeger A, Vain T, Rigal A, Viotti C, Łangowska M, Ma Q, Friml J, Raikhel NV, Hicks GR, *et al.*: **An early secretory pathway mediated by GNOM-LIKE 1 and GNOM is essential for basal polarity establishment in *Arabidopsis thaliana***. *Proc Natl Acad Sci USA* 2015, **112**:E806–E815.

20. Kania U, Nodzyński T, Lu Q, Hicks GR, Nerinckx W, Mishev K, Peurois F, Cherfils J, De Rycke R, Grones P, *et al.*: **The inhibitor Endosidin 4 targets SEC7 domain-type ARF GTPase exchange factors and interferes with subcellular trafficking in eukaryotes**. *Plant Cell* 2018, **30**:2553–2572.

21. Dejonghe W, Sharma I, Denoo B, De Munck S, Lu Q, Mishev K, Bulut H, Mylle E, De Rycke R, Vasileva M, *et al.*: **Disruption of endocytosis through chemical inhibition of clathrin heavy chain function**. *Nat Chem Biol* 2019, **15**:641–649.

22. Zhang C, Brown MQ, van de Ven W, Zhang Z-M, Wu B, Young MC, Synek L, Borchardt D, Harrison R, Pan S, *et al.*: **Endosidin2 targets conserved exocyst complex subunit EXO70 to inhibit exocytosis**. *Proc Natl Acad Sci USA* 2016, **113**:E41–E50.

23. Zhao Y, Hong X, Chen X, Hu C, Lu W, Xie B, Zhong L, Zhang W, Cao H, Chen B, *et al.*: **Deregulation of Exo70 facilitates innate and acquired cisplatin resistance in epithelial ovarian cancer by promoting cisplatin efflux**. *Cancers* 2021, **13**:3467.

24. Yan X, Wang Y, Xu M, Dahhan DA, Liu C, Zhang Y, Lin J, Bednarek SY, Pan J: **Cross-talk between clathrin-dependent post-Golgi trafficking and clathrin-mediated endocytosis in Arabidopsis root cells**. *Plant Cell* 2021, **33**:3057–3075.

25. * Rodriguez-Furlan C, Campos R, Toth JN, Van Norman JM: **Distinct mechanisms orchestrate the contra-polarity of IRK and KOIN, two LRR-receptor-kinases controlling root cell division**. *Nat Commun* 2022, **13**:235.

The combined application of ES16, ES2, and BFA reveals how endomembrane pathways, thereby controlling root cell division and root cell patterning, differentially regulate the polarity of the receptor-kinases.

26. Dejonghe W, Russinova E: **Plant chemical genetics: from phenotype-based screens to synthetic biology**. *Plant Physiol* 2017, **174**:5–20.

27. Norambuena L, Tejos R: **Chemical genetic dissection of membrane trafficking**. *Annu Rev Plant Biol* 2017, **68**:197–224.

28. Simon GM, Niphakis MJ, Cravatt BF: **Determining target engagement in living systems**. *Nat Chem Biol* 2013, **9**:200–205.

29. Kawatani M, Osada H: **Affinity-based target identification for bioactive small molecules**. *MedChemComm* 2014, **5**:277–287.

30. Dejonghe W, Russinova E: **Target identification strategies in plant chemical biology**. *Front Plant Sci* 2014, **5**:352.

31. Qin W, Yang F, Wang C: **Chemoproteomic profiling of protein–metabolite interactions**. *Curr Opin Chem Biol* 2020, **54**:28–36.

32. Cheng Y-S, Zhang T, Ma X, Pratuangtham S, Zhang GC, Ondrus AA, Mafi A, Lomenick B, Jones JJ, Ondrus AE: **A proteome-wide map of 20(*S*)-hydroxycholesterol interactors in cell membranes**. *Nat Chem Biol* 2021, **17**:1271–1280.

33. * Dejonghe W, Vaidya AS, Alfred SE, Cutler SR: ***In planta* labeling using a clickable ER-disrupting probe suggests a role for oleosins in *Arabidopsis* seedling ER integrity**. *ACS Chem Biol* 2021, **16**:2151–2157.

The authors discovered a small molecule, eroonazole, as a selective disrupter of the endoplasmic reticulum (ER) structure and function and revealed that oleosins are the direct targets by using a clickable eroonazole photoaffinity probe in the AP-MS experiment. This study demonstrates the role of oleosins in the ER and the utility of bio-orthogonal photoaffinity probes for target identification in plants.

34. Lomenick B, Hao R, Jonai N, Chin RM, Aghajan M, Warburton S, Wang J, Wu RP, Gomez F, Loo JA, *et al.*: **Target identification using drug affinity responsive target stability (DARTS)**. *Proc Natl Acad Sci USA* 2009, **106**:21984–21989.

35. Lomenick B, Jung G, Wohlschlegel JA, Huang J: **Target identification using drug affinity responsive target stability (DARTS)**. *Curr Protoc Chem Biol* 2011, **3**:163–180.

36. Piazza I, Kochanowski K, Cappelletti V, Fuhrer T, Noor E, Sauer U, Picotti P: **A map of protein-metabolite interactions reveals principles of chemical communication**. *Cell* 2018, **172**:358–372.

37. ** Piazza I, Beaton N, Bruderer R, Knobloch T, Barbisan C, Chandat L, Sudau A, Siepe I, Rinner O, de Souza N, *et al.*: **A machine learning-based chemoproteomic approach to identify drug targets and binding sites in complex proteomes**. *Nat Commun* 2020, **11**:4200.

For the identification of drug targets and binding sites, the authors devised the LiP-Quant approach by incorporating a compound dosage dilution series and a machine learning-derived scoring system into the existing LiP-MS method. They successfully identified the direct target of a fungicide compound by LiP-Quant. This study presents a novel chemoproteomic approach that enables the systematic investigation of protein–small-molecule interactions in complex eukaryotic proteomes.

38. West GM, Tucker CL, Xu T, Park SK, Han X, Yates 3rd JR, Fitzgerald MC: **Quantitative proteomics approach for identifying protein-drug interactions in complex mixtures using protein stability measurements**. *Proc Natl Acad Sci USA* 2010, **107**:9078–9082.

39. Zhang X, Wang Q, Li Y, Ruan C, Wang S, Hu L, Ye M: **Solvent-induced protein precipitation for drug target discovery on the proteomic scale**. *Anal Chem* 2020, **92**:1363–1371.

40. Huang L, Li X, Zhang W, Ung N, Liu N, Yin X, Li Y, Mcewan RE, Dilkes B, Dai M, *et al.*: **Endosidin20 targets the cellulose synthase catalytic domain to inhibit cellulose biosynthesis**. *Plant Cell* 2020, **32**:2141–2157.

41. Huang L, Li X, Li Y, Yin X, Li Y, Wu B, *et al.*: **Endosidin2-14 targets the exocyst complex in plants and fungal pathogens to inhibit exocytosis**. *Plant Physiol* 2019, **180**:1756–1770.

42. Martinez Molina D, Jafari R, Ignatushchenko M, Seki T, Larsson EA, Dan C, Sreekumar L, Cao Y, Nordlund P: **Monitoring drug target engagement in cells and tissues using the cellular thermal shift assay**. *Science* 2013, **341**:84–87.

43. Savitski MM, Reinhard FBM, Franken H, Werner T, Fälth Savitski M, Eberhard D, Martinez Molina D, Jafari R, Bakszt Dovega R, Klaeger S, *et al.*: **Tracking cancer drugs in living cells by thermal profiling of the proteome**. *Science* 2014, **346**:1255784.

44. Becher I, Werner T, Doce C, Zaal EA, Tögel I, Khan CA, Rueger A, Muelbaier M, Salzer E, Berkers CR, *et al.*: **Thermal profiling reveals phenylalanine hydroxylase as an off-target of panobinostat**. *Nat Chem Biol* 2016, **12**:908–910.

45. Gaetani M, Sabatier P, Saei AA, Beusch CM, Yang Z, Lundström SL, Zubarev RA: **Proteome integral solubility alteration: a high-throughput proteomics assay for target deconvolution**. *J Proteome Res* 2019, **18**:4027–4037.

46. ** Lu Q, Zhang Y, Hellner J, Giannini C, Xu X, Pauwels J, Ma Q, Dejonghe W, Han H, Van de Cotte B, *et al.*: **Proteome-wide cellular thermal shift assay reveals unexpected cross-talk between brassinosteroid and auxin signaling**. *Proc Natl Acad Sci USA* 2022, https://doi.org/10.1073/pnas.2118220119.

By applying CETSA/TPP-MS as a proof-of-concept to live *Arabidopsis* cells by means of bikinin, the well-known inhibitor of plant-specific GSK3s, the authors identified the auxin efflux carrier PIN1 as an indirect bikinin target, which interacts with and is phosphorylated by GSK3s. This study demonstrates the applicability of CETSA/TPP-MS in live plant cells and benchmarks the approach in plants.

47. De Rybel B, Audenaert D, Vert G, Rozhon W, Mayerhofer J, Peelman F, Coutuer S, Denayer T, Jansen L, Nguyen L, *et al.*: **Chemical inhibition of a subset of *Arabidopsis thaliana* GSK3-like kinases activates brassinosteroid signaling**. *Chem Biol* 2009, **16**:594–604.

48. Veyel D, Sokolowska EM, Moreno JC, Kierszniowska S, Cichon J, Wojciechowska I, Luzarowski M, Kosmacz M, Szlachetko J, Gorka M, *et al.*: **PROMIS, global analysis of PROtein-metabolite interactions using size separation in *Arabidopsis thaliana***. *J Biol Chem* 2018, **293**:12440–12453.

49. Sokolowska EM, Schlossarek D, Luzarowski M, Skirycz A: **PROMIS: global analysis of PROtein-metabolite interactions**. *Curr Protoc Plant Biol* 2019, **4**, e20101.

50. Lavecchia A, Di Giovanni C: **Virtual screening strategies in drug discovery: a critical review**. *Curr Med Chem* 2013, **20**: 2839–2860.

51. Maia EHB, Assis LC, Alves de Oliveira T, Marques da Silva A, Gutterres Taranto A: **Structure-based virtual screening: from classical to artificial intelligence**. *Front Chem* 2020, **8**:343.

52. Vaidya AS, Helander Jonathan DM, Peterson Francis C, Elzinga D, Dejonghe W, Kaundal A, Park S-Y, Xing Z, Mega R, Takeuchi J, *et al.*: **Dynamic control of plant water use using designed ABA receptor agonists**. *Science* 2019, **366**, eaaw8848.

53. Anderson AC: **The process of structure-based drug design**. *Chem Biol* 2003, **10**:787–797.

54. Baud MGJ, Lin-Shiao E, Cardote T, Tallant C, Pschibul A, Chan K-H, Zengerle M, Garcia JR, Kwan TTL, Ferguson FM, *et al.*: **A bump-and-hole approach to engineer controlled selectivity of BET bromodomain chemical probes**. *Science* 2014, **346**:638–641.

55. Uchida N, Takahashi K, Iwasaki R, Yamada R, Yoshimura M, Endo TA, Kimura S, Zhang H, Nomoto M, Tada Y, *et al.*: **Chemical hijacking of auxin signaling with an engineered auxin–TIR1 pair**. *Nat Chem Biol* 2018, **14**:299–305.

56. Hayashi K-i, Neve J, Hirose M, Kuboki A, Shimada Y, Kepinski S, Nozaki H: **Rational design of an auxin antagonist of the SCF^{TIR1} auxin receptor complex**. *ACS Chem Biol* 2012, **7**:590–598.

57. * Vaidya AS, Peterson FC, Eckhardt J, Xing Z, Park S-Y, Dejonghe W, Takeuchi J, Pri-Tal O, Faria J, Elzinga D, *et al.*: **Click-to-lead design of a picomolar ABA receptor antagonist with potent activity in vivo**. *Proc Natl Acad Sci USA* 2021, **118**, e2108281118.

Based on the insight into the interaction beween ABA and its receptor complex, the authors designed ABA antagonists by modifying the synthetic high-affinity agonist OP and rapidly synthesized a potent ABA receptor pan-antagonist, ANT, via click chemistry-based ligand diversification. The methodology presented in this study broadens the chemical space available for developing potent chemical probes for the manipulation of the ABA signaling in basic research as well as in agriculture.

58. ** Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, Tunyasuvunakool K, Bates R, Žídek A, Potapenko A, *et al.*: **Highly accurate protein structure prediction with AlphaFold**. *Nature* 2021, **596**:583–589.

The authors provide the first computational method enabling the regular prediction of protein structures with atomic accuracy. The study presents a machine-learning approach that incorporates physical and biological knowledge that predicts the 3D structures of proteins and protein complexes.

59. Baek M, DiMaio F, Anishchenko I, Dauparas J, Ovchinnikov S, Lee GR, Wang J, Cong Q, Kinch LN, Schaeffer RD, *et al.*: **Accurate prediction of protein structures and interactions using a three-track neural network**. *Science* 2021, **373**:871–876.

60. Tong AB, Burch JD, McKay D, Bustamante C, Crackower MA, Wu H: **Could AlphaFold revolutionize chemical therapeutics?** *Nat Struct Mol Biol* 2021, **28**:771–772.

61. Tunyasuvunakool K, Adler J, Wu Z, Green T, Zielinski M, Žídek A, Bridgland A, Cowie A, Meyer C, Laydon A, *et al.*: **Highly accurate protein structure prediction for the human proteome**. *Nature* 2021, **596**:590–596.