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

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Abstract

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.

Keywords

Endomembrane trafficking, chemical genetics, small molecule, endosidins, mechanism of action, target identification, chemoproteomics, virtual screening, rational design, artificial intelligence

Short title

Strategies for developing precise chemical probes

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-labelled 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].

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 targets 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 contribution 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 it is an essential gene; and second, the small molecules identified by 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-labelled 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* labelling 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 stable covalent interaction (Figure 2).

Thus far, this 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]. Whereas DARTS detects the abundance change at the protein level, 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* 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 in plants to identify small molecules that can perturb specific processes has been the phenotype-based forward chemical genetics [26] (Figure 1). Based on 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-GEFs* are essential genes 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-6-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 modelling. 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 unravelling 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 modelling, 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, which generates 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 no conflict of interests.

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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.

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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 bioactivity 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*.

Table 1

Features of different chemoproteomics approaches applicable for target identification							
Approach	Sample source	Target validation y 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 molecule)							
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]

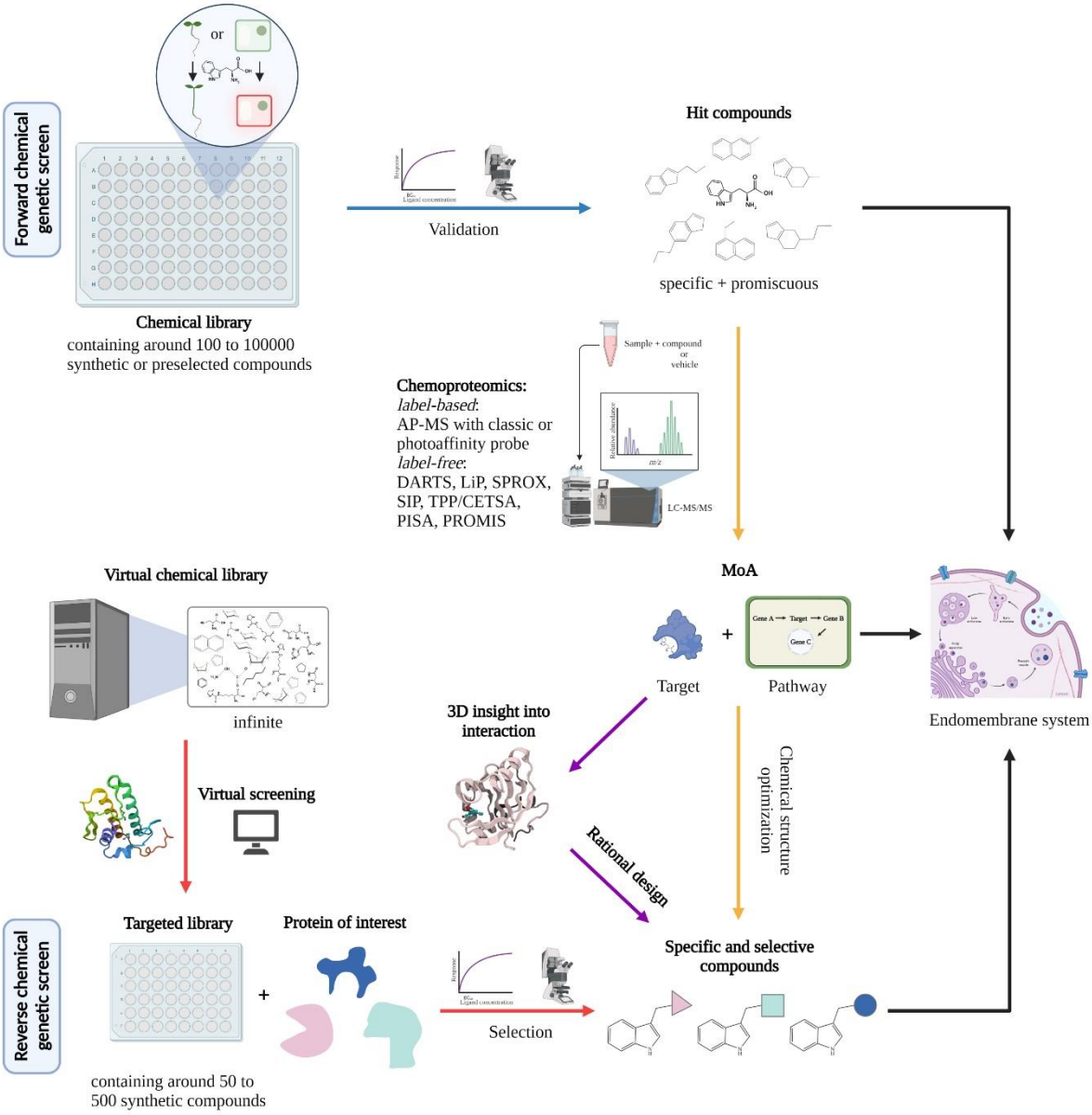

Figure 1. Routes to develop precise chemical probes to dissect membrane trafficking. In plants, a predominant route (blue line) usually starts with a forward chemical genetic screen based on a phenotype or a molecular reporter closely linked to a cellular process under study as the readout. A variety 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 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.

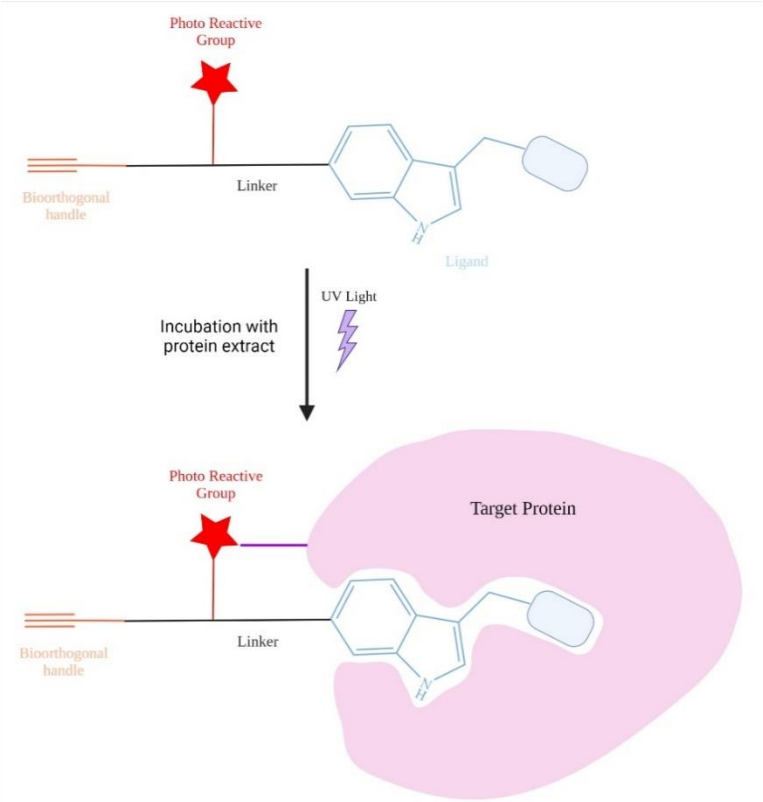

Figure 2. Bioorthogonal photoaffinity probe principle. In plants, a predominant route (blue line, Figure 1) usually starts with a forward chemical genetic screen based on a phenotype or a molecular reporter. Based on SAR, the small molecule of interest (ligand in light blue) is modified to incorporate 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.