Emerging strategies for the identification of protein–metabolite interactions

Marcin Luzarowski and Aleksandra Skirycz*
Max Planck Institute of Molecular Plant Physiology, 14476 Potsdam, Germany

* Correspondence: skirycz@mpimp-golm.mpg.de

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Abstract

Interactions between biological molecules enable life. The significance of a cell-wide understanding of molecular complexes is thus obvious. In comparison to protein–protein interactions, protein–metabolite interactions remain under-studied. However, this has been gradually changing due to technological progress. Here, we focus on the interactions between ligands and receptors, the triggers of signalling events. While the number of small molecules with proven or proposed signalling roles is rapidly growing, most of their protein receptors remain unknown. Conversely, there are numerous signalling proteins with predicted ligand-binding domains for which the identities of the metabolite counterparts remain elusive. Here, we discuss the current biochemical strategies for identifying protein–metabolite interactions and how they can be used to characterize known metabolite regulators and identify novel ones.

Keywords: Complexes, metabolites, methods, proteins, signalling, small molecules.

Introduction

The concept of small-molecule signalling in plants dates back to Charles Darwin, who, besides being an acclaimed evolutionist, was a prominent botanist. Based on a series of elegant experiments studying plant growth towards a unidirectional source of light, Darwin and his son Francis concluded that their results ‘seem to imply the presence of some matter in the upper part [of the seedling] which is acted on by light, and which transmits its effects to the lower part’ (Darwin, 1881). It took 50 years to identify this ‘matter’ as the plant hormone auxin (Went and Thimann, 1937). In plants subjected to a unidirectional source of light, auxin synthesized in the shoot apex is transported down the stem in a way that causes it to accumulate on the shaded side. This accumulated auxin induces cell enlargement, leading to the observed curvature.

Since the isolation of auxin in the 1920s, other plant hormones have been identified, most recently a group of terpenoid lactones named strigolactones (Umehara et al., 2008). Indeed, signalling function has been assigned to small molecules other than hormones. Both primary and secondary metabolites have been implicated in the regulation of biological processes, including growth, development, and environmental responses (Box 1).

Their chemical and functional diversity notwithstanding, all known signalling molecules require a receptor to exert their function. In the vast majority of cases, receptors are proteins,
Box 1. Key developments in protein–metabolite signalling

- **DORN1 serves as a receptor of extracellular ATP in plants**
  Biotic and abiotic stress conditions induce ATP release into the extracellular matrix, which in turn leads to an increase in cytoplasmic calcium concentrations followed by the activation of MAPK signalling and stress-related gene expression. In a recent study, Choi et al. (2014) used forward genetics screening to identify the first plant receptor for extracellular ATP, which they named DORN1 (DOes not Respond to Nucleotides). DORN1 is a legume-type lectin receptor kinase.

- **Limited proteolysis, followed by state-of-the-art mass spectrometry analysis, enables systematic identification of protein–metabolite interactions and ligand-binding sites**
  Piazza et al. (2018) described a chemoproteomic workflow, named LiP-SMap, which combines limited proteolysis with mass spectrometry, enabling the systematic identification of protein partners of the metabolite of choice in a native cellular lysate. LiP-SMap enables the identification of extensive networks of known and previously unknown metabolite–protein interactions. Moreover, the authors demonstrated that LiP-SMap can be used to delineate ligand-binding sites. Although this study was performed using *E. coli*, LiP-SMap is a generic strategy that can be successfully applied to other organisms, including plants.

- **TAP enables the simultaneous analysis of protein, lipid and polar metabolite interactors of a protein of choice**
  Li et al. (2010) and Luzarowski et al. (2017) demonstrated that TAP can be used for parallel analysis of protein, lipid (Li et al., 2010), and polar metabolite (Luzarowski et al., 2017) interactors of a protein of choice. Initially developed for yeast cells (Li et al., 2010), TAP is a generic strategy and was successfully used to retrieve small-molecule interactors of NDPK kinases in plants (Luzarowski et al., 2017).

- **PROMIS is the first method to enable cell-wide analysis of the endogenous protein–metabolite and protein–protein complexes**
  Veyel et al. (2018) described an approach for cell-wide analysis of protein–metabolite and protein–protein complexes, exploiting size exclusion chromatography separation, followed by quantitative metabolomics and proteomics analysis of the obtained fractions. Co-elution is used to define putative interactors. As a proof of concept, the authors reproduced multiple reported binding events and identified putative feedback and feed-forward regulation in pantothenate and methylthioadenosine metabolic pathways in *Arabidopsis thaliana* cell cultures, respectively.

- **Kin10 kinase is a receptor of trehalose-6-phosphate, an important signal of cellular sucrose status**
  Zhai et al. (2018) recently reported a mechanism behind trehalose-6-phosphate (T6P) action, which involves the direct binding of T6P to Kin10 kinase and a consequent decrease of Kin10 affinity towards its protein partner and activator, GirK1. Rather than using a genetic or biochemical strategy to identify T6P putative partner(s), the authors selected Kin10 based on its previously reported involvement in T6P signalling.

either membrane-bound or soluble, although nucleic acid receptors are also known. The non-covalent and reversible small-molecule–receptor interaction serves as a trigger for the signalling cascade. The mode of action varies; interaction often results in a conformational change in the receptor, affecting its activity, localization, and/or interactivity, the latter altering the way in which impending signalling cascades are initiated.

Receptor identity is challenging to determine experimentally. For example, it took 80 years from the discovery of auxin to isolate the auxin receptor TIR1 from *Arabidopsis* (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). To date, and in contrast with auxin, the receptor identities for many of the signalling molecules remain unknown (Box 2). Hence, searching for receptors of known signalling molecules is an obvious research target. This is valid in the opposite direction as well: there are numerous signalling proteins containing one or more predicted ligand-binding domains for which the identities of the small-molecule counterparts remain unknown (Box 2). Finally, it is necessary to mention the intriguing and elusive small-molecule signals that have been proposed based on genetic evidence, although their chemical identities and receptors are not yet known (Box 2).
In this review, we will provide a brief background on the more classical genetic-driven strategies for identifying small-molecule protein receptors, followed by a more exhaustive comparison of the recent biochemical approaches (Box 3). We will discuss how these can be used to track both protein receptors and their ligands, as well as to identify novel small-molecule regulators (Box 4). Finally, we will attempt to address overarching questions about the complexity, functionality, and evolutionary conservation of the protein–metabolite interactome, with an emphasis on the regulatory interactions.

**Box 2. Examples of (i) metabolites implicated in signalling for which protein partner(s) are unknown, (ii) proteins implicated in signalling characterized by the presence of a conserved ligand-binding domain for which small-molecule partner(s) are unknown, and (iii) putative small-molecule signals of unknown chemical identity and protein partners**

### Examples of metabolites implicated in signalling with unknown protein receptor

| Metabolite                  | Function                                                                 | References                                      |
|-----------------------------|--------------------------------------------------------------------------|-------------------------------------------------|
| β-cyclocitral               | Involved in high light acclimation. Component of retrograde signalling. Regulates root growth and architecture | (Ramel et al., 2012; Hou et al., 2016)           |
| N-hydroxy-pipeolic acid     | Inducer of system-acquired resistance                                     | (Chen et al., 2018)                             |
| Diadenosine polyphosphates  | Involved in plant responses to the environment; ‘alarmones’              | (Pietrowska-Borek et al., 2011)                 |
| Catecholamines (e.g. dopamine, norepinephrine, tyramine) | Regulate growth and development. Participate in defence reactions. Important for plant–plant communication | (Kulma and Szopa, 2007; Soares et al., 2014; Ramakrishna and Roshchina, 2018) |
| Serotonin                   | Mediates morphogenesis, vegetative growth, and abiotic and biotic stress survival | (Erland and Saxena, 2017)                       |
| Quercetin/kaempferol        | Regulate auxin transport                                                  | (Yin et al., 2014; Silva-Navas et al., 2016)    |
| 3′5′-cAMP                   | Implicated in the regulation of cell cycle progression                    | (Ehsan et al., 1998; Gehring, 2010; Donaldson et al., 2016) |

### Examples of proteins implicated in signalling with unknown putative ligand

| Protein                              | Function                                                                 | References                                      |
|--------------------------------------|--------------------------------------------------------------------------|-------------------------------------------------|
| Homeodomain–leucine-zipper (HD-Zip) transcription factors containing a putative lipid-binding START domain | 23 members involved in different aspects of plant development (e.g. PROTOGERMAL FACTOR2, GLABRA2, PHABULOSA, PHAVOLUTA, and REVOLUTA) | (Schrick et al., 2014) |
| B2R1-BAM transcription factors containing a β-amylase (BAM)-like domain | BAM7 and BAM8; putative metabolic sensors                              | (Soyk et al., 2014)                             |

### Examples of putative metabolite signals of unknown chemical identity

| Signal                          | Function                                                                 | References                                      |
|---------------------------------|--------------------------------------------------------------------------|-------------------------------------------------|
| Small-molecule component of the Sussex signal | Involved in adaxial/abaxial differentiation; identity speculated. Meristem-derived lipophilic ligand | (Kuhlemeier and Timmermans, 2016) |
| P450 CYP78A5/KLUH-derived signal | Mobile growth factor. Involved in regulation of organ size and regulation of cell proliferation | (Anastasiou et al., 2007)                       |
| Bypass signal                   | Root-to-shoot communication. Mediates growth (cell proliferation) arrest in the shoot apical meristem and interferes with cytokine signalling. Carotenoid derived | (Lee et al., 2016)                             |

**Identification of plant hormone receptors: the power of genetics**

Forward genetic approaches, in which a mutant population is screened for a phenotype of interest followed by mapping of causal mutations, provide an elegant strategy for identifying protein targets of the bioactive small molecules (Fig. 1) (Stockwell, 2000; Zwiewka and Friml, 2012). Because forward genetics depends on the presence of a strong and easy-to-screen phenotype, such approaches are well suited...
Box 3. Biochemical strategies used to study protein–metabolite interactions either *in vivo* or close to *in vivo* conditions (cell-free lysate)

| Method | Experimental concept | Strengths | Limitations | Starting material | Examples and protocols |
|--------|----------------------|-----------|-------------|-------------------|------------------------|
| Small molecule to protein | | | | | |
| Affinity chromatography | Protein affinity towards an immobilized small-molecule ligand | Proteome-wide; generic<sup>a</sup> | Requires small-molecule modification<sup>b</sup>; High rate of false positives | Cell-free lysate | (Ong et al., 2009, 2012; Kosmacz et al., 2018) |
| Stability of proteins from rates of oxidation (SPROX) | Protein susceptibility to oxidation | Proteome-wide; does not require small-molecule modification<sup>a</sup>; generic<sup>a</sup> | Not all binding events affect susceptibility to oxidation (false negatives). Competition with endogenous metabolites<sup>c</sup> | Cell-free lysate | (West et al., 2008; Strickland et al., 2013; Tran et al., 2014) |
| Cellular thermal shift assay (CETSA)/ thermal proteome profiling (TPP) | Protein susceptibility to temperature denaturation | Proteome-wide; does not require small-molecule modification<sup>a</sup>; generic<sup>a</sup> | Not all binding events affect protein stability (false negatives). Competition with endogenous metabolites<sup>c</sup> | Cells (in vivo) and cell-free lysate | (Martinez Molina et al., 2013; Savitski et al., 2014; Huber et al., 2015; Reinhard et al., 2015; Reckzeh et al., 2019) |
| Drug affinity responsive target stability (DARTS)/limited proteolysis-small molecule mapping (LIP-SMap) | Protein susceptibility to proteolysis | Proteome-wide; does not require small-molecule modification<sup>a</sup>; generic<sup>a</sup>; identification of ligand-binding site | Not all binding events affect protein susceptibility to proteolysis (false negatives). Competition with endogenous metabolites<sup>c</sup> | Cell-free lysate | (Lomenick et al., 2009, 2011; Piazza et al., 2018) |
| Capture compounds | Chemical functionalization of a small molecule | Proteome-wide, generic<sup>a</sup>; enables small-molecule visualization<sup>d</sup>; captures transient and weak binding events | Requires small-molecule modification<sup>a</sup>; laborious | Cells (in vivo) and cell-free lysate | (Lenz et al., 2010; Xia and Peng, 2013; Haberkant and Holthuis, 2014) |
| Protein to small molecule | | | | | |
| Tandem affinity purification | Co-purification of epitope-tagged protein in a complex with small-molecule ligands | Metabolome-wide; retrieves both protein (direct and indirect) and small-molecule partners (direct and indirect); generic<sup>a</sup> | High rates of false positives. Requires protein tagging<sup>e</sup> | Cell-free lysate | (Li et al., 2010; Li and Snyder, 2011; Maeda et al., 2013, 2014; Luzarowski et al., 2017, 2018) |
| Untargeted (interactome-wide) | | | | | |
| Protein–metabolite interactions using size separation (PROMIS) | Size separation of small-molecule–protein complexes. Interaction is defined by co-elution | Proteome- and metabolome-wide; does not require small-molecule modification<sup>a</sup> or protein tagging; generic<sup>a</sup>; captures protein–protein and protein–metabolite interactions | Co-elution is an indication but not a lyase proof of interaction | Cell-free lysate | (Veyel et al., 2018) |

<sup>a</sup> Can be used for both drugs and metabolites and across organisms.

<sup>b</sup> Chemical modification may affect protein binding (strength and specificity). Not all compounds can be easily modified.

<sup>c</sup> When used for metabolites, lacks a true ‘no-ligand’ control due to the presence of metabolites in the cellular lysate. Circumvented by an *a priori* filtration step.

<sup>d</sup> For example, through the addition of a fluorescence tag.

<sup>e</sup> Presence of an epitope tag may affect ligand binding (strength and specificity).

General consideration: Methods relying on either proteomic or metabolomic identification are confined to the proteins and metabolites, respectively, that can be accurately detected, quantified, and/or annotated.
Box 4. Examples of experimental workflows for the identification and functional characterization of small-molecule–protein interactions

• **Workflow 1: From small molecule to protein**
  Step 1 (Binding conditions). Single-step size filtration experiments (Veyel *et al.*, 2017) allow fast separation of unbound from protein-bound small molecules and provide an ideal method for finding the best starting material\(^1\) and lysis condition\(^2\) so the metabolite of interest is present in the protein complexes. To determine binding conditions, native cell lysate is prepared using cell-disruption techniques allowing efficient extraction of the complexes (Goldberg, 2008). To separate unbound from protein-bound small molecules, native cell lysate is loaded on to a size filtration unit (e.g. Amicon 10kDa MWCO). Free small molecules are washed through the 10kDa MWCO membrane, whereas protein-bound small molecules remain on the filter. Protein-bound small molecules are detected using metabolomics.

  Step 2 (Target identification). A combination of at least two, and preferably more, independent methods, such as PROMIS, AC/AP, or TPP, is the best strategy for the identification of protein targets with high confidence (Figs 2, 3, and 5) (Kosmacz *et al.*, 2018; Veyel *et al.*, 2018; Reckzeh *et al.*, 2019). For instance, when combining PROMIS, AC/AP, and TPP, the following requirements should be fulfilled: (i) for PROMIS, the small molecule should co-elute with its protein partner; (ii) for AC/AP, the target protein should be significantly enriched after incubation with the small molecule immobilized on the affinity beads; (iii) for TPP, the melting temperature of the target protein should increase upon binding of the small molecule.

  Step 3 (Validation). An important next step in a small-molecule–protein interaction study is validation of the interaction. This is classically done *in vitro* using recombinant protein and biophysical methods, such as microscale thermophoresis, isothermal titration calorimetry, and/or surface plasmon resonance (Peters *et al.*, 2009; Duff *et al.*, 2011; Seidel *et al.*, 2012; Khavrutskii *et al.*, 2013; Jerabek-Willemsen *et al.*, 2014; Levanon *et al.*, 2014; Patching, 2014; Nguyen *et al.*, 2015). Additional *in vivo* validation (e.g. taking advantage of TAP\(^3\)) will considerably strengthen the evidence (Fig. 2A) (Luzarowski *et al.*, 2018).

  Step 4 (Structural analysis). Deciphering the structures of protein–ligand complexes (e.g. using X-ray crystallography) provides crucial information for a functional understanding of the interaction (Turnbull and Emsley, 2013).

  Step 5 (Biological function). In the ideal scenario, the ligand-binding site is mutated, and the phenotypic/physiological and molecular consequences of the abolished interaction are studied *in planta*.

  \(^1\) Plant species, organ identity, developmental stage, environmental conditions.

  \(^2\) Ionic strength, pH, addition of detergent in order to enrich for membrane proteins.

  \(^3\) Based on the assumption that if small molecule A used as a bait will ‘fish out’ protein B, protein B used as a bait will ‘fish out’ metabolite A.

• **Workflow 2: From protein to small molecule**
  Step 1 (Target identification). A combination of independent methods, such as TAP and DRaCALA (Fig. 2A), is the best strategy for identifying small-molecule ligands with high confidence. Steps 2–5 (Validation/Structural analysis/Biological function). As described above.

• **Workflow 3. Identification of novel small-molecule regulators.**
  Step 1 (Reconnaissance/Scouting). Single-step filtration and/or PROMIS enable identification of the protein-bound small molecules specific to, for example, a specific environmental perturbation, developmental stage, or genetic background. These constitute putative metabolite regulators.

  Step 2 (Annotation). Unknown compounds are annotated using a combination of isotope labelling (Giavalisco *et al.*, 2011) and analysis of the fragmentation pattern (Böttcher *et al.*, 2008; Rojas-Chertó *et al.*, 2011; Kueger *et al.*, 2012), and validated using a reference compound (Neumann and Böcker, 2010; Kueger *et al.*, 2012).

  Steps 3–6 (Target identification/Validation/Structural analysis/Biological function). As described above.
for unravelling the identity of plant hormone receptors. For example, brassinosteroid (BR)-deficient mutants exhibit a dwarf phenotype with thick hypocotyls. Li and Chory (1997) screened approximately 80,000 ethyl methanesulfonate (EMS)-mutagenized M2 seedlings and identified 200 BR-deficient mutants. Next, they tested whether the dwarf phenotype associated with BR deficiency could be rescued by treatment with BRs. Of the 200 BR mutants, 18 showed no response to BRs, indicating a mutation in the BR-sensing protein, which was then mapped to BRI1, a plasma membrane-localized leucine-rich repeat receptor kinase (Li and Chory, 1997).

However, forward genetics is not restricted to plant hormones. In a more recent study, Choi et al. (2014) succeeded in identifying the plant receptor responsible for sensing extracellular ATP by screening EMS mutant populations for individuals unresponsive to extracellular ATP signals. The ATP-insensitive dom1 mutant was mapped to a lectin receptor kinase. Binding of ATP to DORN1 triggers an increase in the cytoplasmic calcium concentration followed by the activation of MAPK signalling and expression of stress-related genes. Another example of the application of forward genetics comes from the work of Ranf et al. (2015), which reports the identification of the plant lipopolysaccharide (LPS) receptor, LORE protein, a membrane-localized S-domain receptor kinase. LPS is a bacterial endotoxin, and the LPS–LORE interaction is involved in building up a plant’s innate immunity to Pseudomonas infection.

One of the problems of forward genetics is that it fails to identify a protein receptor if the receptor is part of a large and functionally redundant protein family or if the protein is required for plant survival (Toth and Van der Hoorn, 2010). This limitation can be overcome by combining chemical genetics and forward genetics approaches. In chemical genetics, changes in the phenotype are caused by treatment with chemical compounds rather than by the introduction of mutations (Fig. 1) (Stockwell, 2000; Alaimo et al., 2001; McCourt and Desveaux, 2010; Bjornson et al., 2015). The application of bioactive small molecules allows temporary, fast, and reversible alteration of the phenotype, targeting either a specific part of the signalling pathway or all receptors at once (Rigal et al., 2014). In the past, chemical genetics helped to identify the receptor of the plant hormone abscisic acid (Park et al., 2009), and more recently it aided in the identification of the functions of the individual strigolactone receptors of Striga (Toh et al., 2015).

However, while genetic approaches are elegant, they are also time-consuming, laborious, and their utility is restricted to small molecules that produce easy-to-score phenotypes. Alternative approaches rely on biochemical methods, which enable not only the identification of protein receptors of signalling small molecules but also the reverse—that is, the identification of metabolite binders of the signalling proteins. Moreover, recent methods for the cell-wide characterization of protein–metabolite interactions may be used to establish the identity of hypothesized and novel signalling metabolites.

Biochemical strategies for studying protein–metabolite interactions

Recent progress in mass spectrometry-based proteomics and metabolomics, which has enabled the reliable quantification of thousands of proteins and small molecules in a single sample, has contributed to the increasing number of biochemical methods for studies of protein–metabolite interactions. In simple terms,
biochemical strategies can be divided into two categories: (i) targeted, in which a metabolite or protein is used as bait to retrieve interacting proteins or metabolites, respectively, and (ii) untargeted, which provide a comprehensive image of the protein–metabolite interactome in the cell. Key targeted and untargeted strategies will be introduced here (Box 3).

**From small molecule to protein**

Affinity chromatography/affinity purification (AC/AP) is the oldest of the biochemical approaches described here. It uses compounds chemically coupled to the matrix (e.g. agarose beads) to capture interacting proteins from native cellular lysate (Fig. 2B). Affinity-based methods are still commonly used and are often integrated with protein-labelling strategies to improve protein target discovery (Ong et al., 2009, 2012). A recent example of the successful application of AC/AP is the identification of the interaction between a small-molecule RNA degradation product, 2′,3′-cyclic adenosine monophosphate (cAMP), and the RNA-binding protein Rbp47b. The 2′,3′-cAMP–Rbp47b interaction facilitates stress granule formation in Arabidopsis seedlings subjected to a combination of dark and heat stress (Kosmacz et al., 2018). Unfortunately, because small-molecule immobilization may affect both binding affinity and specificity, AC/AP is not suitable for all compounds. In such cases, alternative approaches such as those described below need to be used. Moreover, AC/AP is prone to high rates of false positives. Non-specific protein binders are excluded by

**Fig. 2.** Mapping targets of a protein or a small molecule of choice using affinity chromatography. (A) To investigate small molecules interacting with a protein of choice, cells expressing a tagged protein or the tag only (empty vector control) are lysed. The cell lysate is incubated with an affinity matrix, to enrich for the tagged protein. The beads are then washed and protein–protein–metabolite complexes are eluted from the beads. Finally, proteins and metabolites are extracted and quantified using mass spectrometry. Empty vector control lines are used to exclude false positives. (B) To identify targets of a small molecule of choice, cell lysate is incubated with the molecule of choice covalently linked to an affinity matrix, or empty beads as a control. The beads are then washed and protein–protein–metabolite complexes are eluted from the beads. Proteins are then extracted and quantified using mass spectrometry. Empty beads are used to exclude false positives.
using negative controls (e.g. empty beads), multiple washing steps with chemically related compounds, and/or elution with increasing ligand concentrations.

The above-mentioned limitations have been addressed by more recent strategies that monitor the changes in protein properties caused by ligand binding, including changes in the rate of oxidation [stability of proteins from rates of oxidation (SPROX)] (West et al., 2008; Strickland et al., 2013; Geer and Fitzgerald, 2016), thermal stability [cellular thermal shift assay (CETSA)/thermal proteome profiling (TPP)] (Martinez Molina et al., 2013; Savitski et al., 2014; Reckzeh et al., 2019), or susceptibility to proteolysis [drug affinity responsive target stability (DARTS)/limited proteolysis–small-molecule mapping (LiP-SMap)] (Fig. 3) (Lomenick et al., 2009, 2011; Piazza et al., 2018). Initially used to monitor drug binding to recombinant proteins (Vedadi et al., 2006), all of these approaches have recently been extended to native cellular lysates, enabling wide-scale analysis of the protein targets of both drugs and metabolites (Tran et al., 2014; Huber et al., 2015; Piazza et al., 2018). In brief, native cellular lysate is incubated with (treatment) or without (no-ligand control) an excess of the ligand under study, followed by either oxidation, heat treatment, or limited proteolysis. Note that in the case of endogenous metabolites, an additional filtration step, in which the native lysate
is passed through a size filtration column to remove free metabolites, is recommended for obtaining a no-ligand control sample. SPROX, CETSA/TPP, and DARTS/LiP-SMap all monitor changes in protein properties caused by ligand binding based on their individual features. For instance, LiP-SMap, in addition to delineating putative protein partners, will also provide information on the ligand-binding site (Piazza et al., 2018), whereas CETSA does not require cell lysis and can be performed on intact cells (Martinez Molina et al., 2013; Savitski et al., 2014). In such cases, equal aliquots of cells, treated by a ligand or a vehicle (as a control), are subjected to temperature gradients. Subsequently, intact cells are subjected to lysis, and the thermal profiles of the proteins in the lysate are assessed (e.g. using proteomics). This is important because, unfortunately, cell lysis may disturb some of the interactions (false negatives) while also resulting in non-specific interactions (false positives) (Evans et al., 2005).

While these techniques have been used in microbial and animal cells for a number of years, SPROX, CETSA/TPP, and DARTS/LiP-SMap have only recently been adapted to plant cells. Published examples in plants include (i) DARTS validation of the interaction between the drug endosidin2 and its protein target, Arabidopsis exocyst complex subunit EXO70 (Zhang et al., 2016), (ii) thermal proteome profiling characterization of the Arabidopsis Mg-ATP interactome (Volkening et al., 2019), and (iii) DARTS validation of the association between the drug endosidin4 and Arabidopsis ADP-ribosylation factor guanine nucleotide exchange factors (ARF-GEFs) (Kania et al., 2018).

An alternative and powerful strategy that circumvents the need for a lysate exploits small-molecule derivatives that, upon binding, covalently label their protein targets (Xia and Peng, 2013; Haberkant and Holthuis, 2014) (Fig. 4). These so-called capture compounds can be designed to have up to three different functionalities. A bioactive compound of interest confers specificity; a reactive group is responsible for covalent attachment of the compound to the proteins; and a sorting group enables ‘click chemistry’-guided attachment of the purification tag so that the compound with the bound proteins can be pulled from a lysate for subsequent analysis (Kolb et al., 2001; Haberkant and Holthuis, 2014; McKay and Finn, 2014). However, similar to affinity-based approaches, chemoproteomic target identification is limited by the ability to synthesize fully potent derivatives of the compound of interest (Haberkant and Holthuis, 2014; Peng et al., 2014; Saliba et al., 2015). Recent examples of the use of capture compounds include the in vivo validation of an interaction between a tomato protein receptor, FLAGELLIN-SENSING3, and flgII-28, a region of bacterial flagellin (Hind et al., 2016), and the identification of numerous novel salicylic acid-binding proteins using the photoreactive salicylic acid analogue 4-AzidoSA (Manohar et al., 2014).

From protein to small molecule

Alongside methods that identify the protein receptor(s) of a pre-selected small molecule, a number of approaches have been developed that enable identification of small-molecule ligands of a protein of choice. Unfortunately, the majority

![Diagram of protein–metabolite interactions](https://example.com/diagram.png)
of these methods rely on the availability of a recombinant protein and exploit compound libraries rather than complex metabolic extracts. Recent examples include the differential radial capillary action of ligand assay (DRAcALA) (Roelofs et al., 2011; Seminara et al., 2019) and ligand-detected nuclear magnetic resonance (NMR) (Pellecchia et al., 2008; Cala et al., 2014).

A unique method that circumvents the above-mentioned limitations and allows metabolome-wide identification of small-molecule interactors in close to in vivo conditions is an adaptation of the AP/tandem affinity purification (TAP) protocol, which is conventionally used to look for protein–protein interactions (Fig. 2A). In brief, the protein of interest is epitope-tagged and expressed in the organism of choice. Protein and metabolite complexes are then immunoprecipitated from native cell lysate using antibodies that are designed to recognize the epitope and are immobilized to the matrix, such as agarose beads (Li et al., 2010). Both proteins and metabolite partners are analysed using a mass spectrometry-based platform. Originally, TAP was used in yeasts to look for lipid binders of proteins ranging from enzymes to kinases (Li et al., 2010; Maeda et al., 2013). More recently, AP and TAP protocols have been adapted to plant cells (Luzarowski et al., 2017, 2018; Dixon and Edwards, 2018). Importantly, the two plant studies demonstrated that AP/TAP could be used to pull out not only lipids but also semi-polar and polar metabolites. It is important to remember that AP/TAP pulls out complete complexes composed of both direct and indirect protein and metabolite interactors. Although it is a powerful approach, AP/TAP is prone to false positives due to the presence of the epitope tag and non-specific binding to the matrix. Non-specific interactors are usually excluded by using multiple negative controls, such as epitope tag–empty vector controls, unrelated proteins, multiple purification steps, and/or subcellular localization filters.

**Untargeted proteome-wide approaches**

Targeted methods constitute an elegant way to identify interactors, but they are limited to either protein or small-molecule bait, and thus they are not suitable for interactome-wide studies. PROMIS (PROtein–Metabolite Interactions using Size separation) is a novel strategy that addresses this limitation and enables proteome- and metabolome-wide analysis of protein–protein and protein–metabolite complexes, starting with a native cell lysate (Fig. 5) (Veyel et al., 2018). In brief, protein–protein and protein–small-molecule complexes are separated by size exclusion chromatography, followed by quantitative metabolomics and proteomics analysis of the obtained fractions. Co-elution is then used to define putative interactors. The main advantages of PROMIS are that it obviates the need for small-molecule or protein modification and that it operates in nearly in vivo concentrations. However, by its nature, co-elution is an indication but not a proof of interaction. On average, every metabolite will co-elute with several hundred proteins, only one of which may constitute a true binder. This is why PROMIS should be seen more as an exploratory approach charting a map of the interactome that must be combined with orthogonal approaches to define the exact composition of the complexes. It is likely that, similar to gene expression studies, the integration of multiple PROMIS datasets will be sufficient to refine interactions.

It should be noted that PROMIS is not the only method that has been developed to study protein–metabolite interactions that exploits co-elution. However, previous methods focused on looking for protein targets of a single, pre-defined drug compound, or looking for small-molecule binders of a single recombinant protein. For instance, drug companies have used size filtration to screen compound libraries for novel ligands by exploiting size differences between free and protein-bound...
compounds. Briefly, a recombinant protein is incubated with a mixture of compounds; non-binding small molecules are separated using a single-step size filtration column and binders are identified using metabolomics (Chen et al., 2015). In addition to size filtration, ion exchange chromatography has also been shown to be capable of separating unbound (free) compounds from those that are in a complex with proteins. The resulting approach was dubbed target identification by chromatographic co-elution (TICC) (Chan et al., 2012). TICC is based on a characteristic shift in the chromatographic elution profile of a compound when it is bound to a protein target. In a proof-of-principle experiment, TICC was used to validate known drug–protein interactions (Kd range micromolar to nanomolar), starting with a native cellular lysate either supplemented with a drug of interest or prepared from drug-treated cells.

In the future, a combination of size filtration with ion exchange chromatography, where protein–metabolite complexes are first separated on the basis of their size and the subsequently obtained fractions (selected based on, for example, the presence of the metabolite of interest) are subjected to further ion exchange separation, may be used for accurate identification of protein binders.

**Future perspectives**

We have presented a brief overview of the biochemical methods that facilitate metabolome- and/or proteome-wide identification of small-molecule–protein interactions. Moreover, and to complement this overview, in Box 4 we outline possible experimental strategies that start with a small molecule or protein of interest or are aimed at the identification of novel small-molecule regulators.

One of the most exciting observations that can be made from the published studies is the unprecedented complexity of the protein–metabolite interactome. Using TAP, Li et al. (2010) found that 70% of the ergosterol biosynthetic enzymes and, remarkably, 20% of the 103 tested yeast protein kinases bound lipid molecules, with many of the interactions being unexpected and of a regulatory nature. Analogously, a LiP-SMap analysis (in Escherichia coli) of just 20 metabolites, comprising amino acids, cofactors, and sugar phosphates, resulted in a network comprising 1678 interactions, of which more than 1400 were novel (Lomenick et al., 2009; Piazza et al., 2018). Finally, PROMIS analysis of Arabidopsis cell cultures revealed as many as 4229 unique metabolic features eluting in the protein–containing fractions, displaying one or several discrete peaks across the separation range, indicating the presence of thousands of novel binding events (Veyel et al., 2018).

Overall, these results demonstrate the complexity of the small metabolite–protein interactome, which occurs in both prokaryotes and eukaryotes, and advocate for an extensive, yet under-studied, role of metabolites in the regulation of protein activities. As in any biological network, some metabolites are expected to act globally and control many proteins, whereas others will act more specifically and target a limited number of proteins. Conversely, while some proteins will have tens of small-molecule binders, others may have none or just a few.

The big emerging question concerns the functionality of the detected interactions. Assuming that all of the identified interactions are true, are they all functional? Are some of the interactions merely a result of chemical similarities and the limited specificity of the protein–binding pockets? Testing the biological role of the identified interactions is often not a trivial task, as it requires a combination of structural biology, biochemistry, and, most of all, genetics approaches. Ligand binding can have multiple consequences, ranging from altering protein activity to changing a protein’s affinity towards its protein partners, as in the case of the recently described binding of trehalose-6-phosphate to KIN10 kinase (Zhai et al., 2018).

Another highly interesting aspect of interactome studies is the extent of evolutionary conservation between protein–metabolite interaction networks. For instance, many signalling compounds are shared between animals and plants. Good examples include neurologically active compounds, such as dopamine, serotonin, and glutamine, which appear to bind and thus engage a different set of regulators (Soares et al., 2014; Roshchina, 2016; Erland and Saxena, 2017; Ramakrishna and Roshchina, 2018).

In summary, we expect that advances in biochemical and mass spectrometry methods will result in a rapid increase in the number of identified protein–small-molecule interactions. This will be followed by the further development of experimental approaches aimed at the structural and functional characterization of these interactions, with major consequences for our understanding of cellular functions, as well as technological advances in terms of drug and agrochemical discoveries.

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