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Special Focus Issue – Mini Review

Probing Plant Receptor Kinase Functions with Labeled Ligands

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Plant cells rely on numerous membrane-localized receptor kinases for communication with each other as well as with the environment to ensure coordinated growth, development and survival. Additionally, several families of small secreted peptides have been identified in plants that possibly regulate various developmental processes through activation of specific membrane receptor kinases. In research efforts to elucidate the underlying signaling processes in plants, it has become obvious that knowledge of both the extracellular ligand and its corresponding receptor(s) is a prerequisite to understand cell communication. Of more than 1,000 orphan peptides in plants, only a few have been functionally characterized and paired with their respective receptors. Here, we discuss recent advances made in the field of receptor–ligand pairing and visualization in plant cells by means of chemically labeled ligands. We summarize and compare various chemical strategies from the mammalian field that can be employed to extend our understanding of the plant receptor kinase functions in plants.

Keywords: Chemical cross-linking • Ligands • Receptor kinases • Signaling peptides • Synthetic peptides.

Abbreviations: AFCS, Alexa Fluor 647–castasterone; ASA, 4-azidosalicylic acid; BR, brassinosteroid; BG, *O*-6-benzylguanine; CEP, C-terminally encoded peptide; CLE, CLAVATA3/EMBRYO SURROUNDING REGION-RELATED; CLV, CLAVATA; FITC, fluorescein isothiocyanate; flg22, flagellin 22; FLS2, FLAGELLIN-SENSING2; LRC, ligand-based receptor-capture; PAL, photoaffinity labeling; RGF, root meristem growth factor; PPR, pattern recognition receptor; RK, receptor kinase; TAMRA, 5′-carboxytetramethylrhodamine; UAA, unnatural amino acid.

Introduction

Plants, being sessile, use a battery of cell surface receptor kinases (RKs) to sense and respond properly to environmental changes to regulate development or orchestrate defense responses. Plant RKs consist of an N-terminal extracellular ligand-binding domain, a single membrane-spanning domain and an intracellular C-terminal kinase domain that conveys downstream signals through phosphorylation. Ligand binding at the cell surface leads to RK dimerization, *trans*-autophosphorylation and signaling activation (Hohmann et al. 2017). In mammals, receptor-tyrosine kinases perceive growth regulators, Toll-like receptors detect microbial molecular patterns to activate innate immunity and the G protein-coupled receptor superfamily responds to chemical signals, whereas in plants a single RK superfamily is functionally equivalent to all these mammalian RKs (Zhou and Yang 2016). RKs act as central regulators of hormone perception, growth, development, disease resistance and responses to abiotic stresses (Hohmann et al. 2017, Kimura et al. 2017, Tang et al. 2017, Wolf 2017, Oh et al. 2018). Notably, the *Arabidopsis thaliana* genome encodes >600 RK homologs that constitute one of the largest gene families, representing 2.5% of the total protein-coding genes in Arabidopsis (Shiu and Bleecker 2001). Since the discovery of plant RLKs (Walker and Zhang 1990), concerted efforts have been made to understand their function (Hohmann et al. 2017), but the function of only a handful of RKs is known. In perspective, Arabidopsis codes for >1,000 secreted peptides that could possibly be perceived by these 600 putative RKs (Shiu and Bleecker 2001, Lease and Walker 2006). Like the RKs, these secreted peptides play an essential role in plant growth, development and responses to the environment (Butenko and Aalen 2012, Murphy et al. 2012, De Coninck and De Smet 2016, Oh et al. 2018). As only a few receptor-peptide ligand pairs have been matched (Oh et al. 2018), the identification of the remaining receptor-ligand couples is highly pertinent to understand fully the RK-mediated signaling in plants.

Highly sophisticated scientific tools have enabled biologists to make unprecedented progress in the field of plant biology. Powerful genetics coupled with the use of molecular probes and advanced microscopy provide a real-time description of molecular events in living cells. However, for the discovery of new biological functions, the genetic perturbation of a protein function is still fundamental to generate observable phenotypes. By means of genetic approaches, several peptide–ligand pairs have been identified (Endo et al. 2014). One recent example is the matching of the two diffusible peptides, designated CASPARIAN STRIP INTEGRITY FACTORS1/2 as ligands of the leucine-rich repeat RK SCHENGEN3/GASSHO1, that regulates lignification of the Casparian strip (Doblas et al. 2017, Nakayama et al. 2017). Such studies are often hampered by gene redundancy and low RK and peptide expression in specific cells or during specific developmental stages, hence not always generating scorable phenotypes. Furthermore, poor solubility of membrane receptors as well as unstable transient RK–ligand interactions create experimental hurdles for classical approaches, such as affinity purification and yeast two-hybrid

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Table 1 Available chemical tools to study receptor–ligand functions

Chemical labeling	Label ligand	Receptor–ligand pair	Model system	*In vivo* cell imaging	Reference
Photoaffinity	Biotin-tagged photoaffinity –castasterone (BPCS)	BRI1–BR	Arabidopsis	No	Kinoshita et al. (2005)
	[125I]ASA–PSK	PSKR–PSK	Arabidopsis	No	Matsubayashi and Sakagami (2000); Matsubayashi et al. (2002)
	[125I]Pep1	PEPR1–Pep1	Arabidopsis	No	Yamaguchi et al. (2006)
	[125I]ASA–RGF1	RGFR1/2/3–RGF	Arabidopsis	No	Shinohara et al. (2016)
	[125I]ASA–CEP1	CEPR1/2–CEP1	Arabidopsis	No	Tabata et al. (2014)
	[125I]ASA–[Ara3]CLV3	BAM1/CLV1–CLV3	Arabidopsis	No	Shinohara and Matsubayashi (2015)
	[125I]ASA–CIF1	GSO1/SGN3/GSO2–CIF	Arabidopsis	No	Nakayama et al. (2017)
Chemiluminescence	Acri-flg22	FLS2–flg22	Arabidopsis	No	Chinchilla et al. (2006)
	Acri-PIPPo	HAE/HSL2–PIPPo	Arabidopsis	No	Stenvik et al. (2008)
Biotin	Biotin–flg22	FLS2–flg22	Rice	No	Shinya et al. (2010)
Fluorescence	AFCS	BRI1–BR	Arabidopsis	Yes	Irani et al. (2012)
	TAMRA-Pep1	PEPR1/2–Pep1	Arabidopsis	Yes	Ortiz-Morea et al. (2016)
	TAMRA-flg22	FLS2–flg22	Arabidopsis	Yes	Mbengue et al. (2016); Jelenska et al. (2017)
Self-labeling	Halo-tag	HAE/HSL2–PIPPo	Arabidopsis	No	Butenko et al. (2014)
	SNAP-tag	G protein-coupled receptors	Human cells	No	Comps-Agrar et al. (2011)
Ligand-based receptor-capture	TriCEPS	Insulin and transferrin receptors	Human cells	No	Frei et al. (2012); Frei et al. (2013)
	ASB	Insulin and transferrin receptors	Human cells	No	Tremblay and Hill (2017)

screens (Uebler and Dresselhaus 2014). Moreover, structural data of various RKs in the past few years have facilitated the modeling of RK–ligand interactions, subsequent receptor activation and receptor interactions with downstream signaling cascades (Bojar et al. 2014, Morita et al. 2016, Zhang et al. 2016a, Zhang et al. 2016b, Hohmann et al. 2017). Nonetheless, because of the limited success of the above-described approaches, several chemical tools and probes are used to dissect the interaction and functioning of RK–ligand pairings in the cellular milieu (Smith and Collins 2015, Ortiz-Morea et al. 2016, Jelenska et al. 2017). Synthetic chemistry has generated diverse chemical compounds, including agonists and antagonists, and tagged/labeled analogs of important phytohormones, such as brassinosteroids (BRs) (Lv et al. 2014, Malachowska-Ugarte et al. 2015, Winne et al. 2017) and small signaling peptides (Mbengue et al. 2016, Ortiz-Morea et al. 2016, Jelenska et al. 2017) that can be used to provide key insights into many biological processes, such as RK signaling. This review focuses on the use of chemically labeled ligands (**Table 1**) to probe the function of RKs in plants and highlights some of the successes as well as limitations of this approach.

Chemical Cross-Linking Approaches for Receptor Fishing

Photoaffinity labeling (PAL) is a powerful technique used to study protein–protein interactions, to identify unknown receptor-ligand pairs and to detect new or alternative binding sites in proteins (Smith and Collins 2015) (**Fig. 1A**). In this method, the ligand is covalently modified with a photoreactive group that upon irradiation produces a reactive species that covalently cross-links the ligand to its target macromolecules. The tag used for the target identification can be a fluorescent dye, a radioisotope or a partner for a specific binding event, such as, biotin–avidin. Typically, PAL has been used to decipher the very elusive perception of the phytohormones, BRs. By means of the photoreactive analog of castesterone, a bioactive BR, not only was the binding with the putative receptor BRASSINOSTEROID INSENSITIVE1 (BRI1) confirmed, but the binding domain on BRI1 was also mapped (Kinoshita et al. 2005). Other examples are the identification of the phytosulfokine receptor–ligand and the PEP1 receptor1 (PEPR1)–ligand pairs (Matsubayashi and Sakagami 2000, Matsubayashi et al. 2002, Yamaguchi et al. 2006). With a recently adapted PAL, a high-throughput screening of a custom-made RK expression library of 88 members was carried out to identify the receptors of the hormonal peptides, C-terminally encoded peptide (CEP) and the root meristem growth factor (RGF) in Arabidopsis (Tabata et al. 2014, Shinohara et al. 2016). The 125I-labeled photoreactive 4-azidosalicylic acid (ASA) fused to RGF1 was synthesized without compromising the optimal biological activity and was used for the screen. Competitive displacement of the bound [125I]ASA–RGF1 by an excess of unlabeled ligand validated that RGF1 specifically interacted with RGF RECEPTOR1 (RGFR1), RGFR2 and RGFR3. Furthermore, the triple *rgfr* mutant was insensitive to the exogenous RGF peptide, hence confirming that the three RKs perceive the RGF peptides in the proximal meristem (Shinohara et al. 2016). Moreover, PAL led to the identification and characterization of the receptors for the Arabidopsis CEP that was derivatized to the photoreactive form [125I]ASA–CEP1 with a biological activity comparable with that of the native CEP1. The [125I]ASA–CEP1 photolabeled two related RKs, designated CEP RECEPTOR1 (CEPR1) and CEPR2, and the double loss-of-function *cepr1cepr2* mutant was insensitive to CEP1 (Tabata et al. 2014). The receptors for the Casparian strip integrity

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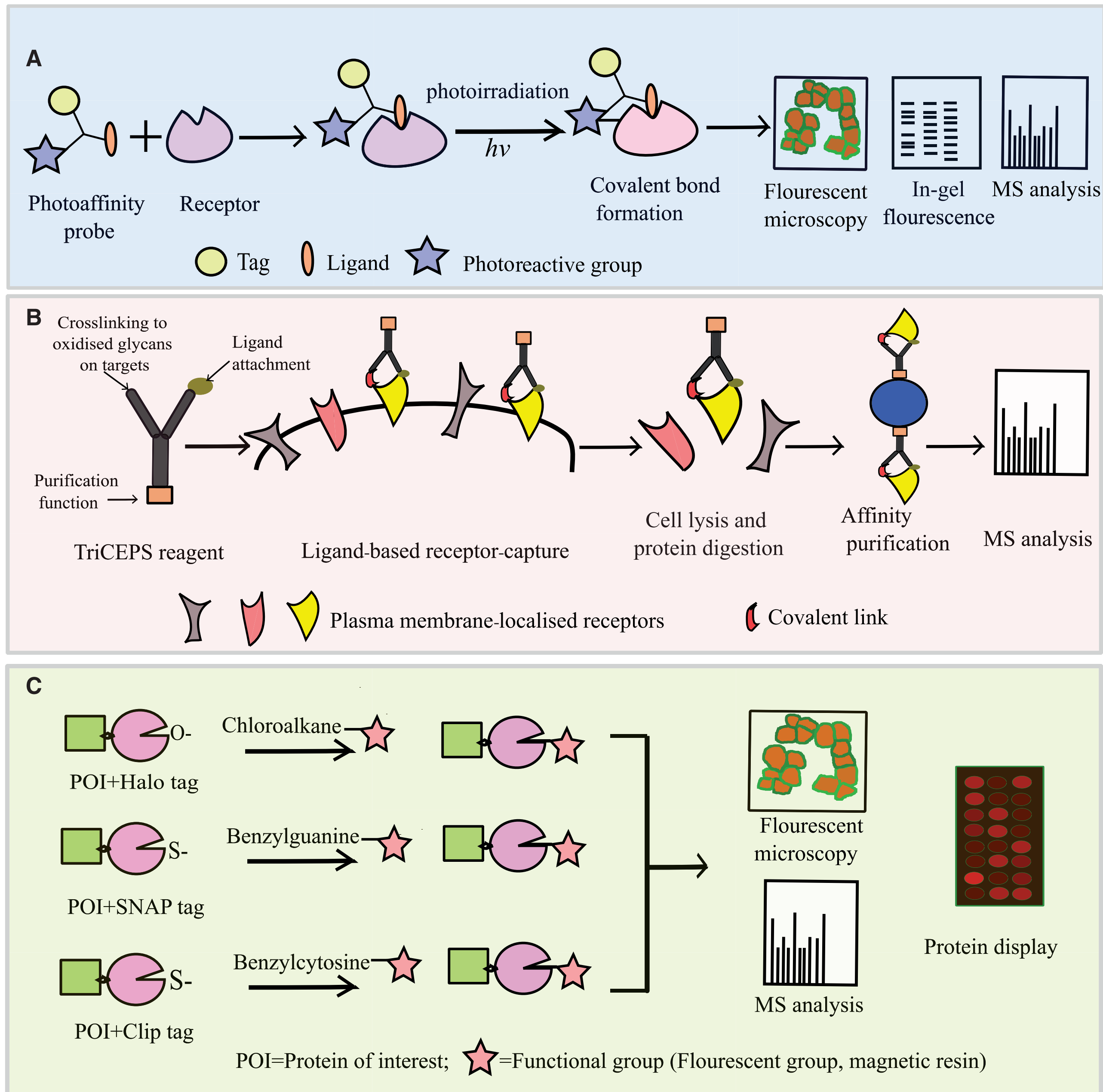

Fig. 1 Ligand labeling techniques used for receptor–ligand pairing. (A) Photoaffinity labeling. A typical photoaffinity labeling (PAL) experiment starts from synthesis of the photoaffinity probe formed by linking the ligand with a suitable photoreactive group and a suitable reporter tag (e.g. fluorescent dye, biotin, etc.). The sample is treated with an excess of photolabeled probe, followed by irradiation with UV light, leading to covalent cross-linking between the probe and target protein. The captured protein is then purified by affinity purification and can be further analyzed by mass spectrometry (MS). Depending on the reporter tag, the captured protein can also be utilized for other applications, such as fluorescent imaging and in-gel fluorescence. (B) Ligand-based receptor-capture technology enabled by TriCEPS reagent. The TriCEPS reagent contains an *N*-hydroxysuccinimide ester for coupling with the ligand, a hydrazine group to react with aldehydes introduced into carbohydrates of the receptors by oxidation and a biotin for affinity purification of the receptor-ligand complex. Aldehydes are introduced into the oligosaccharide chains of cell surface glycoproteins by gentle oxidation, and the oxidized sample is incubated with the ligand-coupled TriCEPS. The ligand binds to its receptor, and the hydrazine group of the TriCEPS forms a covalent link to the carbohydrate aldehyde on the receptor. The targeted glycoproteins can be identified by affinity purification with the biotin group of TriCEPS, followed by MS. (C) Self-labeling tags. Proteins of interest are tagged with the genetically encoded markers HaloTag, SNAP-tag or CLIP-tag, and the fusion protein is expressed in the cells. The self-labeling reaction is carried out by incubation with an appropriate substrate (chloroalkane, benzylguanine and benzylcytosine, respectively) fused to a functional group leading to an irreversible covalent labeling of the tagged protein with a synthetic probe. The tagged protein can then be utilized for research, such as protein isolation and purification, protein visualization and function, and molecular interactions.

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factor (CIF) were also identified by high-throughput screening with PAL using the RK expression library (Nakayama et al. 2017).

Besides identification of receptor–ligand pairs, PAL has been utilized to differentiate between direct receptor–ligand binding and indirect ligand–co-receptor interaction. *CLAVATA3* (*CLV3*) encodes a small secreted peptide that is specifically expressed in the shoot apical meristem to regulate the transcription factor WUSCHEL and to maintain the balance between stem cell differentiation and division (Somssich et al. 2016). The CLV3 peptide is recognized by four membrane-localized receptors, namely CLV1, CLV2, CORYNE and the RECEPTOR-LIKE PROTEIN KINASE2 (RPK2). Although the binding of CLV3 to CLV1 had been established, whether the CLV3 peptide bound directly to CLV2 or RPK2 remained ambiguous. With the PAL technique, the binding of CLV3 to CLV2 and RPK2 was re-evaluated (Shinohara and Matsubayashi 2015). The arabinosylated CLV3 ([Ara3]CLV3) peptide was derivatized with the ^{125}I-labeled photoreactive ASA to produce [^{125}I]ASA–[Ara3]CLV3, of which the biological activity was similar to that of [Ara3]CLV3. The ligand cross-linking activity of [^{125}I]ASA–[Ara3]CLV3 was confirmed by a positive PAL in the microsomal fractions of Bright Yellow-2 (BY-2) tobacco (*Nicotiana tabacum*) cells overproducing CLV1-HaloTag (HT), but not CLV2-HT and RPK2-HT, implying that CLV2 and RPK2 do not bind [Ara3]CLV3 and that they may act as co-receptors in CLV3 signaling. With the same PAL strategy, one member of the BARELY ANY MERISTEM (BAM) RK family, a close paralog of CLV1, was identified as a direct receptor of CLV3. As the *clv1bam1* double mutant was insensitive to exogenous CLV3, CLV1 and BAM1 were sufficient for the recognition of the CLV3 signaling in the shoot apical meristem (Shinohara and Matsubayashi 2015). Recently, PAL has been coupled with the innovative technology of engineering synthetic bifunctional CLAVATA3/EMBRYO SURROUNDING REGION-RELATED (CLE) peptides (Hirakawa et al. 2017). These peptides were synthesized by careful swapping of the pre-existing variation in the natural CLE peptide subfamily that exhibits activities beyond the original peptides by interacting with multiple receptors. Binding of the bifunctional CLE peptide to CLV1 and the TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF) receptor was confirmed by PAL (Hirakawa et al. 2017). The synthetic peptide led to the functional elucidation of the specific residues of the parent peptide necessary for the activity. Such hybrid peptides could be used as a powerful tool to exploit the natural genetic diversity, to program artificial cell signaling and to be instrumental in the production of new valuable plant species.

Nonetheless, several technical problems are associated with the use of radiolabeled peptides, namely they are expensive, have a short half-life and require special facilities and optimal handling to avoid potential hazards (Shinya et al. 2010). To overcome these shortcomings, other synthetically labeled peptides can be utilized. Acridinium esters have been applied to form a non-radioactive and cost-effective chemiluminescent label that rapidly degrades in the presence of hydrogen peroxide with the concomitant emission of light that allows the quantification of the label at attomolar amounts in a luminometer (Butenko et al. 2014). The robustness of this label was first checked by establishing the binding of the well-known FLAGELLIN-SENSING2 (FLS2)-flagellin 22 (flg22) interaction in tomato (*Solanum lycopersicum*) cells (Meindl et al. 2000, Chinchilla et al. 2006) with the acridinium–flg22 fusion. Further, the acridinium label functionality was recognized by the binding of the acridinum-conjugated peptide PIPPo, a member of the INFLORESCENCE DEFCIENT IN ABSCISSION (IDA) peptide family, with the RK HAESA (HAE) and HAESA-LIKE2 (HSL2) that control floral organ abscission in Arabidopsis (Stenvik et al. 2008, Butenko et al. 2014). These acridinium-labeled synthetic peptides were key elements in the development of bioassays for matching receptor–ligand pairing. Synthetic peptides were used to elicit the ectopically expressed receptors in *Nicotiana benthamiana* and the generated oxidative burst response was recorded as a read-out in the IDA peptide HAE/HSL2 receptor system (Butenko et al. 2014). This sensitive non-radioactive methodology could reveal a direct correlation between peptide activity and receptor affinity. However, the prior knowledge of potential receptors and the required generation of oxidative burst responses from the receptor–ligand pairing seem to restrict the use of this technique to universal receptor–ligand pairing analysis.

Alternatively, an option could be affinity cross-linking of biotinylated ligands for the identification of their receptors. Without radioactive compounds, not only are biotinylated ligands convenient to use, but also biotin-tagged receptors can be purified by a simple affinity purification. To demonstrate the assay functionality, the receptor molecule was purified with the FLS2-flg22 interaction as a model system with biotinylated flg22 and *FLS2*-overexpressing transgenic rice (*Oryza sativa*) cells (Shinya et al. 2010). However, chemical biotinylation often yields heterogeneous products that result in an impaired function. An attractive alternative is the exploitation of enzymatic biotinylation by means of the *Escherichia coli* biotin ligase (BirA), which is highly specific and conjugates one single biotin on a 15-amino-acid peptide tag, designated an AviTag peptide. The AviTag can conveniently be added genetically at different positions of the target protein (Fairhead and Howarth 2015). Still, these chemical cross-linking techniques do not take into account the holistic view of the ligand–receptor interactions that depends on the protein complex formed by co-receptors and/or interacting proteins. Large-scale applications of these technologies to study plant RK biology are yet to be seen.

Dissection of the Intracellular Dynamics of Receptor–Ligand Complexes by Fluorescently Labeled Ligands

Fluorescent ligands are important tools for probing the spatiotemporal dynamics of receptor-mediated signaling in living cells. They have a broad utility spectrum because they can be applied to several plant species in a reversible manner. For the first time in plants, the endocytic route of receptor–ligand complexes was mapped in living cells and the interplay between

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trafficking and BR signaling was assessed by means of Alexa Fluor 647–castasterone (AFCS), a bioactive fluorescently labeled BR analog (Irani et al. 2012, Winne et al. 2017). The AFCS internalization pattern in the BR receptor-null and overexpressing mutants revealed that BR receptors mediate the AFCS internalization. Disruption of endocytosis, either pharmacologically or genetically, prevented the AFCS uptake, suggesting that the receptor-AFCS complexes follow a conserved clathrin-mediated endocytosis. These results highlight the usefulness of fluorescently labeled ligands as a tool to investigate hormone transport, real-time visualization and trafficking of receptor-ligand complexes (Irani et al. 2012). Although several other fluorescently tagged BR derivatives are available (Raichenok et al. 2012, Lv et al. 2014), they all share the drawback that they are derived from a covalent binding between functional group(s) of BRs and that some properties of the resultant fluorescent biomolecule differ from those of the parent molecule. Another class of fluorescent BRs utilizes the BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) dyes that are attached to BRs through the amidation of the terminal carboxylic group with amine (Malachowska-Ugarte et al. 2015). Even though the BR–BODIPY conjugates retain all functional BR groups and strongly cross-react to antibodies raised against BRs, their activity in plants and their direct binding to the BR receptors remains to be tested.

Recently, exogenous fluorescently labeled peptides have been used to track endocytosis of different RKs in Arabidopsis cells (Mbengue et al. 2016, Ortiz-Morea et al. 2016, Jelenska et al. 2017). The 23-amino-acid-long peptide *At*Pep1 was conjugated to 5′-carboxytetramethylrhodamine (5′-TAMRA) at the N-terminus to generate the biologically active TAMRA–pep1. Live-cell imaging revealed that TAMRA–pep1 and its receptor PEPR1 co-internalized in epidermal root cells of Arabidopsis and the intracellular accumulation of TAMRA–pep1 required clathrin (Ortiz-Morea et al. 2016). Similar observations were made with the TAMRA-labeled fragment of bacterial flg22 that binds the FLS2 receptor (Mbengue et al. 2016, Jelenska et al. 2017). In epidermal leaf cells, TAMRA–flg22 co-localized with FLS2–green fluorescent protein (GFP) in the plasma membrane and mobile vesicles, demonstrating that the ligand-bound FLS2 undergoes endocytosis. TAMRA–flg22 was also used to establish that entry into the plant cell via endocytosis together with the FLS2 receptor is needed for the flg22 delivery to distal plant organs following vascular connections (Jelenska et al. 2017). Nevertheless, a serious drawback of these fluorescence-based ligands is the fluorescence quenching that often results from conformational changes in the fluorophore-conjugated ligand after binding to the receptor or from modifications in the oligomeric state of the ligand. In an effort to fine-tune the labeling process, a method has been devised that uses fluorescein isothiocyanate (FITC)-labeled cell surface proteins (Breen et al. 2016). Extensive proteolysis of the FITC–protein has been shown to eliminate fluorescence quenching and allows comparison of the fluorescence of the sample under study with that of a known concentration of the proteolyzed FITC–protein for quantitative analysis of the cell surface receptors.

What Can We Learn from the Mammalian Systems?

Several innovative technologies that enable the unbiased detection of ligand–receptor pairing in mammalian systems are underway. For example, ligand-based receptor-capture (LRC) allows the selective identification of cell surface glycosylated receptors for a given ligand (Frei et al. 2013) (**Fig. 1B**). This technology utilizes the chemoproteomic reagent TriCEPS, that consists of three independent functionalities, an ester moiety for conjugation to a free ligand-containing amino group, a hydrazine moiety for binding to *N*-glycosylated receptors and a biotin tag to allow affinity purification of the captured glycopeptides for quantitative mass spectrometry (MS). The LRC-TriCEPS technology has been validated for the insulin, transferrin, apelin and epidermal growth factor receptors (Frei et al. 2012, 2013). The usefulness of the TriCEPS method, however, is limited, because it can only detect *N*-glycosylated receptors, and the nature of the receptors is unknown beforehand. The TriCEPS method has been improved by the development of the heterotrifunctional label transfer reagent designated ASB, which combines an aldehyde-reactive aminooxy (A) group for cross-linking to cell surface receptors, a sulfhydryl (S) group forming disulfide bonds between ligands and cross-linkers, and a biotin (B) allowing MS to quantify streptavidin-bound proteins (Tremblay and Hill 2017). Until now, these methods have not been applied to samples of plant origin, but it will be interesting to see whether similar methods could be adapted for large-scale LRC in plants.

A key element in understanding the function of membrane proteins, including RKs, is the visualization of their localization and trafficking routes in living cells. For the past two decades, proteins have been fused to fluorescent tags. More recently, in mammalian cells, the protein of interest is coupled to a self-labeling tag molecule that covalently reacts with a small molecule fluorophore-containing substrate. The most commonly used self-labeling enzyme tags are the SNAP(f), CLIP(f) and Halo tags (Keppler et al. 2003, Gautier et al. 2008, Los et al. 2008) (**Fig. 1C**). The HaloTag is a haloalkane dehalogenase and reacts with primary alkylhalides, whereas SNAP and CLIP tags are variants of the *O*-6-alkylguanine-DNA alkyltransferase and react with *O*-6-benzylguanine (BG) and *O*-2-benzylcytosine derivatives, respectively (Liss et al. 2016). These self-labeling enzyme tags are genetically fused to the protein of interest and react covalently with an exogenous substrate that is linked to a fluorescent dye in an irreversible manner. In addition to their specificity and prospect for fast labeling reactions, these techniques possess the advantage that the reactive group that covalently binds to the tag can be bound to a wide variety of fluorophores (and other molecules, such as affinity tags). As a result of this chemical flexibility, a broad range of experiments that require different protein labeling can be carried out by simply changing the substrate (Thorn 2017).

The major drawbacks are the added complexity by the use of an external fluorescent substrate and the required washing to reduce the background. For example, in SNAP-tag probes for the study of the endocytosis and recycling of cell surface

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proteins, including RKs, the covalent nature of the BG binding to the SNAP tag hinders the removal of excess probes from the cell surface to reveal the intracellular endocytosed protein pool. However, the SNAP-tag system has been modified to introduce a cleavable disulfide bond between the BG moiety and the fluorophore, thus allowing the splitting of the excess fluorescently labeled probes of the cell surface by a cell-permeable reducing agent without affecting the population of internalized BG-labeled SNAP-tag fusion proteins sequestered within the endosomes (Cole and Donaldson 2012).

Another drawback of all the enzyme labeling techniques mentioned above is the mass increase of the enzyme–protein fusion of approximately 18–33 kDa that possibly hinders the protein function and impairs the protein–ligand interaction. Alternatively, the smallest tags available are the fluorescent unnatural amino acids (UAAs) that can be incorporated into proteins during translation, as done in a neuroreceptor of a *Xenopus* oocyte (Rodriguez et al. 2006). These protein-linked UAAs provide exciting opportunities to study proteins as either spectroscopic probes, UV-inducible cross-linkers or functional groups for bioorthogonal conjugations or post-translational modifications (Neumann 2012). However, the establishment and incorporation of UAAs in proteins of interest is still very challenging even in mammalian cells due to the low labeling efficiency and inefficient membrane permeability. This technique has still not been adopted in plants. Another approach, that needs to be applied to plant RKs, is the peptide-based recognition tags for protein labeling, in which a 0.6–6 kDa small-peptide tag is attached to the protein with the flexibility to introduce arbitrary reporters to facilitate routine and high-throughput analyses of RKs (Lotze et al. 2016).

Future Perspectives

All of the above-described methods for receptor-ligand pairing have strengths and limitations, and therefore combinatorial approaches using self-labeling tags, reagents, such as TriCEPS/ASB, along with affinity purifications with cross-linkers could be promising options to elucidate fully the highly complex nature of RK–ligand interactions in plants. However, the widespread application of such techniques in cells with cell walls, such as plants, fungi and bacteria, has not been achieved, because the permeability of extrinsic fluorophores is an issue. Nonetheless, among self-labeling tags, the HaloTag technology has been shown to be applicable in different types of intact plant cells (Lang et al. 2006). Constant efforts are made to develop new tags and fluorophores with stronger signal, sensitivity and wider application to different organisms. It is pertinent to mention here that small structural changes in a ligand could have a tremendous effect on its binding affinity for the receptor, its permeability, transport, diffusion rate as well as its modifications by the metabolic machinery. Hence, a close collaboration between chemistry and plant biology is vital to produce new chemical tools that can be employed to enhance our understanding of plant RK signaling.

Bioinformatics-based approaches offer an alternative avenue for the exploration of ligand–receptor interactions, as they are relatively fast and offer user-friendly in silico screenings. Molecular docking methodologies are of great importance in receptor–ligand matching studies, and the advent of the state-of-the-art docking algorithms has increased the correct prediction of the structure and the binding energy of a receptor–ligand complex (Kooistra et al. 2016, Smith et al. 2016). Moreover, because the crystal structures of several receptor proteins are available, the opportunities in structure-based ligand discoveries are enormous. Structural studies point toward the conserved receptor binding and activation mechanisms of several peptide hormones. Thus, this knowledge can be utilized to match peptide–RK pairs and to elucidate their activation mechanism (Han et al. 2014, Zhang et al. 2016a, Zhang et al. 2016b). However, in the structural biology of membrane proteins, the hurdles are associated with the overexpression and stability of plant membrane proteins due to their inherent nature. Nevertheless, these virtual screening methodologies can be complemented with the chemical tools described above to confirm the hits obtained from the structure-based studies. Hence, scientists can fully appreciate and exploit the potentials of predictive computational tools to study receptor–ligand interactions. The ongoing development of innovative methodologies complemented with the efforts to revisit the established techniques offer a broad spectrum of approaches to match several orphan peptides to their receptors and to elucidate the functions of RK–peptide complexes.

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