Topical Review on Protein-Protein Interaction Techniques

Techniques for the Analysis of Protein-Protein Interactions in Vivo\[OPEN\]

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Identifying key players and their interactions is fundamental for understanding biochemical mechanisms at the molecular level. The ever-increasing number of alternative ways to detect protein-protein interactions (PPIs) speaks volumes about the creativity of scientists in hunting for the optimal technique. PPIs derived from single experiments or high-throughput screens enable the decoding of binary interactions, the building of large-scale interaction maps of single organisms, and the establishment of cross-species networks. This review provides a historical view of the development of PPI technology over the past three decades, particularly focusing on in vivo PPI techniques that are inexpensive to perform and/or easy to implement in a state-of-the-art molecular biology laboratory. Special emphasis is given to their feasibility and application for plant biology as well as recent improvements or additions to these established techniques. The biology behind each method and its advantages and disadvantages are discussed in detail, as are the design, execution, and evaluation of PPI analysis. We also aim to raise awareness about the technological considerations and the inherent flaws of these methods, which may have an impact on the biological interpretation of PPIs. Ultimately, we hope this review serves as a useful reference when choosing the most suitable PPI technique.

From protein synthesis, maturation, and degradation to vesicle budding, trafficking, and fusion and from receptor dimerization, signaling cascades, and gene regulation to metabolism and catabolism, almost all cellular functions depend on and are executed by complex protein-protein interactions (PPIs). While complete biochemical pathways and even some protein complexes involved in these processes had been deciphered by the 1960s, the knowledge about PPIs at that time was limited. Owing to the fledgling state of molecular biology in those days, methods for studying PPIs and the development of such techniques have emerged only slowly. The advent of gene fusion and protein labeling, together with the engineering of fluorescent probes covering the entire spectrum of light, provided the initial spark for a technological revolution in the study of PPIs (Braun and Gingras, 2012).

In this Topical Review, we provide an overview of current tried-and-tested-tools for investigating the mechanistic relationships between potential protein partners that are easily accessible to a plant scientist working in a standard molecular biology laboratory. Each of these methods comes with its own set of benefits and drawbacks. A detailed introduction to the biology underlying each method, novel improvements, and potential pitfalls are included, allowing readers to choose the most suitable method for their research. Some actual examples from the plant sciences have been included, but a detailed discussion of the biological questions that were addressed has been omitted, as the techniques take center stage in this review.

In order to help researchers decide which method is most suitable, we summarize the main properties and issues inherent to each technique featured in this review (Table I; Fig. 1). In general, the following points should be considered when designing a PPI analysis method.

1. Technology. Each PPI technique utilizes a unique concept for combining probes and reporting interactions. The mode of action of a PPI technique can influence the likelihood of a positive readout of a given protein couple as well as its interpretation.

2. Interaction dynamics. Interactions between proteins can be weak or strong, transient or stable, non-permanent or permanent. Kinetics, thermodynamics, stoichiometry, and cofactors can affect a PPI and therefore are important factors to consider prior to PPI analysis and when choosing an appropriate detection system.

3. Environmental conditions. Non-native environments (e.g. yeast) can severely affect the expression, translation, subcellular localization, posttranslational modifications, lifetime, and turnover of a protein and therefore may preclude positive interaction analysis. Verifying protein expression by immunoblot analysis is imperative, especially when a negative interaction is reported.
(4) Expression control. The overexpression of a protein might be necessary to enable the detection of an otherwise weak or transient interaction, but it also might lead to artificial results. Balancing expression through induction or repression is crucial for meaningful analysis.

(5) Reporter output. Information about an interaction is quickly conveyed through the action of a reporter (directly through the output of probes, such as fluorescence) or with a temporal delay (indirectly through transcriptional regulation, such as reporter gene expression).

(6) Controls. A positive interaction is only credible if a corresponding negative control using a closely related ortholog or point mutation of the original protein is performed. Control proteins should localize to identical compartments, turnover and maturation must not be different, and their spatiotemporal distribution with the binding partners must be equal compared with the positive interactor. It is crucial to analyze the same protein couple using at least two (or preferably three) different PPI techniques.

The PPI methods described in this review include methods that are preferentially used in the plant sciences (Figs. 1 and 2C), namely, the yeast two-hybrid system, the split-ubiquitin system, FRET-related techniques, BiFC, and the split-luciferase complementation assay. CoIP, an ex vivo approach that is a viable alternative PPI method of choice in plant research (Fig. 2C; Table II).

An even bigger technological breakthrough occurred at the beginning of the 1990s, which was a game changer for the development of in vivo PPI techniques and cell biological methods in general. Toward the end of the 1960s, the mechanisms underlying and components facilitating the phenomenon of bioluminescence were discovered. Among the first substances to be isolated was the luciferase aequorin, a light-producing enzyme from the jellyfish Aequorea victoria (Shimomura et al., 1962). Intriguingly, while the living jellyfish emits green light, the Ca\(^{2+}\)-dependent oxidation of the substrate coelenterazine, which aequorin catalyzes, leads to the emission of blue light in vitro. From the extracts of jellyfish, Osamu Shimomura also purified a very brightly autofluorescing protein, which he named GFP. He postulated “an energy transfer from the light emitter of aequorin to GFP.” In other words, the energy of the blue light produced by the chemiluminescent aequorin is transferred to GFP, which in turn emits red-shifted green light (Morise et al., 1974). In fact, this proposed mechanism probably represents the first description of bioluminescence resonance energy transfer, which is currently used as a technique to study PPIs (Boute et al., 2002). Bioluminescence resonance energy transfer combines a light-producing luciferase, an energy-receiving fluorescent protein, and the phenomenon of FRET, a theory postulated some 30 years earlier by the physicist Theodor Förster (Förster, 1946).

Figure 2A highlights the increase in the number of scientific articles that feature PPIs as either a topic or title keyword and were published from the last decade of the 20th century to the present. While the mention of PPIs in scientific publications (PPI as topic) has increased almost exponentially in the past 20 years, the number of articles featuring the term PPI in the title (likely articles that focus on technology development) has increased in a steady, linear manner. The increase in publications coincides with three major technological and scientific advances: (1) the invention of the yeast (*Saccharomyces cerevisiae*) two-hybrid system in 1989; (2) the cloning of GFP in 1992; and (3) the publication of the yeast genome sequence in 1996.

The yeast two-hybrid method, developed by Stanley Fields and Ok-kyu Song, was the first and remains one of the most prominent systems for detecting PPIs (Fields and Song, 1989; Fields, 2009). As of February 2016, a keyword search in Thomson Reuters Web of Science yielded almost 60,000 publications with the topic yeast two-hybrid (Table II). The invention of the yeast two-hybrid technique not only triggered thousands of studies on PPIs but also spearheaded the development of alternative PPI methods and, in conjunction with the availability of fully sequenced genomes and high-throughput PPI techniques, initiated the subdiscipline of interactomics (see Box I). While the annual number of publications featuring yeast two-hybrid methods appears to have stalled recently (Fig. 2B), this technique remains the uncontested number-one PPI method of choice in plant research (Fig. 2C; Table II).

Work in the 1980s and 1990s focused on cloning and recombinant expression of aequorin (Prasher et al., 1985, 1992). Doug Prasher, together with Roger Tsien and Roger Heim, started engineering artificial fluorescent proteins by introducing mutations in the original GFP (Heim et al., 1994; Heim and Tsien, 1996). These novel fluorescent proteins revolutionized our understanding of subcellular processes, enabling biologists to label organelles, perform live imaging of subcellular protein movement, measure Ca\(^{2+}\) concentrations, pH, redox state, and membrane voltage, and, most importantly, monitor PPIs in vivo and in real-time (Ehrhardt, 2003; Fricker et al., 2006).

The engineering of fluorescent proteins with different spectral properties also enabled FRET to be used to study PPIs in vivo some 50 years after its initial description. As shown in Figure 2B, by the end of the 20th century and in the wake of the innovations that followed the development of fluorescent proteins, the number of publications featuring FRET as a keyword increased rapidly and continued to do so. Notably, FRET is exploited not only to detect PPIs but also to determine the properties of the protein environment as unimolecular protein sensors. FRET has a range of applications in...
physics, chemistry, and materials science, which also accounts for the large number of publications.

**Categories of PPI Techniques**

Since these groundbreaking discoveries, dozens of novel tools or improvements to established techniques have been developed for the detection of PPIs. These techniques have been categorized in various ways, such as in vivo versus in vitro, biophysical versus biochemical, split complementation versus two hybrid, and (with regard to the reporter output) enzymatic (e.g. chromogenic or prototrophic) versus fluorescent markers. Fluorescent reporters refer to inherent or complemented

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**Table 1. Properties of the in vivo PPI techniques discussed in this review**

<table>
<thead>
<tr>
<th>Method</th>
<th>Organism/System</th>
<th>Reporter</th>
<th>Risk of Artifacts&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Quantification&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Large Scale&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Comments</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>False Positives</td>
<td>False Negatives</td>
<td>HT</td>
<td>Unbiased Screening</td>
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<tr>
<td>Yeast two-hybrid</td>
<td>Yeast</td>
<td>Survival on depleted medium or enzymatic</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Split-ubiquitin system</td>
<td>Yeast</td>
<td>Survival on depleted medium or enzymatic</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>FRET-SE</td>
<td>Plant</td>
<td>Fluorescence</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>FRET-AB</td>
<td></td>
<td>Fluorescence increase in donor upon acceptor bleaching</td>
<td>−</td>
<td>+++</td>
<td>++</td>
<td>−</td>
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<tr>
<td>FRET-FLIM</td>
<td></td>
<td>Fluorescence decay</td>
<td>−</td>
<td>+++</td>
<td>+++</td>
<td>−</td>
</tr>
<tr>
<td>BiFC</td>
<td>Plant</td>
<td>Fluorescence</td>
<td>+++</td>
<td>+</td>
<td>+&lt;sup&gt;c&lt;/sup&gt;</td>
<td>++</td>
</tr>
<tr>
<td>Split-luciferase</td>
<td>Plant</td>
<td>Bioluminescence, enzymatic</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
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<tr>
<td>CoIP</td>
<td>Plant</td>
<td>Immunostaining</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

<sup>a</sup> Estimates range from low (−), moderate (+), and high (+++) to very high (+++).<br>
<sup>b</sup> Ranges are from not applicable (−), possible (+), and suitable (++) to highly suitable (+++).<br>
<sup>c</sup> Credible, unbiased quantification impossible using classical BiFC; reliable semiquantitative detection of PPIs possible using the ratiometric BiFC (Grefen and Blatt, 2012a).<br>
<sup>d</sup> Screening for novel interactors possible when combining CoIP with mass spectrometry (MS) analysis.
Figure 1. Protein-protein interaction techniques. I. Two-hybrid techniques (2H) use two functional proteins or domains as probes. Their inherent functionality is symbolized by dashed arrows, which can represent either DNA-binding or transcriptional activity for the GAL4 DNA-binding and transactivation domains in yeast two-hybrid analysis (A) or fluorescence (B). II. PCA (ProtA-Cub) uses a column-based assay with an antibody and a fluorescent protein (C). D. USPs (Ubiquitin Specific Peptidases) can be used in conjunction with bait and prey proteins (D). E. nYFP and cYFP are used for fluorescence imaging (E). F. NLuc and CLuc are used for luminescence imaging (F).
fluorescence as opposed to enzymatic, chromogenic color, or fluorescence output, which requires the generation of substrate turnover (luciferase or lacZ expression).

The in vivo approach (the focus of our attention) assumes that the host organism in which the PPI takes place is still alive when the analysis is performed. In some techniques involving the use of prototrophic reporters, such as in yeast-based systems, the survival of the organism is both a prerequisite and proof of the detection of an interaction. However, the greatest advantage of in vivo techniques is that they preserve the native surroundings in which the interaction takes place and is monitored.

PPI techniques that involve fluorescent reporter output are often performed in living cells as well, but in principle, the measurements do not necessarily require live-cell imaging. Immunolabeling is a feasible way of fixing an otherwise fast-moving or transient interaction, but it involves terminating the life of the cell. Clearly, this particular finishing touch should not alter the technique’s in vivo label. After all, the interaction is allowed to take place in vivo, and only during the detection of this interaction is death of the cell acceptable. This argument is self-evident when we look at CoIP, which can be seen as a chimera of in vitro and in vivo techniques: while the detection of an interaction clearly takes place in vitro in the true sense of the word, the establishment of the interaction itself is allowed to occur in an in vivo (i.e. living organism) environment. Accordingly, this approach is referred to as ex vivo.

Inherent to most PPI techniques is the fusion of two separate molecules (probes) attached to the proteins of interest (POIs), leading to their reporting of the interaction. There are two categories of probes based on the nature of their reassembly or interaction: probes used for PCA versus two-hybrid analyses (Fig. 1). The latter is naturally connected to the popular yeast two-hybrid method (Fields and Song, 1989). As the name implies, the probes for the two-hybrid system contain two discrete domains, each of which is functional in its own right, whereas the PCA probes consist of two nonfunctional fragments of a reporter protein; its complementation indicates a positive interaction (Stynen et al., 2012).

The split-ubiquitin system is the pioneering, classic example of a PCA method (Johnsson and Varshavsky, 1994; Müller and Johnsson, 2008), a categorization that is important for obtaining an in-depth understanding of the methods, their underlying biology, and potential limitations. While the two-hybrid system in principle can be converted to a yeast one-hybrid system, allowing the detection of protein-DNA interactions (Gstaiger et al., 1995), the PCA approach requires both split reporter fragments for a meaningful output; each fragment by itself is non-functional, an important difference. In recent years, the split-ubiquitin system has been referred to as a membrane yeast two-hybrid method (Kittanakom et al., 2009), which is biologically misleading, as the split-ubiquitin system is a PCA and not a two-hybrid technique. Worse still, this label downplays the split-ubiquitin system as an independent method that is superior in some aspects to the two-hybrid approach.

General Considerations about PPIs

The expression of POIs in yeast, as well as in in planta-based assays, is often driven by constitutive, strong promoters. Aberrant amounts of protein in the cell type examined may be a consequence of the use of such strong promoters. For yeast-based techniques, this problem may be negligible, considering that the PPI is monitored in a heterologous environment and is already flawed due to issues with codon bias, posttranslational modifications, and protein folding, stability, and turnover. However, for in planta approaches, where the advantage of homologous expression is implicit, using endogenous promoters should help reduce artifacts associated with aberrant expression.

From a thermodynamics perspective, it is evident that the concentration of a protein is a major driving force for any PPI (see Box II); hence, when artificially raising cellular protein concentrations, non-interactors might be forced to associate. Interaction data based on the use of protein overexpression should always be critically evaluated and compared between at least two (or better yet three) different methods. Nevertheless, overexpression or molecular crowding might still be

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Figure 1. (Continued.)

upon excitation such as in Förster resonance energy transfer (FRET) sensors (B). The proximity of the probes suffices for reporter output to be detected, as indicated by the black arrow. A, Example of the yeast two-hybrid technique and its reporter output (i.e. growth on depleted [interaction-selective] medium). AD, Activation domain; BD, DNA-binding domain; UAS, upstream activating sequence. B, Cartoon of a typical FRET interaction and its visualization through sensitized emission (see “FRET: Lifetime Versus Intensity” for details). C, Strictly speaking, coimmunoprecipitation (CoIP) can be counted as a two-hybrid technique when both bait and prey are fused to epitope tags (e.g. GFP). An interaction is reported through immunoblotting of the extract prior to column loading (L) and, after washing, the eluate (IP). M, Marker, II. Protein fragment complementation assays (PCAs), such as the split-ubiquitin system (D), bimolecular fluorescence complementation (BIFC; E), and split-luciferase complementation (F), use two non-functional protein fragments as probes. Only upon reassembly is their intrinsic function (enzymatic, fluorescence, and so on) restored, which is used to reveal an interaction (black arrow). D, Diagram of a split-ubiquitin assay; interactions are monitored through growth on depleted medium, which is similar to yeast two-hybrid analysis. E, Cartoon of BIFC analysis and its (suboptimal) detection through confocal microscopy. See “BIFC: From Cherry Picking to Quantification” for further details on the appropriate analysis, quantification, and controls for this particular PPI technique. F, Split-luciferase complementation assay and its application on a 96-well plate, which enables high-throughput analysis of plant protoplasts by directly measuring luminescence after the addition of substrate (luciferin). + and − refer to typical positive and negative interactions, respectively, throughout.
circumvented in an in vivo environment through gene silencing or targeted degradation, attenuating potential occurrences of false-positive interactions.

It is crucial to check the native spatiotemporal expression pattern of a protein and its supposed partner. This includes its expression in particular tissues and at specific developmental stages as well as in particular subcellular compartments. Only if the two POIs have the chance to meet at some point in time or space is it sensible to perform PPI analysis under non-native conditions.

We now summarize a few general points that should be considered before initiating work with any of the described PPI techniques. Particular attention is given to the following factors, which are likely to influence the detection of a PPI.

**Organismal factors** are particularly important, as heterologous expression can be influenced by codon bias and posttranslational modifications or the lack thereof. Construct generation (cloning system), expression strength (choice of promoters, replication origin), maintenance in the organism of choice (marker), and probe-related issues (linkers, tags and their orientation) can be summarized as **structural factors**. Equally important for successful and reliable interaction analysis are **experimental factors**, such as the need to include rigorous controls, assess their suitability, and choose alternative methods to corroborate the results.

**Organismal Factors**

Expressing a plant protein heterologously in yeast, for example, can result in low translation efficiency due to suboptimal codon usage (Grefen, 2014). Codon optimization, however, can be a two-edged sword, as it was recently shown that rare codons found in gene sequences of secretory and membrane proteins can slow down protein synthesis, thereby improving cotranslational translocation (Chartier et al., 2012; Pechmann et al., 2014; Yu et al., 2015). More worrying still is the recent finding that using different synonymous codons modulates translation rates.
Box I: An Omics Perspective of PPIs

The relatively low cost for sequencing whole genomes and comparing them between ecotypes and species allows for rapid data accumulation. Changes in transcript levels or epigenetic signatures enable in-depth views of evolutionary or developmental effects to be obtained. The suitability of some PPI techniques for high-throughput and/or large-scale approaches, such as CoIP-MS (Miernyk and Thelen, 2008) and yeast two-hybrid analysis, has led to the emergence of another omics discipline: interactomics.

This term, which was originally coined by Bernard Jacq in 1999 (Sanchez et al., 1999), describes the effort to assemble virtually all binary PPIs in an organism and/or compare them with those of other species (Kiemer and Cesareni, 2007). Whether it is sufficient to look exclusively at pairwise interactions is debatable, but such analysis is the easiest way to begin monitoring network structures. Interactome data sets can be acquired in silico through prediction algorithms (based on physicochemical properties, evolutionary conservation, coexpression analysis, or a combination of these, including integration of functional data) or experimentally via high-throughput PPI techniques (Morzy et al., 2008; Braun et al., 2013).

Again, the yeast two-hybrid system has pioneered this particular side branch of PPI studies. Yeast is an extremely powerful organism for high-throughput PPI analysis either through mating-based approaches or sequential cloning of cDNA libraries. It is also easy to collect and identify positive interactions, since survival of the yeast can be used as a reporter. Nonetheless, every method comes with flaws such as the detection of false positives (which will be discussed in detail below), and applying these techniques at a large scale inadvertently multiplies inherent issues, raising valid concerns over the authenticity of large lists of interactors (Hart et al., 2006).

While the initial screens and arrays (Uetz et al., 2000; Ito et al., 2001) were predicted to contain almost 50% false positives (von Mering et al., 2002), recent improvements have yielded more reliable interaction data (Yu et al., 2008). Nevertheless, interactome data should be treated critically; they should be verified experimentally for individual candidates, and only when confirmed should they be used to inform hypothesis generation. There are a number of online resources where interactome data can be accessed (Table Box I; Szklarczyk and Jensen, 2015). For example, the interactomes of Arabidopsis (Geisler-Lee et al., 2007; Lee et al., 2015), rice (Oryza sativa; Gu et al., 2011; Sapkota et al., 2011), and maize (Zea mays; Zhu et al., 2016) have been assembled through prediction algorithms and experimental evidence or a combination of the two.

Screening Approaches

Using PPI techniques to screen for novel interaction partners of a POI is a many scientists’ preferred way to venture into new avenues of research. Unbiased approaches to obtain novel interactors of a POI include cDNA library screens using the yeast two-hybrid system (Gietz, 2006), the split-ubiquitin system (Dirnberger et al., 2008), and the (now more affordable) CoIP-MS technique (Pertl-Obermeyer et al., 2014). It is imperative, however, to critically test an interaction couple individually and independently (with alternative techniques) before investing too much time and effort in analysis.

Hunting for novel binding partners also can begin with a limited number of putative candidates using large but defined collections (arrays) of open reading frames as potential preys (Miller et al., 2005; Lalonde et al., 2010). These arrays feature a defined choice of protein groups or subfamilies, the screening of which has been executed successfully with different techniques, notably the membrane proteome of Arabidopsis using the mating-based split-ubiquitin system (Jones et al., 2014) and CoIP-MS to analyze SNARE protein interactions (Fujiiwara et al., 2014b).

and alters the cotranslational folding of protein domains (Buhr et al., 2016). These points emphasize the importance of biochemical verification of protein expression especially (but not only) in negative controls, as the failure to report an interaction does not necessarily mean that a protein couple does not interact. Negative results could simply be due to modified expression or even a lack thereof.

Equally problematic in heterologous expression systems is the need for correct postranslational modifications (Duan and Walther, 2015; Hurst and Hemsley, 2015). A plethora of postranslational modifications are available, such as phosphorylation, glycosylation, and lipidation, all of which depend on recognition of the host organism by the molecular machinery. Posttranslational modifications can influence a PPI either directly through the modification of interaction sites (e.g. phosphorylation) or indirectly by shifting localization (e.g. palmitoylation; Hurst and Hemsley, 2015). Another important postranslational modification is the glycosylation pattern. Each eukaryotic kingdom has its own glycosylation signature, which likely influences the functionality and interaction patterns of recombinantly expressed proteins (Dell et al., 2010). This may result in the failure to detect a potential PPI due to either the absence or incorrect glycosylation signature in a POI or to its mistargeting, misfolding, and degradation, highlighting the fundamental importance of testing PPIs with (at the very least) two different techniques.

Structural Factors

Vector Systems

Numerous vector systems are available for both yeast- and plant-based PPI techniques. Comprehensive lists of vector systems for each technique can be found in reviews that focus on individual methods. Rather than trying to assemble a list of available vectors for each system, we would like to emphasize that the introduction of novel cloning techniques has made it quite easy to construct expression cassette/vector systems for any of the PPI techniques listed here. Ultimately, it might be less time consuming to produce such systems than to receive outdated plasmids with
incomplete sequence information or verification. Producing such vectors also would enable the researcher to combine the latest improvements (tags, fluorophores) and choose the best promoter (endogenous, constitutive, repressible, or inducible) while, most importantly, enabling dual or triple expression from one stretch of DNA (plasmid or transfer DNA [T-DNA]), optimizing the desired PPI technique for the POIs. With a range of novel, rapid, easy-to-use multicloning techniques to choose from, such as GreenGate (Lampropoulos et al., 2013), GoldenGate (Engler et al., 2008), Gibson Assembly (Gibson et al., 2009), Multisite Gateway (Sasaki et al., 2013), GoldenGate (Engler et al., 2008), Gibson Assembly (Gibson et al., 2009), Multisite Gateway (Sasaki et al., 2013), and the 2in1 system (Grefen and Blatt, 2012a), building a multieexpression cassette has become an attractive option. In general, multiple gene expression from a single plasmid should be the preferred choice, especially in any transient (plant) transformation. For example, mixing of two individual Agrobacterium tumefaciens strains only yields approximately 80% cotransfection, in addition to high expression variability, which is most likely a consequence of unequal gene dosage (see Fig. 6A below; Hecker et al., 2015).

In yeast, it is possible to coexpress two fusion proteins from two individual plasmids through the use of two different prototrophic selection markers. Additionally, as yeast is capable of homologous recombination (in vivo cloning), it is possible to create almost any desired vector or construct (Joska et al., 2014), which is extremely useful when working with genes that appear to be toxic. For example, for unknown reasons, some DNA sequences, such as that of Arabidopsis thaliana Histidine Kinase 5 (AHK5; Mira-Rodado et al., 2012), cause problems during cloning in Escherichia coli, resulting in transposon-inserted or point-mutated open reading frames (Horák et al., 2008). Strategies to counter these issues include using an E. coli strain that reduces the copy number during log-phase growth (CopyCutter or Biozym) or simply performing in vivo cloning in yeast.

**Expression Control: Promoters and Replication Origins (ORIs)**

Choosing a promoter to control heterologous gene expression is of great importance, particularly in yeast, where overexpression can potentially interfere with metabolism, leading to lethality or other strong phenotypes. A number of different promoter systems have been established for yeast, ranging from repressible or inducible to weak or strong constitutive expression.

Most systems feature the use of the alcohol dehydrogenase promoter, a strong constitutive promoter that is

### Table 1: Large-scale interactome analyses and databases

<table>
<thead>
<tr>
<th>Plant Species</th>
<th>Method</th>
<th>Objective of the Study</th>
<th>No. of Interactions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabidopsis thaliana</td>
<td>Yeast two-hybrid</td>
<td>Aux/IAA-ARF</td>
<td>213</td>
<td>Piya et al. (2014)</td>
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<tr>
<td></td>
<td>Affinity purification-MS</td>
<td>Interactome of <em>A. thaliana</em> Qa-SNAREs</td>
<td>518</td>
<td>Fujiwara et al. (2014)</td>
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<tr>
<td></td>
<td>Yeast two-hybrid</td>
<td>Abscisc acid signaling network</td>
<td>&gt;500</td>
<td>Lumba et al. (2014)</td>
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<tr>
<td></td>
<td>Computational</td>
<td><em>Pseudomonas syringae</em> interactome</td>
<td>11,000</td>
<td>Sahu et al. (2014)</td>
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<tr>
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<td>Split-ubiquitin system</td>
<td>Membrane-linked Interactome Database (MIND1)</td>
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<td></td>
<td>Computational</td>
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<td></td>
<td>Computational</td>
<td>AIPID (A. thaliana protein interactome database)</td>
<td>28,062</td>
<td>Cui et al. (2008)</td>
</tr>
<tr>
<td>Brassica rapa</td>
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</tr>
<tr>
<td></td>
<td>Yeast two-hybrid</td>
<td>MADS box</td>
<td>272</td>
<td>de Foter et al. (2005)</td>
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<tr>
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<td>MADS box</td>
<td>119</td>
<td>Lesberg et al. (2008)</td>
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<td>Solanum lycopersicum</td>
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<td>Global interactome</td>
<td>4,587</td>
<td>Geisler (2011)</td>
</tr>
<tr>
<td></td>
<td>Coffeea canephora</td>
<td></td>
<td>37,112</td>
<td>Ho et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>Oryza sativa</td>
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<td>37,112</td>
<td>Ho et al. (2012)</td>
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<tr>
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<td>Computational</td>
<td>PRIN (predicted rice interactome network)</td>
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<td></td>
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<td>Zea mays</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hordeum vulgare + TAP-MS</td>
<td>14-3-3 family</td>
<td>500</td>
<td>Schoonheim et al. (2007)</td>
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<tr>
<td>Physcomitrella patens</td>
<td>Computational</td>
<td>Predicated interactome</td>
<td>67,740</td>
<td>Schuette et al. (2015)</td>
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</tbody>
</table>
Box II Table: $K_d$ values of two-hybrid and PCA techniques featured in this review

<table>
<thead>
<tr>
<th>Method</th>
<th>$K_d$ of Probes</th>
<th>$K_d$ of PPI</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast two-hybrid</td>
<td>n.a.</td>
<td>&lt;50 nM</td>
<td>Estojak et al. (1995)</td>
</tr>
<tr>
<td>Split-ubiquitin</td>
<td>7 μM</td>
<td>&lt;100 μM</td>
<td>Jourdan and Searle (2000)</td>
</tr>
<tr>
<td></td>
<td>1 μM</td>
<td>&lt;1 μM</td>
<td>Müller and Johansson (2008)</td>
</tr>
<tr>
<td>Split-luciferase (Nanolum)</td>
<td>190 μM</td>
<td>&lt;1 μM</td>
<td>Dixon et al. (2016)</td>
</tr>
<tr>
<td>BiFC</td>
<td>0 μM</td>
<td>&lt;1 μM</td>
<td>Magliery et al. (2005)</td>
</tr>
<tr>
<td>FRET</td>
<td>110 μM (YFP)</td>
<td>&lt;10 μM</td>
<td>Piehler (2005)</td>
</tr>
<tr>
<td></td>
<td>74 μM (mYFP[A206K])</td>
<td>&lt;10 μM</td>
<td>Zacharias et al. (2002)</td>
</tr>
</tbody>
</table>

*DNA-binding and transactivation domains do not show intrinsic association; hence, a $K_d$ value is not applicable (it would be very high).*  
*The $K_d$ of the probes was determined for the reassociation of wild-type versions of Nub and Cub.*  
*It is estimated that the Nubl13A mutation increases the $K_d$ to 1 μM and that the Nubl13G mutation value might be even higher, allowing the split-ubiquitin system to detect transient interactions up to $K_d$ values of 100 μM (Müller and Johansson, 2008).*  
*The split fluorescent protein fragments are known to spontaneously and irreversibly reassociate; hence, the $K_d$ is negligibly small (Magliery et al., 2005).*  
*$< 100$ μM.  
*$< 1$ μM.  
*$< 1$ μM.  
*$< 10$ μM.  
*Each of these values is an approximation.*

Box II Figure: Kinetics and affinities of PPI techniques. The $K_d$ is inversely correlated to the binding affinity. PPIs can exist as permanent or transient interactions. While transient interactions can be described as weak due to the higher probability of complex dissociation, strong transient or permanent interactions bear higher intrinsic binding affinity and greater temporal stability. More details are given in the text and references (Barasjanzky et al., 2005; Perkins et al., 2010; Kastritis et al., 2011).

Box II: Structure and Physics of PPIs

The heterologous cellular environment can become challenging for protein function. Difficulties arise related to the presence of unspecific aggregation while retaining a high degree of specificity for second-order homomeric or heteromeric PPIs at low cellular concentrations (Reichmann et al., 2007; Marsh and Teichmann, 2015). Van der Waals forces, ionic interactions, hydrogen bonds, interactions with water molecules, and hydrophobic effects form the physicochemical foundations of PPIs (Keskin et al., 2008). Taking a more macromolecular view, the combination of interface shape, its amino acid composition, hydrogen bonds, the solvation free energy gain from interface formation, and the eventual binding of the side chains all play important roles in PPIs (Sowmya et al., 2015). Single changes at the amino acid level often do not influence an interaction substantially due to the large surface areas that contribute to most interaction sites (Reichmann et al., 2007; Aakre et al., 2015). Nevertheless, there are indeed cases where single point mutations can disrupt PPIs (Karnik et al., 2013a; Zhang et al., 2015).

The affinity between two interacting proteins, A and B, can be quantified through the $K_d$. The $K_d$ describes the concentration of protein A at which it is bound to half of all available sites on protein B at equilibrium (Kastritis and Bonvin, 2013). In other words, the smaller the $K_d$ (femtomolar or picomolar range) of a protein pair AB, the higher the affinity of A to B, as fewer molecules of A are needed for 50% of all A molecules to bind to B (Perkins et al., 2010). $K_d$ values can be determined experimentally or predicted, but they are influenced by exogenous factors such as temperature, pH, and salt concentration in the solvent (Kastritis et al., 2011).

PPIs also can be classified as obligate or non-obligate PPIs (Nooren and Thornton, 2003). Obligate PPIs are permanent, usually show low $K_d$ values, and form strong heterotopic or homotopic interactions, as is the case for potassium channel tetramers (Ward et al., 2009) and transporter oligomers such as proton pumps (Schumacher and Krebs, 2010). Subunits or monomers of these complexes are individually unstable and non-functional; hence, the term obligate for structural stability is dictated by their interaction. While there are also examples of permanent, non-obligate interactions such as enzyme-inhibitor complexes (avidin-biotin is a classic example), the vast majority of non-obligatory interactions are of a transient nature and may or may not be weak (Nooren and Thornton, 2003). PPIs can be transient in terms of their cellular longevity but still might be very strong in terms of binding affinity. For example, the ternary SNARE complexes, which facilitate vesicle fusion through a tight but reversible interaction, show high binding affinities ($K_d < 50$ nM; Rice et al., 1997). Examples of weaker, transient interactions in the micromolar range include enzyme-substrate and receptor-ligand interactions (Chinchilla et al., 2007; Jiang et al., 2013).

$K_d$ values can be used to describe the detection limit of a PPI technique or the range in which it shows the highest sensitivity and stringency. The table in this box lists ranges of $K_d$ values from the literature for the methods described in this review. For two-hybrid techniques such as yeast two-hybrid and FRET, in which the probes (trans-activating and DNA-binding domains or monomeric fluorescent proteins) do not carry intrinsic binding affinity toward each other, $K_d$ values of individual POI couples are sufficient to describe the affinity range of the technique. The more the values are experimentally determined for individual PPI couples, the more reliably the PPI technique can be assessed for its suitability to detect a certain PPI.
used in its full-length (ADH1; ~1,500 bp) or truncated (ADH1*; ~700 bp) version. Notably, expression from the full-length ADH1 promoter decreases during the late exponential, ethanol-producing growth phase, whereas the truncated version, while slightly weaker in expression, is not repressed in the presence of ethanol (Ruohonen et al., 1995). This observation should be considered when growing yeast for too long in liquid medium for immunoblot analysis. The ability of yeast to switch promoters on or off depending on the carbon source available in the medium or by application of exogenous factors also can be exploited for temporal control (Weinhandl et al., 2014).

Inducible promoters that are commonly used include the Gal-inducible GAL1 promoter (Johnston, 1987) and the copper-inducible CUPI promoter (Butt et al., 1984). However, growth is considerably slower on Gal than on Glc, and copper addition must be carefully balanced, as this metal is toxic to yeast. Also, the leakiness of a promoter can preclude genetic analysis, as basal traces of protein expression can lead to an interaction in a sensitive detection assay. A potential alternative involves the use of a repressible promoter. MET25, for example, is a strong promoter in the absence of Met, but it shuts down in the presence of Met (Sangsga et al., 1985). Notably, complete supplement mixture usually contains 134 μM Met (20 mg L⁻¹), which significantly reduces gene expression (Grefen, 2014).

For many years, the strong cauliflower mosaic virus 35S promoter (Ow et al., 1987) has been incorporated in numerous plant expression vectors and used for stable as well as transient transformation (Blatt and Grefen, 2014). Unfortunately, particularly in stable transformants, the expression of a transgene driven by this promoter is often silenced and, even if successful, the strong overexpression can cause artifacts in PPI analysis, as discussed above. However, if native expression of a protein is low due to restricted spatial or temporal expression, constitutive promoters such as RPS5a or UBO10 can be used to allow sufficient expression and, in turn, detection of an interaction (Weijers et al., 2001; Grefen et al., 2010b). An alternative to constitutive expression would be to regulate expression through chemical treatment (estradiol, ethanol, or dexamethasone), stimulus induction (heat shock or light), or the use of tissue-specific expression, all of which allow an interaction to be restricted spatiotemporally and, hence, putting a PPI closer to its native context (Borghi, 2010; Siligato et al., 2016).

The origin of replication is another important factor that can be used to regulate the strength of expression, albeit primarily when using yeast plasmids (James, 2001). Yeast maintains three different types of origins of replication: autosomal replication sequence, 2μm, and integrating. While the autosomal replication sequence often is used in combination with a centromere sequence to guarantee plasmid stability over multiple generations, these plasmids are maintained in low abundance of one to two copies per cell. To enhance the expression of a POI even further, a 2μm plasmid can be used, allowing yeast cells to keep up to 50 copies. The most native-like maintenance and expression would be achieved using an integrating plasmid, which stably inserts into the yeast chromosome. Such plasmids, however, are rarely used for the expression of heterologous plant proteins and the analysis of their interactions.

**Linkers**

When contemplating the assembly of custom-built cloning cassettes or vectors for a PPI technique, it is crucial to choose the correct linker between the protein and the tag (Chen et al., 2013). If the linker is too short or rigid, an interaction of the POIs might not enable reassembly or might limit the area in which the probes can meet, preventing detection (Wehr and Rossner, 2016). The short linker sequences left by Gateway cloning after recombination may not be sufficiently flexible to allow for probe interaction (Grefen and Blatt, 2012a).

The orientation of the linker and tag is an additional and important factor to consider, as tagging of either the N or C terminus of a protein can potentially mask signal peptides, targeting sequences, or even interaction interfaces, precluding the identification of binding partners (Grefen and Blatt, 2012b). While masking of a signal peptide or targeting sequence might prevent interaction due to artificial mislocalization, occlusion of a large, often hydrophobic targeting sequence can result in the aggregation of proteins and their subsequent degradation. Before a PPI is tested, sequence analysis of the protein using TargetP, PSORT, or other in silico tools could be used to obtain information about targeting sequences and to determine tag orientation accordingly.

The topology of the POIs also can be an important factor, as some PPIs discussed below demand specific orientation of the termini. The classical split-ubiquitin analysis, for example, requires the bait protein to be membrane bound to prevent leakage of the fusion protein into the nucleus, where the attached transcription factor would cause reporter gene activity; however, at the same time, the C terminus must face the cytosol (see below). In addition, it is important to note that, when verifying interactions using different methods, the absence of an interaction in one method could easily be due to the tag orientation, which could be circumvented by testing all possible tag combinations of a binary interaction couple (Stellberger et al., 2010).

Subcellular localization is a point of concern when performing meaningful analysis of a detected PPI; however, deliberate targeting can be an advantage, and some techniques are easier to perform in certain compartments. For example, FRET acceptor photobleaching can become unreliable when detecting a PPI in a highly motile organelle or compartment (see below).

**Experimental Factors: Controls**

A final but central point before commencing any PPI analysis is to select meaningful negative and positive
controls. These controls should be chosen according to the method used, and the location in which the interaction is supposed to take place in the heterologous system versus its native localization must be taken into account. In other words, for a protein couple that interacts in the vicinity of the plasma membrane, the negative control should not be an obligatory nuclear protein. The best negative control is usually a point mutation of one of the proteins under investigation, provided that the mutation does not lead to its degradation or increased turnover (Grefen et al., 2010a; Kodama and Hu, 2012). The gold standard for negative controls should be that the expression of the fusion protein is verified via immunoblot analysis.

THE YEAST TWO-HYBRID TECHNIQUE: WHERE IT ALL BEGAN

It is interesting to observe the impact that a technological article can have on the community. The ingenious yet simple method devised by Stanley Fields and Ok-Kyu Song in 1989 is certainly an example of such an article, which revolutionized the study of PPIs (Fields and Song, 1989; Fields, 2009).

The principle is straightforward (Figs. 1A and 3A). The transcriptional activator GAL4 contains an N-terminal DNA-binding domain and a C-terminal transactivation domain interspaced by a structural region. Both domains, the DNA-binding (amino acids 1–147) and transactivation (amino acids 768–881) domains, are independently stable and functional and do not require the central body of the protein. In other words, each one is able to fulfill its nuclear function despite being displaced from the rest of the GAL4 protein: the DNA-binding domain still binds the GAL1 upstream activating sequence, and the transactivation domain, if brought into the vicinity of the TATA box, is sufficient to activate transcription. When two POIs that interact are fused to the DNA-binding and transactivation domains, respectively, their association creates a chimeric transcription factor, turning on gene expression downstream of the GAL1 upstream activating sequence (Fields and Song, 1989).

This system has also been successfully established using different split probes. The DNA-binding domain of the bacterial repressor LexA can be fused to the GAL4, bacterial B42, or Herpes simplex VP16 transactivation domain. The different domains feature properties that allow yeast two-hybrid analysis to be modulated. While the GAL4-DNA-binding domain contains a nuclear localization signal, LexA does not, which results in less bait protein entering the nucleus (Brent and Finley, 1997). This could potentially reduce the number of false positives but also might prevent the detection of a weak interaction. Other approaches to manipulating the system include adjusting the strengths of various activation domains. For example, while VP16 is a very strong transcriptional activator (Uhlmann et al., 2007), B42 is rather weak and can counteract toxic effects associated with strong activation via the GAL4 transactivation domain (Gill and Ptashne, 1988).

The choice of DNA-binding and transactivation domains also has implications for the choice of the reporter gene cassettes incorporated in the yeast strain. While the GAL4-DNA-binding domain binds upstream of the GAL1, GAL2, or GAL7 promoter, LexA requires the presence of the lexA operon of E. coli upstream of the reporter genes. These can code for chromogenic enzymes such as β-galactosidase (lacZ, for selection via blue/white coloring of colonies), prototrophic markers (e.g. HIS3 or ADE2, enabling survival on depleted growth medium), or both (James, 2001). For example, the yeast strain PJ69-4A (James et al., 1996) carries the HIS3 reporter driven by the GAL1 promoter, ADE2 driven by the GAL2 promoter, and lacZ driven by the GAL7 promoter. Since the GAL4-DNA-binding domain can bind to the upstream activating sequences of all three promoters but with different affinities, the combination of multiple reporters provides higher stringency, significantly reducing the number of false positives. While the HIS3 and ADE2 reporters enable qualitative analysis via survival on selective growth medium, the lacZ gene allows for semiquantitative readout of interactions through enzyme assays using substrates such as 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside acid (X-Gal) and 2-Nitrophenyl β-D-galactopyranoside (ONPG) (Serebriskii and Golemis, 2000; Möckl and Auerbach, 2004). Additionally, the haploid yeast strain PJ69-4A also exists as the isogenic partner strain PJ69-4a (James, 2001). Rather than performing numerous, tedious dual transformations (two plasmids at once), it is possible to first transform a set of different bait constructs in one mating type and a set of preys in the other mating type; then, by mixing the two haploid mating types, large numbers of binary interactions can be evaluated.

Screening and False Positives

Both the yeast two-hybrid and split-ubiquitin systems are exceptionally well suited for high-throughput approaches and complementary DNA (cDNA) library screening (Fig. 3B; Table 1, Box I). Important points to consider are the choice of library (polyA-cDNA, normalized, tissue or development specific) and whether sequential transformation or a mating-based approach will be used. One of the biggest limitations of the yeast two-hybrid system is the obligatory nuclear localization of the interaction partners. Most of the problems that complicate the analysis of the results of yeast two-hybrid assays are a direct consequence of this prerequisite. For example, membrane proteins must be truncated to allow for their forced relocation to the nucleus. Large cytoplasmic proteins may be prevented from entering the nuclear pores (Kabachinski and Schwartz, 2015), and their truncation can often render them non-functional, leaving the significance of any interaction result in question.

The false positives that usually come up in a cDNA library screen arise from three different types of proteins and their interactions (Fig. 3C; Parchaliuk et al.,
Figure 3. The yeast two-hybrid system and its modifications. A, The original yeast two-hybrid analysis detects an interaction between two proteins fused to either the DNA-binding domain (BD; bait) or the DNA transactivation domain (AD; prey) of GAL4, respectively. An interaction reconstitutes the DNA-binding and transactivation domains to activate reporter genes such as ADE2, HIS3, and lacZ. B, A cDNA library screen allows the identification of novel binding partners (green crayon-like shape), whereas non-interacting proteins (opaque square and sphere) fail to activate the reporter genes. C, Examples of false-positive results that might be detected in yeast two-hybrid cDNA library screens that should be excluded by appropriate testing (see “The Yeast Two-Hybrid Technique: Where It All Began” and Box I): (1) unrelated prey fusions that bind to the bait; (2) transcriptional activators that are sufficient to trigger reporter gene activity; and (3) enzymes that overcome the selection pressure on depleted medium by restoring prototrophy. D, An extension of the classic yeast two-hybrid system is yeast three-hybrid analysis, in which two non-interacting or weakly interacting proteins (bait I and bait II) are bridged or stabilized by a third protein (cyan; prey/bridge). E, Reverse yeast two-hybrid detects the interference of a known interaction couple and can be used to screen for inhibiting proteins or molecules (red cones). Transcript activation of a selection marker such as URA3 renders yeast sensitive to 5-fluoroorotic acid. If the interaction is prevented, the reporter genes are not activated and the yeast can survive on 5-fluoroorotic acid. UAS, Upstream activating sequence.
1999): (1) proteins that bind unspecifically to the bait; (2) random transciptional activators; and (3) enzymes that restore the prototrophy of the yeast. (1) Unrelated proteins, such as spatiotemporally misexpressed proteins, may bind unspecifically to the bait and cause reporter gene activity [Fig. 3C, (1)]. Such false positives can be counted through the appropriate choice of a library. Rather than using a library from the whole organism, the use of tissue- or developmental stage-specific libraries can help reduce the number of false positives. Green tissue-derived libraries will contain a large number of chloroplast-related protein sequences, which can lead to numerous false positives when using a non-chloroplast-localized bait POI. (2) Intrinsic transcriptional activity independent of the interaction also can generate false positives through interaction with the bait protein [Fig. 3C, (2)]. As these effects might be less efficient than that of a bona fide interactor, adjusting the expression levels of bait and/or library prey can yield more stringent conditions and reduce the number of false positives in this category. (3) In some cases, enzymatic gene products from a cDNA library have the potential to restore prototrophy in yeast [Fig. 3C, (3)]. Using at least two auxotrophic markers such as ADE2 and HIS3 provides a check against this possibility, as two different enzymes would need to be replaced to facilitate the steps in the biochemical pathways of His and adenine production. If only HIS3 is used as a marker, the addition of 3-AT, an inhibitor of HIS3, can help titrate screening conditions and reduce the likelihood of false positives.

**Ternary Interaction Analysis**

The simplicity of the yeast two-hybrid system almost immediately sparked the development of several modifications and improvements. Most importantly, the system can be used to detect trimeric interactions by simply coexpressing a third protein partner, leading to the development of the yeast three-hybrid screening approach. Here, an interaction can be (1) outcompeted, (2) enhanced, or (3) facilitated/bridged (Fig. 3D; Zhang and Lautar, 1996). The system has been used successfully in the plant field; examples of each of these binding modes can be found in the literature (Suzuki et al., 2009; Maruta et al., 2016). The yeast three-hybrid system is particularly elegant when using a controllable promoter for the third protein partner, allowing the trimeric interaction to be switched on or off through the addition of an inducer or inhibitor (Tirode et al., 1997).

An elegant, alternative solution to screening for inhibitors is reverse yeast two-hybrid analysis (Fig. 3E; Leanna and Hannink, 1996). This system is based on the expression of a reporter gene that can be used as a negative selection marker. By reintroducing CYH2 (Leanna and Hannink, 1996) or URA3 (Vidal et al., 1996) into yeast cells, the cells are rendered sensitive to cycloheximide, an inhibitor of protein biosynthesis, or to 5-fluoroorotic acid, a precursor of the toxic uracil analog 5'-fluorouracil, respectively. If an interaction is prevented through a mutation in the bait, prey, or third protein partner, the yeast cells can grow on cycloheximide or 5-fluoroorotic acid. When assaying for third binding partners, it is important to keep in mind that an ortholog of the third protein might actually be present in yeast and facilitate bridging in lieu of absence of the plant candidate. A solution to this problem would be to knock out the corresponding gene in the reporter strain used for the yeast three-hybrid analysis, provided that it is not essential for survival and/or does not cause severe phenotypes.

There are a range of further modifications and improvements to the yeast two-hybrid system. A comprehensive list of references for these can be found in an excellent review by Patrick Van Dijck and coworkers (Stynen et al., 2012). One notable example is the application of a two-hybrid approach in plant cells. The plant two-hybrid approach allows an interaction in protoplasts to be detected using GUS expression as a readout (Ehlert et al., 2006).

**THE SPLIT-UBIQUITIN SYSTEM: A FULL-LENGTH ALTERNATIVE**

Despite its great popularity, the greatest disadvantage of the classical yeast two-hybrid system is the obligatory nuclear localization of the proteins and, hence, their site of interaction. Artifacts may arise from the truncation and mislocalization of otherwise non-nuclear proteins. Five years after the initial description of the yeast two-hybrid system, an alternative with the potential to overcome these limitations was described. The split-ubiquitin system never managed to gain as much popularity as the yeast two-hybrid system despite providing the same ease of application, yet it allows virtually all protein types to be tested without the need to truncate or mislocalize these proteins and without introducing additional artifacts to those associated with the yeast two-hybrid system.

Ubiquitin is a eukaryotic protein that is highly conserved among species, with an almost identical sequence comprising just 76 amino acids. This protein is involved in a multitude of vital processes through its inherent function (i.e. tagging proteins destined for degradation via ubiquitin-specific proteases). In 1994, Nils Johnsson and Alexander Varshavsky split ubiquitin into two fragments, an N-terminal Nub (amino acids 1–34) and a C-terminal Cub (amino acids 35–76), and fused a HA-tagged protein to its C terminus (Johnsson and Varshavsky, 1994). Coexpression of both split fragments leads to the reassembly of ubiquitin and subsequent cleavage of the hemagglutinin-tagged protein, which can be visualized through immunoblot analysis. Mutating the Ile at position 13 of Nub to an Ala or Gly prevented this reassembly, leading to the establishment of the first PCA system. Later work identified 10 additional single and double point mutations in the Nub peptide that offer continuous levels of affinity between Cub and Nub, an excellent tool set of varying stringency that facilitates the estimation of the dissociation constant (Kd) range of two interacting POIs (Box II; Raquet et al., 2001).
A few years later, Igor Stagljar and coworkers used the chimeric transcription factor PLV as a reporter. PLV consists of Protein A from *Staphylococcus aureus* (containing two IgG-binding sites) followed by the LexA DNA-binding and *H. simplex* VP16 DNA activation domains (Figs. 1D and 4A; Stagljar et al., 1998). The Protein A tag was originally incorporated for immunochemo detection of the fusion construct and cleaved reporter; however, compared with a bait vector depleted of the Protein A tag, its presence also appears to prolong the lifetime of the transcription factor, hence increasing reporter gene activity (C. Grefen, unpublished data). While the incorporation of a transcriptional readout provides a highly stringent detection system, it imposes the use of membrane-anchored or -attached proteins as Cub fusions; otherwise, diffusion of the entire fusion protein into the nucleus would result in reporter gene activation. A later development termed the cyto-split-ubiquitin system circumvents this problem by attaching an N-terminal membrane anchor (the transmembrane domain of Oligosaccharyltransferase 4, OST4) to the Cub fusion, thereby allowing transcription factors or cytosolic proteins to be used as bait (Fig. 4B; Möckli et al., 2007; Karnik et al., 2015).

In addition to the transcriptional readout reporter system, another approach was developed using the complementation of ubiquitin. Here, the Cub moiety is fused to orotidine-5'-phosphate decarboxylase (URA3), implementing an alternative reporting mechanism to nuclear transcription (Fig. 4D). URA3 is preceded by an Arg residue that destabilizes it after cleavage according to the N-end rule of degradation (Fig. 4D; Wittke et al., 1999). Rapid turnover of URA3 renders cells auxotrophic to uracil but resistant to 5-fluoroorotic acid (Müller and Johnsson, 2008). Hence, the URA3-based split-ubiquitin system can be used for either the detection of binary PPIs (when counterselection with 5-fluoroorotic acid is used) or the screening of inhibitors or point mutations that abolish an interaction (when using uracil-depleted medium for selection). While the cost for the chemical compound 5-fluoroorotic acid and the necessary replica plating represent downsides, this reporter system facilitates screening for inhibitors of an interaction at the membrane or in the cytosol or nucleus (Dinrberger et al., 2006).

The split-ubiquitin system has been used extensively in plant research to identify interactions of membrane proteins such as G-proteins (Aranda-Sicilia et al., 2015), phosphate transporters (Fontenot et al., 2015), and aquaporins (Besserer et al., 2012; Hachez et al., 2014), potassium channels (Obrdlik et al., 2004), and their interactions with SNARE proteins (Honsbein et al., 2009; Grefen et al., 2015; Zhang et al., 2015), specifically using the vectors modified by Petr Obrdlik and coworkers (Obrdlik et al., 2004). Improvements to the original vector system included increasing the signal-to-noise ratio through introducing a Met-repressible promoter driving the bait Cub fusion (Obrdlik et al., 2004), incorporating cloning sites for in vivo cloning as well as Gateway recombination technology (Obrdlik et al., 2004; Grefen et al., 2007, 2009), and altering linkers and tags (Grefen and Blatt, 2012b), all of which, together with the development of strains that allow a mating-based approach to be taken (Ludewig et al., 2003), have made the system suitable for high-throughput analysis (Box I; Lalonde et al., 2010; Chen et al., 2012b; Jones et al., 2014).

The split-ubiquitin system also suffers from false-positive results similar to those detected using the yeast two-hybrid system. In particular, in cDNA library screens, typical false positives include ubiquitin-related orthologs and proteolytic enzymes, which probably lead to the recognition of ubiquitin-specific proteases or direct cleavage of the PLV reporter, even in the absence of an interaction or prey (C. Grefen, unpublished data). This type of background activation of ubiquitin-specific proteases also might account for the observation that using the split-ubiquitin system in planta with a destabilized GFP as a reporter, whose cleavage can be detected via immunoblot, yields high background ratios compared with its application in yeast (Rahim et al., 2009). Nevertheless, optimizing the signal-to-noise ratio of this in planta split-ubiquitin system would make this in vivo PPI technique highly valuable.

### Other Applications

The split-ubiquitin system also is suited for trimeric analysis, a method known as the split-ubiquitin system bridge assay (Fig. 4C; Honsbein et al., 2009, Grefen and Blatt, 2012b; Grefen, 2014). The possibility of analyzing membrane proteins as potential enhancer, bridging, or inhibiting factors becomes increasingly interesting, for example, in the analysis of (ligand-induced) receptor oligomerization. Apoplastic proteins, peptides, small molecules, or drugs can be screened for their potential to inhibit or increase the interactions of membrane-bound receptors (Dinrberger et al., 2008; Grefen, 2014; Wehr and Rossner, 2016).

A novel modification, termed SPLIFF, enables spatiotemporal examination of PPIs (Fig. 4E; Moreno et al., 2013). The bait POI is C-terminally fused with mCherry followed by the Cub peptide. A destabilized GFP is fused to the C terminus of the Cub moiety. Bait and prey fusions are expressed in yeast strains of different mating types. Upon mating, the GFP is cleaved off and quickly degraded, increasing the mCherry-to-GFP ratio at the site of interaction. Time-lapse microscopy of the yeast enables the PPI to be monitored with high spatial and temporal resolution (Dünkler et al., 2015). If the high background reported for plant cells when using the split-ubiquitin system could be reduced and if the expression of the bait fusion is controlled by inducible promoters, SPLIFF would represent an interesting in planta tool for monitoring interactions in real-time.

### FRET: LIFETIME VERSUS INTENSITY

The discovery and subsequent engineering of fluorescent proteins revolutionized cell biological detection systems 20 years ago (Heim and Tsien, 1996). For the...
first time, protein tagging with fluorescent proteins allowed the subcellular and spatiotemporal distribution of proteins to be resolved and their dynamics to be studied in vivo. Expanding the fluorescent palette also contributed to further advancements in developing biosensors, subcellular markers, and optimized fluorescent proteins for interaction studies (Day and Davidson, 2009).

However, as much as the development of high-end confocal microscopes and fluorescent proteins has boosted the analysis of proteins on a subcellular scale, assessing colocalization and PPIs remain two separate issues (Shaw and Ehrhardt, 2013). Colocalization between two POIs using fluorescent proteins with different spectroscopic properties might suggest their involvement...
in the same pathway or protein complexes. However, spatial resolution is dependent on the diffraction limit of light microscopy, which is in the range of 200 nm. Considering this distance and (for simplicity) just one dimensionally, the size of a GFP molecule, which is roughly 3 × 4 nm (Yang et al., 1996), would allow 48 fluorescent protein molecules to fit between two POIs at either end. In other words, while image analysis would reveal the presence of both fusion proteins in the same spot, dozens of other protein molecules could be situated between them. Hence, the colocalization of fluorescence determined by standard light microscopy alone cannot provide information about protein complex formation.

A solution to this problem is provided by the physical phenomenon of FRET ( Förster, 1946), a process that, for example, enables the light-harvesting complex to transfer the energy of absorbed photons to the reaction center of photosynthesis. This non-radiative energy transfer is based on long-range dipole-dipole coupling between two molecules. Hence, using “fluorescence” instead of “Förster” is not only a misnomer by convention but also is misleading (Figs. 1B and 5A). FRET occurs when the energy relaxation of an excited donor fluorescent protein is not emitted via its own fluorescence but instead is emitted through non-radiative transfer of this energy to a nearby acceptor molecule; incidentally, the acceptor does not have to be a fluorescent protein but can simply be an energy sink for the donor (Lakowicz, 2006).

For FRET to occur, three prerequisites must be met: (1) the spectral overlap of donor emission and acceptor absorption must be sufficient for effective energy transfer; (2) the distance between fluorophores must be below 10 nm (see below and Fig. 5A); and (3) the dipole orientation of the fluorophores must be aligned, ideally in parallel fashion. Additional factors that influence energy transfer efficiency include the quantum yield of the donor (the ratio of photons emitted per photons absorbed) and the extinction coefficient of the acceptor, which describes the capacity of a molecule to attenuate energy. The distance at which 50% of the energy is transferred is called the Förster radius (R0). In general, the energy transfer is dependent on the inverse sixth power of the distance between donor and acceptor, and as a consequence, FRET is limited to distances of 1 to 10 nm (Sun et al., 2011). This distance is considerably lower than the diffraction limit of light, yet the efficiency of energy transfer allows optical analysis of PPI dynamics to be performed in vivo with high spatiotemporal resolution.

While FRET is often used to infer protein interactions, its detection actually informs on the vicinity and/or orientation, which can be visualized using another theoretical calculation (Lalonde et al., 2008). The cylindrical GFP has a volume (V) of roughly 30 nm3 (V = πr2h; where r = 1.5 nm and h = 4 nm). The 10-nm radius of a hypothetical sphere in which FRET occurs equals a volume of approximately 4,200 nm3 (V = 4/3πr3; where r = 10 nm), indicating that almost 140 GFP molecules could be considered within that area. For POIs tagged with GFP, this number would be far smaller, which demonstrates that even when FRET is detected, it does not necessarily prove a direct physical interaction but instead could result from the proximity of proteins, such as in a multiprotein complex. However, in a twodimensional space, such as a membrane domain, far fewer molecules can fit into the FRET radius. As a result, the observance of FRET with membrane proteins is more likely due to direct interaction, whereas FRET with soluble proteins has a probability, albeit small, to be caused by vicinity.

The use of FRET-based methods to detect PPIs appears to have outstripped the yeast two-hybrid system (Fig. 2B; Table II). One reason for the popularity of FRET is that, in addition to its use for detecting PPIs, it also can be used with genetically encoded, unimolecular sensors, and it can be used in native species in vivo (Frommer et al., 2009; Uslu and Grossmann, 2016). A range of sensor molecules have been developed or optimized for use in plants, enabling the detection of calcium (Krebs et al., 2012; Wagner et al., 2015), phosphate (Mukherjee et al., 2015), zinc (Lanquar et al., 2014), and abscisic acid (Jones et al., 2014a) as well as physiochemical states of the cell such as pH (Michard et al., 2008) and membrane voltage (Greif et al., 2015). One great advantage of using intramolecular FRET sensors lies in the theoretically equal stoichiometry, rendering ratiometric readouts independent of sensor concentration.

Detecting FRET through Sensitized Emission, Acceptor Photobleaching, and Fluorescence Lifetime Microscopy

There are a number of ways to detect FRET, each with its own set of benefits and pitfalls. For brevity, we will focus on the three most commonly used technologies: (1) sensitized emission; (2) acceptor photobleaching; and (3) fluorescence lifetime imaging (FLIM). More specialized methodologies are described in reviews focusing on FRET (Clegg, 2009; Sun et al., 2011; Ishikawa-Ankerhold et al., 2012).

Measuring the increase in intensity in acceptor emission when exciting the donor is the basic principle behind sensitized emission FRET. In practice, this can be done by exciting the donor in the range of its absorption maximum while measuring emission in the range of the acceptor’s emission spectrum. This method is straightforward, but it must be carefully executed, as unequal loading of the fluorescent proteins will give dramatically different results. Figure 5B shows the absorption (dashed lines) and emission spectra (solid lines) of mTurquoise2 and mVenus (modified from Hecker et al., 2015). The 4 weighted spectral overlap, which is depicted in dark gray, is one of three important factors for efficient FRET. However, the overlap between absorption (magenta) and emission spectra (green) of the donor and acceptor defines what is referred to as spectral bleed through or cross talk. Cross talk arises from the fact that all fluorescent proteins absorb and emit photons across a range of wavelengths rather than at a specific wavelength, like a laser. Therefore, even if detection is restricted to the peak emission of...
one fluorescent protein, it does not preclude the emission of other wavelengths. The same applies to excitation: even wavelength regions that are very low in absolute percentage of absorbance will lead to fluorophore excitation when high-excitation light intensity and/or large amounts of fluorescent protein are used. Only when wavelengths are chosen that lie completely outside of the emission and/or excitation region of a fluorescent protein can spectral bleed through be avoided. A simple test for bleed through is to observe the fluorescent proteins alone, at different concentrations, and to record the full spectral characteristics of each fluorophore.

Two types of spectral bleed through are common: (1) acceptor spectral bleed through (Fig. 5B, magenta), which occurs when excitation of the donor does not exclude excitation of the acceptor, causing an emission of the acceptor that is not due to FRET; and (2) donor spectral bleed through (Fig. 5B, green), where emission of the donor leaks into the emission spectrum of the acceptor. To distinguish FRET from bleed-through artifacts using sensitized emission, great care must be taken when choosing the controls, including donor only, acceptor only, and donor and acceptor together, as well as the analysis of spectral data (Müller et al., 2013).

Figure 5. FRET. A, Simplified Jablonski diagram. A fluorescent protein (here depicted as the cyan-colored crystal structure of a CFP attached to a POI in gray) that absorbs light (blue wavy arrow) causes an electron to be raised to an excited singlet state, \( S_1^* \) (blue solid arrow). Internal conversion (black wavy arrow) causes relaxation to the \( S_0 \) ground state (cyan solid arrow). Crossing of the energy barrier between \( S_1 \) and \( S_0 \) can cause the emission of a photon at a longer wavelength (red shifted, Stokes shift; i.e. fluorescence). If an acceptor molecule is in close enough proximity (here, YFP attached to a red-colored interacting protein), the energy can be transferred non-radiatively to the acceptor molecule. Relaxation of the acceptor molecule, in turn, can lead to even more red-shifted fluorescence. B, Absorbance/fluorescence spectrum of mTurquoise2 and mVenus. The dark gray surface depicts the \( \Lambda^4 \) weighted overlap integral. Acceptor and donor spectral bleed through are shown in magenta and green, respectively (see “FRET: Lifetime Versus Intensity” for details; image modified from Hecker et al., 2015). C, Example of a FRET acceptor photobleaching experiment. A nucleus expressing two interacting proteins attached to a donor and an acceptor fluorophore. After bleaching (right), the acceptor fluorescence is almost completely lost, but an increase in donor fluorescence can be detected (image from Hecker et al., 2015, modified for visualization purposes only). D, Exemplary fluorescence lifetime decay curve of a putative donor molecule when FRET is occurring (red solid curve; \( \tau_1 \)) or in the absence of FRET (cyan solid line; \( \tau_2 \)). Fluorescence lifetime \( \tau \) is the average time that a fluorescent protein resides in the excited state and at which fluorescence intensity decreased to \( 1/e \) of its initial value.
Uncontrolled gene dosage can easily be misinterpreted as a specific signal in sensitized emission assays. For example, donor emission in the acceptor peak channel when the acceptor is absent or in lower abundance could be interpreted as the presence of the acceptor. Conversely, when the abundance of the acceptor exceeds that of the donor and is excited by the same light source, more emission in the acceptor would be taken as an indication of FRET when none exists. One solution in these circumstances is to adjust the relative amount of fluorescent proteins by controlling gene dosage (Hecker et al., 2015).

Another way to detect FRET is to determine the increase in donor intensity after photobleaching of the acceptor (Fig. 5C). The rationale behind this approach lies in the loss of energy transfer from the donor to the acceptor when bleached, resulting in an increase in donor fluorescence intensity and lifetime. Acceptor photobleaching is a straightforward method that does not require high-end microscopes, is not affected by spectral bleedthrough, and allows the sample analyzed to serve as its own control.

However, a number of pitfalls should be considered when applying acceptor photobleaching. Bleaching of the acceptor also can bleach the donor, causing its photoconversion or making it generally toxic to the specimen (Ishikawa-Ankerhold et al., 2012). As bleaching occurs in the excited state of a fluorescent protein, the laser line used to bleach the acceptor must not overlap with the excitation spectrum of the donor. Another issue is the temporal delay in recording; in images between prebleaching and postbleaching, POIs or organelles can move in or out of the focal plane, thereby affecting fluorescence collection and rendering a potential change in donor fluorescence intensity meaningless. Solutions include fixing the tissue or using immunolocalization, but such handling also can influence FRET efficiency (Bücherl et al., 2013; Joosen et al., 2014). As mentioned above, stoichiometric differences in the expression of an acceptor and donor also can reduce confidence in the detection of FRET itself. Again, guaranteeing at least equal gene dosages can increase FRET efficiency and confidence in acceptor photobleaching analysis (Hecker et al., 2015).

FLIM is a method that is unaffected by spectral bleedthrough, differences in donor or acceptor concentration, excitation intensity variance, and photobleaching (Bücherl et al., 2014). The fluorescence lifetime $\tau$ of a fluorescent protein represents the average time it resides in its excited $S_1$ state before relaxation to the $S_0$ ground state through photon emission. A disadvantage of FLIM is that it requires sophisticated hardware add-ons, including a pulsed laser that can emit extremely short laser bursts between which the decay of donor fluorescence can be measured pixel by pixel. Nevertheless, having this information avoids many of the difficulties of spectral bleedthrough, as one needs only look at donor output.

A typical decay curve is depicted in Figure 5D. FRET results in a decrease in $\tau$ for the donor due to energy transfer (Fig. 5D, red curve), whereas without FRET, the value for $\tau$ greater (Fig. 5D, cyan curve). FLIM requires control measurements of the donor without the acceptor as well as a control of the donor with a fluorescently labeled, unrelated but colocalizing protein.

Of all the methods used to detect FRET, FLIM is probably the most reliable, allowing highly dynamic spatiotemporal resolution of PPIs. Even so, FLIM measurements can be affected by environmental factors such as pH, temperature, and the refractive index of the medium. These properties of fluorophores can be exploited when synthesizing biosensors for FLIM analysis (Frommer et al., 2009).

### Optimal FRET Couples

Table III summarizes FRET couples and examples of their use in the plant field since Theodorus Gadella and coworkers first demonstrated that the cyan fluorescent protein (CFP)/yellow fluorescent protein (YFP) couple is suitable for detecting FRET in planta (Gadella et al., 1999). A detailed discussion of individual point mutations, resulting spectral changes, and their implications for FRET can be found elsewhere (Kremers and Goedhart, 2009; Müller et al., 2013).

Despite being outdated, the classic FRET pair CFP/YFP still enjoys great popularity (Tramier et al., 2006; Bhat, 2009). Continuous improvements and optimization of other fluorescent proteins and the inherent flaws of CFP and YFP suggest their replacement with more advanced fluorescent protein couples (Table III; Hecker et al., 2015). Like all GFP-based fluorescent proteins, the original CFP (and YFP) has a weak tendency to dimerize, a feature that can complicate detecting FRET when cluster formation of an acceptor or donor results in the quenching of fluorescence (Zacharias et al., 2002). In addition, CFP has a low quantum yield, biexponential fluorescence decay, and long fluorescence lifetime that renders it extremely sensitive to bleaching (Tramier et al., 2006). YFP is much brighter than CFP but has an intrinsic sensitivity to environmental conditions such as pH and halide concentration that is responsible for artifacts in biosensors (Schwarzländer et al., 2014). Together, the resulting FRET couple has a small $R_0$ and a large differential in brightness, which can mask differences in the concentrations of the donor and acceptor and lead to artificial FRET detection, depending on the method used.

What would characterize an optimal FRET couple? The ideal FRET pair consists of a donor that is monomeric, has a high quantum yield, and is relatively insensitive to photobleaching yet features a long fluorescence lifetime and a large Stokes shift (the distance between absorption and emission maxima). An optimal acceptor is also monomeric, has a high absorption coefficient, and, if acceptor photobleaching is planned, should be photolabile to guarantee at least 90% bleaching. The FRET pair together should have a large spectral overlap, yet ideally with little bleed through, nearly equal, short maturation times, and a high $R_0$ to allow detection of an interaction between large proteins or complexes (Table III).
Future Applications of FRET

FRET analysis is an excellent approach to studying in planta PPI dynamics in real-time, as complex association is not hindered through irreversible complementation. FRET can be expanded to detect ternary interactions by using more than two fluorescently labeled proteins in three-color spectral FRET (Sun et al., 2010), two-step FRET (Seidel et al., 2010), three-chromophore FRET (Galperin et al., 2004), or triple FRET (Peter et al., 2014). In addition to the donor and acceptor, these techniques take advantage of a red-shifted third fluorescent protein, acceptor 2, for analyzing larger protein complexes that introduce a significant increase in $R_0$. A persistent problem, however, is the need for spectral separation of the donor and acceptor 2, because overlap between the two short-circuits the acceptor (Seidel et al., 2010). The combination of FRET and BiFC is not a three-color FRET approach, but it represents a ternary interaction analysis nonetheless. This approach has been applied successfully in planta to detect ternary SNARE complex formation using acceptor photobleaching (Kwaaitaal et al., 2010).

In many ways, FRET is still in its infancy in plant research (Fig. 2; Table II). With the development of FRET pairs that use excitation wavelengths in the range of the green gap of chlorophyll, problems derived from tissue autofluorescence can be overcome. Confocal laser scanning microscopy can be used for detection in FRET analysis, as well as BiFC, a technique currently favored by most plant scientists that we discuss in the following section.

BIMOLECULAR FLUORESCENCE COMPLEMENTATION: FROM CHERRY PICKING TO QUANTIFICATION

A considerable number of biological studies, especially in the plant sciences, feature the PCA technique BiFC as the method of choice when it comes to detecting PPIs (Fig. 2C; Table II). BiFC was originally described in E. coli when Ghosh et al. (2000) demonstrated that a fluorophore such as GFP could be split into N- and C-terminal fragments, which on their own are non-fluorescent (Fig. 1E). Upon interaction, the fused fragments (now in close proximity) can refold, mature, and fluoresce (Ghosh et al., 2000). The method was later adapted for use in mammalian and plant systems (Hu et al., 2002; Bracha-Drori et al., 2004; Walter et al., 2004).

Despite its popularity, BiFC suffers from two major limitations: (1) the irreversible complementation of the split fluorescent protein fragments with estimated half-life times of 10 years precludes the analysis of interaction dynamics (but not necessarily their frequency); and (2) the tendency of most split fragments to spontaneously reassemble autonomously if unhindered (Box II; Magliery et al., 2005; Berendzen et al., 2012; Ohashi et al., 2012). Both flaws have the additive effect of increasing the likelihood of false positives, as the split fragments will eventually reassemble on their own; because this self-assembly is effectively irreversible, false-positive signals will accumulate over time. The challenge has been and remains how to determine when this is the case.

While a range of publications simply show exemplary images of black negative controls and bright positive samples without biochemical validation of expression (as exemplified in Fig. 1E), a growing number of researchers have come to view BiFC with greater caution due to these inherent complications (Lalonde et al., 2008; Grefen and Blatt, 2012a; Horstman et al., 2014). Here, rather than enumerating the different (individual expression) vector systems that have been reported since the original development of BiFC, which unfortunately have done little to tackle the issues associated with the spontaneous reassembly of split fluorescent protein fragments and its irreversibility, this section addresses the issues around the use of BiFC and their potential solutions.

Most in planta BiFC systems rely on overexpression of the split fluorescent protein fusions, conditions

<table>
<thead>
<tr>
<th>FRET Pairs</th>
<th>Donor</th>
<th>Acceptor</th>
<th>Donor Excitation $\lambda_{\text{exc}}$</th>
<th>Donor Emission $\lambda_{\text{em}}$</th>
<th>Donor Quantum Yield</th>
<th>Acceptor Extinction Coefficient</th>
<th>$R_0$</th>
<th>Examples of Fluorescent Protein Pairs Used in Plant Research</th>
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<tr>
<td>T SAPhine</td>
<td>mOrange2</td>
<td>399</td>
<td>565</td>
<td>0.6</td>
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<td>4.66</td>
<td>FLIM, AB Bayle et al. (2008)</td>
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<td>ECFP</td>
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<td>434</td>
<td>527</td>
<td>0.36</td>
<td>72,000</td>
<td>4.72</td>
<td>FLIM Sheerin et al. (2015)</td>
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<tr>
<td>mTRQ1</td>
<td>mVenus</td>
<td>434</td>
<td>527</td>
<td>0.84</td>
<td>105,000</td>
<td>5.73</td>
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<tr>
<td>mTRQ2</td>
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<td>610</td>
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<td>72,000</td>
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<td>0.65</td>
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<td>6.15</td>
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<td>596</td>
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Designing BiFC Experiments

In the following section, we discuss the precautions that should be taken when designing a meaningful BiFC analysis, particularly in terms of those related to (1) construct design, (2) expression system, (3) detection system, and (4) suitable controls.

Construct Design

An important choice to be made is between using an available vector system (preferentially a system that allows cloning of both fusion proteins and a transformation marker from one strand of DNA; Grefen and Blatt, 2012a; Gookin and Assmann, 2014) and building one’s own using available cloning techniques such as GreenGate (Lampropoulos et al., 2013), GoldenGate (Engler et al., 2008), Gibson Assembly (Gibson et al., 2009), Multisite Gateway (Sasaki et al., 2004), and the 2in1 conversion system (Grefen and Blatt, 2012a). For example, expressing fusion proteins from a single T-DNA causes less variability in their expression levels in individual cells (Fig. 6A). By contrast, when two A. tumefaciens strains were mixed with individual plasmids and infiltration into Nicotiana benthamiana leaves, an overall high level of variability of expression was observed, with only approximately 80% of cells cotransfected with both constructs (Hecker et al., 2015). This problem can be overcome through statistical analysis, but the situation is further complicated because, like the FRET assay, unequal gene dosage will yield misleading results, impeding meaningful analysis when using independent T-DNA transformations.

Recently, it was shown that splitting the YFP-derived Venus at amino acid 210 (instead of the classical amino acid 155/156 split) causes a significant reduction in background fluorescence (Ohashi et al., 2012). This split system has been implemented in a plant vector system using the expression of multiple proteins from a single T-DNA, including a Golgi-localized CFP marker as the transformation control (Gookin and Assmann, 2014). However, the binary vector system uses classical cloning sites, requiring tedious, sequential cloning. Furthermore, the initial publication only includes exemplary images, with neither quantification nor immunoblot analysis to confirm protein expression in any of the experiments (Gookin and Assmann, 2014); quantitative confirmation of this split site in planta is still pending.

The other currently available multicistronic expression system features the Gateway-compatible 2in1 system, which allows simultaneous transformation of two independent genes into a single T-DNA. Additionally, this system contains a constitutively expressed, soluble red fluorescent protein (RFP) to be used as both transformation control and ratiometric marker (Fig. 6B; Grefen and Blatt, 2012a). This ratiometric BiFC system has been utilized successfully by a number of different laboratories with a diverse range of PPIs (Karnik et al., 2013b; Lipka et al., 2014; Albert et al., 2015; Kumar et al., 2015; Le et al., 2016; Perrella et al., 2016).

Expression System

Should the experiment be performed in a heterologous or homologous expression system, using transient transfection of leaves or protoplasts, or in stably transformed plants? If stable transformation is attempted, the irreversibility of the interaction at hand should be acknowledged. Many processes in signaling or trafficking are facilitated by short-lived PPIs, where interaction is as important as the eventual release; however, BiFC will literally cross-link the interaction partners in vivo, preventing their release, which might be paramount for their physiological function. A possible exception is when the actual interaction targets the protein complex for degradation, which is not hindered by the complemented fluorescent protein. In general, we presume that most interaction couples will not yield viable transformants independent of the strength or spatial distribution of the expression. In our own experience, the heterodimerization of the Arabidopsis ethylene receptors cannot be detected using BiFC, perhaps due to dominant-negative effects of the irreversibly bound receptor dimers (Grefen et al., 2008). Methods allowing reversible and dynamic interactions, such as FRET-based techniques, are more appropriate for detecting PPIs in stably transformed Arabidopsis lines.

Detection System

The detection of fluorophore complementation should be quantitative instead of being based on a biased choice of exemplary images. As Richard Immink points out in his excellent critique of the current use of BiFC in plant research, “[T]he goal of a BiFC experiment is to obtain strong support of a protein-protein interaction and not just to obtain an image of a fluorescent cell” (Horstman et al., 2014). The goal should be to quantify the complemented fluorescence (or its absence)
Suitable Controls

Validating the PPI with a suitable negative control is key to guarding against false positives. Validation also is essential for instances in which no interaction is apparent. An absence of fluorescence can occur (1) if the cell is untransformed, (2) if the cell is transformed incompletely (with only one of two plasmids), (3) if there is a lack of expression of either or both fusion proteins, or (4) if the interaction is absent. Indeed, some of the main issues with many published BiFC results are the incorrect use of controls (irrelevant or unsuitable), a lack of quantification (e.g. showing images but no effort at statistical analysis), and the failure to verify protein expression, such as by immunoblot analysis (Horstman et al., 2014). It has been suggested that one of the best negative controls is to include an untagged version of one of the two interacting POIs as a competitor (Kodama and Hu, 2012). The rationale is that one should observe a decrease in complemented YFP in the presence of the competitor (Fig. 6C). However, we tested this approach using the 2in1 system (Grefen and Blatt, 2012a) and a competitor tagged with CFP to verify its expression while quantifying the ratio of its intensity to that of the complemented YFP. The use of a competing protein did not lead to a decrease in BiFC (C. Grefen, unpublished data). This result is not surprising given the inherent feature of irreversible YFP complementation. Similarly, Morell et al. (2008) found that BiFC was only blocked if a specific competitor was added before the POIs were induced, and similarly, it could not be reversed after complex formation. An equilibrium generally can be influenced by the presence of a competitor, but irreversible product formation shifts the equilibrium to the product side (to the right in Fig. 6C). Hence, if this analysis is to be of any use, competition must be monitored in a tight time frame, as differences in the kinetics of only YFP formation might be detectable. Steady-state competition experiments only make sense using a truly reversible detection system. Recently, a method was published featuring a reversible BiFC approach based on a phytochromic fluorophore (To et al., 2016). It remains to be seen whether this system can overcome the limitations associated with classical BiFC.

Taken together, when selecting a BiFC system (produced in house or previously reported), the strength of the promoter, tag orientation, type of split fluorophore (physicochemical properties, split sites), and, most of all, careful choice of controls are important factors to consider. To gain credible results from BiFC analysis, future work needs to incorporate what was learned from past experience: it is essential to ensure the expression of all fusion proteins from the same stretch of DNA, to include a fluorochrome marker allowing ratiometric analysis of an interaction (Grefen and Blatt, 2012a), or to use a combination of these approaches with split fluorescent protein against a coexpressed fluorophore that serves as both a transformation control of the cell under study and a reference marker for ratiometric analysis (Fig. 6B; Grefen and Blatt, 2012a). The use of a large sample size also may aid in the quantification of BiFC signals, taking advantage of methods such as flow cytometry, as discussed below.

In Vivo Protein-Protein Interaction Techniques

Box III: The Special Case of PCAs

Split protein fragments often carry inherent affinity toward each other. Hence, determining the suitability of a PCA-based technique for a given PPI depends on the $K_d$ values of the split probes and needs to take account of the threshold of a $K_d$ where the association of these probes becomes the driving force of an interaction rather than the PPI in question (Box II; Müller and Johnson, 2008). The balance between PPI affinities and the affinity of the marker or tag is an inherent issue of the heavily used BiFC technique, where the spontaneous reassembly of the unmodified nonfluorescent fragments and the irreversibility of their reassociation result in a negligibly small $K_d$ (see “BiFC: From Cherry Picking to Quantification”; Do and Boxer, 2011). In fact, an excess of more than 100 μ peptide competition cannot disrupt a reconstituted YFP molecule, which remains intact even when incubated in 2 M urea for several days (Morell et al., 2007). This clearly highlights the notion that a PCA method does not allow conclusions to be drawn on the dynamics of a transient PPI if the $K_d$ of the split probes is orders of magnitude smaller than that of the PPI couple of interest. We stress that useful biological information still can be obtained with BiFC, but the user should be aware that the BiFC readout is related to the $K_{C_{ON}}$ and is normally not related to the $K_{C_{OFF}}$ of a POI and that other factors likely play a large role in BiFC ($K_d$ is the ratio of $K_{C_{OFF}}$ to $K_{C_{ON}}$).

Many additional interdependent factors obscure the results of PCA-based techniques. For example, the localization and concentration of split probes fused to POIs have strong effects on reassembly; PPIs between membrane proteins can report artifactual (positive) results through an increase in the concentration of bait and prey in the two-dimensional membrane space and the resulting increased collision probability. Also, proteostatic effects, including maturation time, stability, and turnover rates of both the POI and split probes, contribute to the signal strength of a PCA method. Not all proteins are equally stable in a cell, which presents issues, especially for in vivo experiments in which POIs may be regulated natively and independently. Furthermore, the split probes fused to the POIs may influence the turnover of the whole fusion protein, resulting in the presence of suboptimal (or at least unequal) amounts of bait and prey fusions. A delay in the maturation of one POI as opposed to the predicted partner affects kinetics during reassembly. Equally important is the steric accessibility of split probes in fusions for reconstitution: an interaction between bait and prey can result in occlusion of the interaction surfaces of the split probes, resulting in a false-negative readout. Such problems might be resolved through the addition of flexible linkers, and they can provide information about protein structure. All of these factors must be considered when choosing the optimal PCA technique.
fragments that might have less intrinsic affinity toward each other (Ohashi et al., 2012).

In any event, fluorescence should be detected as early as possible and not at a time point when all possible interactions are complete (or saturated), as recommended by some. With many proteins expressed using the 35S promoter, the highest expression levels occur after 3 d (Grefen et al., 2010b), an observation that underpins the importance of measuring BiFC fluorescence after 1 d (or a maximum of 2 d) post infiltration; weak expression levels often can be informative in these circumstances. Time-lapse analysis over a time frame of hours could also distinguish true from false interactions and, depending on the split sites of the fluorescent protein fragments, might even allow weak and strong interactions to be distinguished. Using a large sample size also might help sift the chaff from the wheat, for example, by taking advantage of protoplast expression systems and flow cytometry.

**Flow Cytometry Lights the Way for Quantitative BiFC**

Over the past decade, various laboratories have coupled BiFC analysis with flow cytometry for analysis, sorting (fluorescence-activated cell sorting), or both (Miller et al., 2015). Flow cytometry is a technique used to examine a population of particles or cells in free suspension, one by one, typically by collecting and analyzing fluorescence and scattered light. This technique allows individual fluorescence emission intensities...
to be obtained for large populations (n > 10,000) in a very short time (5 min or less). Flow cytometry turns the fact that BiFC is irreversible from a problem into an attribute, as trapped, weak interactions can be detected and quantified using this method (Morell et al., 2007; Berendzen et al., 2012).

Ozalp et al. (2005) found that, due to its irreversibility, BiFC gives an output representing a summation of protein interactions over time. Looking at a large enough population of cells, one can observe variance in signal strength. In order for a BiFC signal to be scored, enough reconstituted fluorescent protein must accumulate to exceed the intrinsic autofluorescence of an individual cell. Articles have been published showing only the percentage of cells with BiFC signals (Llorca et al., 2015), only their intensity (Kim et al., 2012; Wang and Carnegie, 2013), or a combination thereof (Li et al., 2010; Lee et al., 2011; Berendzen et al., 2012). Variance in interaction strengths, both weak and strong, were observed in cross-check glutathione S-transferase pull-down assays (Wang and Carnegie, 2013), lending further support to the notion that BiFC flow cytometry indeed captures varying degrees of interaction strengths and are not artifacts of transfection variance.

Where can we expect to observe weak interactions and how do we know they are genuine? According to Hu et al. (2002), the split fluorescent protein fragments are estimated to assemble with a half-time of 60 s (Hu et al., 2002). Due to the irreversibility of BiFC, it has been proposed that BiFC might be able to capture an enzyme interacting with its substrate (Morell et al., 2007; Miller et al., 2015). In this case, one can expect very weak (infrequent) signals if we presume that the PCA is focused faster than other proteins and, hence, reports turnover in addition to interaction, as observed for the RUM1 IAA protein (von Behrens et al., 2011). Thus, although not thoroughly demonstrated, BiFC flow cytometry appears to be able to capture information pertaining to interaction frequency and binding strength by providing a quantitative measure of BiFC. The combination of flow cytometry and ratiometric BiFC (Grefen and Blatt, 2012a) should be an even more powerful approach, as the intrinsic RFP helps to exclude untransformed protoplasts.

SPLIT-LUCIFERASE COMPLEMENTATION ASSAY: LET THERE BE LIGHT

Originally developed for use in mammalian cells (Ozawa et al., 2001), the split-luciferase assay and its subsequent complementation have since been established successfully in plants to identify a variety of PPIs (Fujikawa and Kato, 2007; Li et al., 2011; Lin et al., 2015; Lund et al., 2015). The split-luciferase complementation assay is a PCA that relies on the complementation of two split fragments of a luciferase protein and its subsequent detection through substrate conversion (Fig. 1F). Light-producing species can be found in almost all phyla of life. From bacteria and fungi to vertebrates, from land-dwelling hexapods to marine mollusks and fishes, bioluminescence has evolved convergently in as many as 40 different species (Haddock et al., 2010). The terms luciferase and luciferin are generic and are used for all classes of light-producing enzymes; their respective substrates are usually preceded by the species name. Photon emission emerges as a side product of oxidative decarboxylation of a luciferin by its corresponding luciferase (Woo et al., 2008; Marques and Esteves da Silva, 2009). Similarities in substrate conversion aside, luciferases differ substantially from each other in terms of structure, size, substrate specificity, and cofactors. Table IV provides an overview of the different systems used in planta, where the luciferases from the North American firefly (Photinus pyralis) and the sea pansy (Renilla reniformis) are utilized exclusively, while in mammalian cells, other luciferases derived from click beetle, Gaussia spp. and nanoLUC also have been used (Remy and Michnick, 2006; Kim et al., 2007; Dixon et al., 2016).

Fiery Versus Renilla: Comparison of Properties

Firefly luciferase is a 62-kD protein comprising a single polypeptide chain folded into a large N-terminal domain and a small C-terminal domain (Conti et al., 1996; Thorne et al., 2010). This enzyme uses D-luciferin as a substrate. In the presence of Mg²⁺, ATP, and oxygen, firefly luciferase catalyzes a two-step reaction, converting D-luciferin into oxyluciferin, AMP, and CO₂, thereby releasing a photon (Marques and Esteves da Silva, 2009). The emission is visualized in the green-yellow spectrum of light (540–570 nm). The reaction creates inhibitory products, leading to an inactivation of firefly luciferase that can be counteracted by CoA (de Ruijter et al., 2003; Fraga et al., 2005; da Silva and da Silva, 2011). The reaction is pH and temperature sensitive and will undergo a red shift if firefly luciferase is exposed to acidic pH or higher temperatures (Seliger and McElroy, 1964; Fraga, 2008).

Renilla luciferase is 37 kDa in size and also consists of a single polypeptide. Therefore, the split forms of this enzyme result in moieties that are considerably smaller than the split firefly PCAs. The size of the split probes can become an important factor when analyzing PPIs of small proteins. Here, large probes could potentially mask interaction sites and prevent the detection of a
PPI. The substrate of Renilla luciferase is coelenterazine, which is catalyzed into coelenteramide and CO₂ under the consumption of oxygen, leading to the emission of blue light (480–510 nm; Matthews et al., 1977; Woo et al., 2008).

Firefly luciferase emits green-yellow to red light, which, however, is less bright than the blue light that Renilla luciferase produces. Also, firefly luciferase requires ATP as a cofactor, which might not be sufficiently accessible in all cellular compartments or might affect endogenous pools, thereby interfering with cellular physiology. Renilla luciferase does not require ATP to catalyze its reaction; however, coelenterazine, the substrate of Renilla luciferase, is unstable and can undergo spontaneous oxidation (Zhao et al., 2004), whereas D-luciferin is sufficiently stable to be used successfully to monitor circadian rhythms (Millar et al., 1992). Interestingly, coelenterazine can be transported in human cells by a P-glycoprotein (Pichler et al., 2004). This family of proteins also exists in plants and plays a role in polar auxin transport (Geisler and Murphy, 2006).

While split-luciferase complementation is a useful in vivo technique, it is equally suitable for in vitro analysis (Ohmuro-Matsuyama et al., 2013). Reconstitution of luciferase is in many cases reversible (Stefan et al., 2007; Li et al., 2011; Lund et al., 2015), and, at least for firefly split-luciferase complementation, the emission of light is dependent on the interaction of the fused partners (Dale et al., 2016). In addition, the short half-life of the luciferases (firefly luciferase, approximately 3 h; Renilla luciferase, approximately 4.5 h; Thorne et al., 2010) makes split-luciferase complementation an ideal system for monitoring dynamic interactions in real-time and makes it suitable for high-throughput analysis (Li et al., 2011; Lund et al., 2015). With regard to free fragments, any possible background from the firefly luciferase moiety is orders of magnitude lower than that of the reconstituted complex or the full-length protein (Dale et al., 2016).

As plants are not bioluminescent organisms, split-luciferase complementation is a very sensitive detection system, giving high signal-to-noise ratios. The intrinsic light production via luciferase avoids the use of exogenous light sources, preventing plants or cells from photodamage or a decline in signal intensity due to bleaching. However, luciferase activity must be measured in the dark, which should be considered, for example, when studying light-dependent processes. Finally, the substrates of luciferase, D-luciferin and coelenterazine, must be exogenously applied. In planta, this can be easily achieved via the incubation of protoplasts or watering (i.e. spraying the plants with dissolved luciferin; Chen et al., 2008).

The advantages of the split-luciferase complementation assay include flexible temporal control with fast turnover and/or reversibility of probe assembly, thereby allowing for highly dynamic PPI analysis in planta. Nevertheless, the technique appears to be neglected among PPI techniques used by plant scientists (Fig. 2C). Implementation of other luciferases (Gaussia spp., click beetle, or nanoLUC), which may be more suitable for the demands of plant systems, will likely pull the split-luciferase complementation assay out of its niche existence. In fact, the nanoLuc split-luciferase complementation system NanoBiT, which has been engineered to be ideal for most PPIs (Dixon et al., 2016), will hopefully be available for plant scientists in the near future.

### IN PLANTA COIP: ONE TO BIND AND ONE TO FIND

Given the range of techniques featured in this review, CoIP appears to be the odd one out, a chimera between an in vivo and in vitro system. Correctly labeled an ex vivo technique, CoIP is often used as an alternative method to one of the above systems, allowing the detection of an interaction in specific tissues or developmental stages, under experimentally determined conditions, and/or in different genetic backgrounds.

Historically speaking, CoIP was adapted from column affinity chromatography (or affinity purification; Cuatrecasas et al., 1968), which is based on the fact that any given biomolecule usually has an inherent recognition site that can be bound by another natural or artificial molecule (e.g. an enzyme binding its substrate or inhibitor; Cuatrecasas et al., 1968; Magdeldin and Moser, 2012). Thus, affinity purification is principally based on molecular recognition between a matrix-bound molecule and the target molecule in an extract that is presented to it (Fig. 1C). This approach was originally used to purify biomolecules of interest from a solution or cell lysate. The technique is simple, involving the setting up of a column and binding of the target molecule, followed by washing and elution, with all steps processed consecutively in the column (Magdeldin and Moser, 2012).

CoIP can be categorized as a specialized or small-scale affinity purification that exploits the antibody-antigen

<table>
<thead>
<tr>
<th>Luciferase</th>
<th>Amino Acids</th>
<th>Expression System</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Firefly</td>
<td>1–398</td>
<td>394–550</td>
<td>N. benthamiana leaves</td>
</tr>
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Table IV. Available split-luciferase fragments and their first use in planta
The principle of CoIP is that the POI or antigen forms an immune complex in a cell lysate with its specific antibody. As the antibody is bound to beads (agarose or magnetic), which are usually coated with proteins (e.g., Protein A) to capture antibodies, the immune complex can be precipitated easily. Unspecifically bound proteins can be washed off using appropriate detergents and salt conditions, allowing purification of the POI in its complex. After elution, the interacting candidates can be analyzed by various assays such as western blotting, ELISA, or MS (Butler et al., 1978; Masters, 2004; Monti et al., 2005). The combination of CoIP and MS analysis is a particularly powerful screening tool for discovering novel interactors or establishing interaction maps of a POI (Miernyk and Thelen, 2008).

CoIP often is performed using native antibodies raised against the POIs or using epitope tags (Qoronfleh et al., 2003). Notably, the discovery that antibodies of the camelid family require only part of the H chain for antigen recognition, the so-called nanobody, of the camelid family require only part of the H chain has greatly enhanced binding specificity while reducing incubation times (Hamers-Casterman et al., 1993; Harmsen and De Haard, 2007; Muyldermans, 2013).

Nevertheless, weak or transient interactions are difficult to detect using CoIP (Lee et al., 2013; Avila et al., 2015), although cross-linking can be used to help stabilize the protein complexes (Garcia-Molina et al., 2014; Pertl-Obermeyer et al., 2014). Additionally, an interaction in CoIP could be due to the presence of a protein complex of multiple interactors, with an apparent binary interaction facilitated by known or unknown cofactors (Hall, 2005). This comes as a blessing in disguise, as it allows higher order protein complexes or ligand-induced dimerization to be dissected, a phenomenon that is neatly exploited when an interaction between two POIs is either induced by an exogenously applied factor (ligand, pathogen, and so on) or disrupted in a knockout line of the endogenous bridging factor (Chinchilla et al., 2007; Albert et al., 2015; Fuchs et al., 2016).

Recently, two novel techniques have been developed that circumvent the limitations of traditional CoIP. Both developments, single-molecule pull down (Jain et al., 2011) and real-time single-molecule CoIP (Lee et al., 2013), use total internal reflection microscopy (Axelrod, 2001) to detect interactions on a coverslip. Both methods involve immobilizing the bait POI on the surface of a coverslip while the prey protein is labeled with a fluorescent tag. Interaction can be visualized and quantified through the emergence of a fluorescent spot. Using multiple prey proteins with different fluorophores allows for the exploration and permutation of higher order protein complexes. Additionally, combining total internal reflection microscopy with photobleaching or lifetime imaging allows for stoichiometric analysis (Gust et al., 2014). These methods have not yet been applied in the plant field but will surely provide a powerful platform in the near future.

**CONCLUSION**

Even if all points outlined in the introduction are taken into account, one is spoiled for choice in selecting the right PPI technique to detect a POI couple. Every method comes with advantages and drawbacks, underpinning the importance of using combinations of alternative detection methods. Our analysis also highlights how vital it is to have a basic understanding of the biology behind each method. An example (one that is far too often overlooked) is the issue of probe affinity in PCAs (Table in Box II). While it might be beneficial to use a method that detects weak, transient interactions by literally cross-linking them, this irreversible fusion will undoubtedly have an impact on the detection system (organism) and, therefore, requires the use of appropriate negative controls.

Certainly, the best negative controls in all PPI experiments are point-mutated POIs using site-directed mutagenesis approaches (Karnik et al., 2013a), as long as the stability, turnover, and localization are not influenced by the mutation. Conversely, if such a mutation does not affect the interaction signal, the user may be justified in his/her suspicions that the PCA detects other factors (see Box III). These factors might provide biologically important insights, but they may not necessarily inform on any interaction.

Some of the challenges of PCA techniques, as mentioned above, need to be assessed when designing new probes for improved PPI detection methods. For example, although not yet tested in plants, the design of the NanoBiT system has involved determining the Kd of the free fragments, characterizing their turnover rates, and documenting differences in steric accessibility (Dixon et al., 2016).

Reversibility in probe interactions is an important feature, as it reduces the rate of false positives and allows kinetic analysis of interactions (Michnick et al., 2007). While two-hybrid methods such as yeast two-hybrid analysis and FRET can be used to study dynamic interactions, PCA-based methods usually suffer from probe affinity and, in the case of BiFC, irreversibility.

Future developments will need to extend PPI methods to accommodate the detection of multiple interaction partners or competitors to help dissecting protein complexes.

For the detection of transient interactions, probe affinity has to be adjusted to allow for kinetic and dynamic analysis of – ideally – single molecule interactions.

Protein expression under native, cell-type-specific or inducible conditions should allow spatiotemporal resolution of interactions thereby distinguishing biologically meaningful interactions from artifacts potentially caused through aberrant (mislocalized) overexpression.

Side effects of unequal gene dosage causing unbalanced stoichiometry of interactions partners need to be avoided and intrinsic controls – not least to aid in quantification – should be implemented.
of assembly. However, even in a reversible PCA method such as the split-ubiquitin system, the finite cleavage of the reporter and its resulting signal amplification preclude dynamic PPI analysis. Fluorescent reporters are the preferred choice for such kinetic studies, as the signal is generally lost upon dissipation of the interaction, at least for FRET or light emission using the PCA split-luciferase complementation techniques.

While one method alone is never sufficient to validate a PPI, even when three different techniques are used, the failure of one of these techniques to show an interaction does not dismiss the other two positive results. Various technical features of the method might be the underlying reason for its failure. For example, if a binary interaction is in reality trimeric and the third method lacks the additional factor, such information would represent a biological explanation worthy of further attention.

OUTLOOK

In this omics age of ours, a gap remains between the sheer scale of the data accumulated and our understanding of basic fundamental processes at the molecular level. As informative as large-scale PPI approaches may be, deciphering binary interactions and elucidating components of small protein complexes, their dynamics, stoichiometry, and structure-function relationships will ultimately provide the molecular and functional details that are necessary to decode cellular mechanisms.

Newly developed, sophisticated, superresolution microscopy techniques such as structured illumination microscopy, stimulated emission deproteinluciferase microscopy, photoactivated localization microscopy, and stochastic optical reconstruction microscopy make it feasible to detect PPIs at the single-molecule level (Gutierrez et al., 2010; Komis et al., 2015; Sydor et al., 2015). In addition, spectromicroscopy techniques (Harter et al., 2012) such as fluorescence cross-correlation spectroscopy (Machán and Wohland, 2014) represent technological improvements that greatly improve the detection of the spatiotemporal resolution of interactions, particularly in (but not limited to) membrane environments. While these novel techniques allow dynamic PPI studies to be performed in an in vivo environment, the greatest limitation to their application at present remains the expensive equipment needed for their execution.

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