Affinity Purification and Mass Spectrometry: An Attractive Choice to Investigate Protein-Protein Interactions in Plant Immunity

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Abstract: The use of affinity purification to isolate protein complexes from biological tissues, followed by mass spectrometry (AP-MS), has ballooned in recent years due to improvements in affinity purification protocols, sizeable increases in nucleic acid sequence data essential for interpreting mass spectra, and technological advances in mass spectrometry. Plant biologists are now exploiting AP-MS to identify plausible protein-protein interactions crucial to plant defense systems. As a result, knowledge of protein interactions in plants has grown. For example, new protein partners have been found to interact with RIN4 and RPS2, two plasma membrane-bound proteins critical for defense responses in Arabidopsis thaliana. Moreover, a nuclear protein complex in A. thaliana that includes the defense signaling protein MOS4 has been affinity purified and many of the identified protein partners found to be conserved with those in a protein complex previously characterized in yeast and humans. In another example, several proteins were identified that interact with a defense signaling GTPase, Rac1, in rice (Oryza sativa). Clearly, AP-MS is an important technique that will continue to provide novel insight into protein-protein interaction networks in plants. Here we review some of these recent discoveries and summarize the different techniques of AP-MS that have been used successfully to identify some of the interacting proteins in the plant defense response to pathogen attack.

Keywords: Plant-pathogen interaction, plant immunity, protein-protein interaction, protein complex, affinity purification, mass spectrometry.

1. INTRODUCTION

Many different types of viruses, bacteria, fungi, oomycetes, phytoplasmas and nematodes are pathogens of plants. To avoid infection by such a wide range of microorganisms, plants deploy an active, but non-adaptive, comprehensive immune system that first detects the presence of the pathogen and then responds through multifaceted biochemical and cellular processes [1]. It is now known that some associations between a pathogen and a plant and the resulting defensive responses within plant cells are predicated on protein-protein interactions [1, 2]. More specifically, protein-protein interactions may help determine the final winner in the competition between the plant and the pathogen. Thus, to understand disease and solve disease problems, it is necessary to discover the plant proteins responsible for defense and elucidate protein-protein interactions.

Over the past two decades many methodologies have been developed and adapted to characterize protein-protein interactions. Yeast two-hybrid (Y2H) [3, 4] and split-ubiquitin [5] are in vitro cell-based assays used to determine if two proteins of interest interact. The benefits of these assays are that they are simple, standardized systems that can be performed in routine molecular biology laboratories familiar with cell culturing. In plant pathology, these assays have been used to test the interactions between a pathogen protein and a plant protein or between two plant proteins [6-12]. One thing that makes these techniques especially powerful is that they are amenable to shotgun or high-throughput application and can be used to identify large networks of interacting proteins. For example, high-throughput Y2H assays were used to reveal an overlapping network of several hundred interacting proteins in rice that regulate cell cycling or confer tolerance to abiotic stress and resistance to disease [13, 14].

Despite the ease and successes of the in vitro cell-based assays, plant biologists are now embracing in vivo methods for studying protein-protein interactions. One such method entails performing affinity purification of a protein followed by tandem mass spectrometry to identify the target protein and any of its co-purified partners (AP-MS) [15, 16]. All together, AP-MS might appear to be a counterintuitive method of choice for routine plant molecular biology labs. Affinity purification is relatively more labor intensive and technically more challenging than methods like Y2H, and mass spectrometry requires considerable advanced training and expertise that is not traditionally taught to plant biologists. Nevertheless, it is evident from several recent publications that AP-MS is an emerging choice for the discovery of interactions between proteins that are part of the plant immune system [17-20]. Herein, we summarize these recent discoveries and discuss some of the aspects of AP-MS that are making it such a successful technique for discovery in the field of plant pathology.
2. OVERVIEW OF AFFINITY PURIFICATION AND MASS SPECTROMETRY

Affinity purification has been successfully applied to purify protein complexes from a variety of organisms (reviewed in [21]) including bacteria [22], mammals [23-25], and insects [26]. Recently, several affinity purification protocols have been adapted for use in plants (Table 1).

In the simplest conceptualization of AP-MS, plant cells are first disrupted to make proteins available for purification and the target protein is then purified from the complex mixture, usually with an antibody. It is presumed that proteins that physically interact with the target protein prior to tissue disruption are co-purified. The proteins can then be separated from each other and identified by mass spectrometry.

Selection of an appropriate affinity purification protocol is subject to numerous criteria. For example, the subcellular localization of the target protein may determine what purification method gives the greatest enrichment in the desired protein complex, e.g., nuclear, chloroplast, membrane, etc. In some cases, chemical cross-linking can be performed in situ prior to tissue disruption to better ensure that the association of interacting partners is preserved [27]. Of course, it is also important to reduce the number of artificial protein interactions in the affinity purified sample, and this can dictate the type of controls used for comparison and the choice of affinity purification technique. When target-specific antiserum is available, multiple rounds of purification can be performed to reduce artificial interactions. Alternatively, it is possible to prepare an in-frame fusion of the target gene to a sequence encoding a generic epitope tag (FLAG, myc, GFP, etc.), express the fusion protein in an organism of interest (reviewed in [28] and Table 1), and use commercial antibodies to the tags to purify the tagged proteins or use other common chemical affinity enrichment methods specific to them (biotin, His, protein A, etc.). The addition of two tags allows consecutive rounds of purification (i.e., tandem affinity purification (TAP)), which theoretically reduces non-specific protein partner binding [16]. Unfortunately, it can be complicated when the fusion proteins are intended to be expressed in plants. Not all plants are amenable to transformation, regeneration may take years for some plants and desired expression levels can be difficult to optimize. However, there are transient assays using virus vectors or tissue culture options that may lower some transformation barriers. Nonetheless, the formation and isolation of authentic protein interactions in the native environment of the plant cell would seem to be preferable over interactions in yeast using Y2H. A counter argument is that Y2H has a better ability to test weak or transient interactions between proteins as opposed to AP-MS that requires the protein interactions be maintained during cell disruption and affinity purification. The advantages and disadvantages of these systems are topics that will continue to be discussed for years.

After proteins and their interacting partners are affinity purified, they are often separated from each other to enable their detection. Proteins can be separated by 1-D or 2-D polyacrylamide gel electrophoresis and excised from the gels. Alternatively, the complete affinity-purified protein sample can be proteolytically digested and the peptides separated by HPLC. This HPLC separation method is inherent to Multidimensional Protein Identification Technology (MudPIT) [29], a technique that has been very useful for resolving small amounts of protein complexes not readily observable on gels [30]. Either way, once proteins and peptides are separated, they can be identified by mass spectrometry or tandem mass spectrometry, which provides information about amino acid order (reviewed in [31, 32]). In most cases, species-specific DNA and protein sequence information is needed to interpret the mass spectra, and the recent increases in this genomic information for many different plant species is one reason why mass spectrometry-based proteomics has become more prevalent in plant biology. Mass spectral data is useful in other ways too. Mass spectra can reveal the presence of post-translational modifications such as phosphorylation that are critical to protein-protein interactions, or be used to measure the abundance or ratios of proteins to each other in a protein complex [30].

The expertise required to operate mass spectrometers and to analyze mass spectra can deter many inexperienced scientists from using AP-MS. However, many institutions have core facilities that can provide most services including protein separation, digestion, mass spectrometry and database searching (peptide/protein identification) [32, 33]. Unfortunately, not all facilities can provide appropriate guidance for experimental design with respect to plants while others may have limited ability to provide other specialized technological analyses like deconvoluting spectra of cross-linked peptides (which can support the notion that two peptides interacted, but might otherwise lead to a false-positive if not interpreted correctly [34-36]), statistically evaluating the data to resolve the amounts of proteins in a complex [30] or determining a probability that two proteins indeed interacted [37, 38].

In summary, it is the combination of transgenic expression in plants (rather than transient yeast expression), affinity purification of protein complexes from plant cells, and subsequent mass spectrometry for protein identification (rather than DNA sequencing or cell culture for interaction confirmation) that makes AP-MS so much more difficult than Y2H. Nevertheless, the following recent publications reveal that AP-MS is within reach of plant biology laboratories and is enabling discovery in ways that might not have been attainable using in vitro cell-based assays.

3. IDENTIFICATION OF PROTEIN-PROTEIN INTERACTIONS IN PLANT IMMUNITY RESPONSES

The bacterial pathogen Pseudomonas syringae produces at least three proteins, AvrRpt2, AvrRpm1 and AvrB, that act to modify the Arabidopsis thaliana protein RPM4 at the plasma membrane and increase virulence [39-41]. This, and the fact that RIN4 physically associates with A. thaliana RPM1 and RPS2 [40] proteins which confer specific disease resistance to P. syringae suggest that RIN4 is part of a complex of interacting proteins that recognize the presence of the pathogen and then communicate a defense response, possibly through a protein-protein interaction network. To identify additional components of this interaction network, Liu et al. [17] purified RIN4 from A. thaliana leaf extracts using RIN4-specific antibody. This was sufficient to isolate and identify it, its known interaction partner RPS2 and six addi-
One of the RIN4 interaction partners was a plasma membrane H+ ATPase. Since H+ ATPases can regulate the opening and closing of the stomatal apertures, a potential entry point into a leaf for a bacterial pathogen, the AP-MS results led to the hypothesis that stomatal control may be part of a pathogen defense protein-protein interaction network. The hypothesis was supported by showing that plants not expressing or over-expressing RIN4 displayed differential ATPase activity in stomata. Thus it is possible that RIN4 can prohibit pathogen entry into the leaf through its protein-protein interactions with the ATPase. The importance of the potential interactions with the five other proteins thought to interact with RIN4 remains to be determined, but given the encouraging results with ATPase, it is possible that the other RIN4 interacting partners may also function in RIN4-associated defense responses.

It should be noted that a number of factors contributed to the success of these AP-MS experiments. First, identifying RPS2 alongside RIN4 was an important positive control since it had previously been shown that RPS2 and RIN4 associate [12, 39-41]. To ensure this, the authors optimized their protocols so RPS2 and RIN4 were co-purified by the RIN4 antibody. Despite the optimization and careful controls used by the authors, other known RIN4-interactors, such as NDR1 and RPM1 [12, 42], were not discovered by AP-MS. It could be that the proteins were not in the purified complex or that the interactions were not resolvable by this method, possibly due to inadvertent bias introduced through optimization.

The study by Liu et al. [17] suggests that while one mode of AP-MS can be useful in detecting some important protein-protein interactions, it may not be sufficient to discover all of them. Different approaches may be required to identify the many possible protein-protein interactions. Case in point, Qi and Katagiri [20] also investigated the RIN4/RPS2 protein-protein interaction network by AP-MS but instead focused on RPS2 rather than RIN4. Because evidence suggests that RPS2 and RIN4 are membrane-bound [39], Qi and Katagiri [20] specifically optimized their AP-MS protocol for the isolation of protein complexes from membranes that included RPS2, unlike Liu et al. who used whole leaf extracts. Qi and Katagiri [20] also presumed that harsh techniques would be necessary to separate an RPS2 complex from the membrane and that strong affinity binding might also be necessary to aid in the purification of membrane proteins solubilized at low amounts. Therefore, instead of using an RPS2-specific antibody to purify an RPS2 protein complex, transgenic plants were made to express a fusion protein of RPS2 with hemagglutinin (HA) and biotin carboxy carrier protein domains (BCCD). They hypothesized that the high degree of affinity between streptavidin and biotin would enable greater selectivity of the tagged protein; however they also feared that wild-type plants would naturally biotinylate proteins and that a background of biotinylated proteins could confound results. To overcome this limitation, the authors crossed plants expressing their fusion protein into plants deficient in 3-methylcrotonyl CoA carboxylase, which effectively reduced the amount of endogenous biotinylation. Also, as opposed to Liu et al. [17], Qi and Katagiri [20] examined fresh rather than frozen leaf tissue to avoid fracturing cells along the surface of the membranes which may have otherwise reduced membrane protein associations [43]. Finally, the proteins in the microsomal membrane fractions were chemically cross-linked to better insure that their interactions were preserved upon protein release from the membrane. The cross-links were chemically reversed after isolation and the proteins identified by mass spectrometry.

Although the RSP2 protein was tagged with both HA and BCCD, Qi and Katagiri never needed to perform TAP as the

<table>
<thead>
<tr>
<th>Species1</th>
<th>TAP Tags/ Cleavage Site</th>
<th>Promoter</th>
<th>Gateway4</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotiana benthamiana</td>
<td>2×protein A and mutated CBP2/TEV1</td>
<td>35S</td>
<td>yes</td>
<td>[16]</td>
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<tr>
<td>A. thaliana</td>
<td>2×IgG-BD,6×His, and 9×myc/thinovirus 3C protease site</td>
<td>2×35S</td>
<td>yes</td>
<td>[59]</td>
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<tr>
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<td>Variable4</td>
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<td>[60]</td>
</tr>
<tr>
<td>A. thaliana</td>
<td>SBP and 2×protein G/TEV1</td>
<td>Variable4</td>
<td>yes</td>
<td>[60]</td>
</tr>
<tr>
<td>A. thaliana</td>
<td>HA and biotin carboxyl carrier protein domain/ PreScission site</td>
<td>2×35S or proRPS23</td>
<td>yes</td>
<td>[20]</td>
</tr>
</tbody>
</table>

Note:  
1. Plant in which the tagged protein was evaluated;  
2. several mutations were introduced to the nuclear localization signal (NLS) in the calmodulin-binding protein (CBP) domain to improve the usefulness of the tag;  
3. TEV, tobacco etch virus protease cleavage site;  
4. different promoters can be used to drive the fusion protein expression;  
5. the AtRPS2 gene promoter;  
6. compatible with Gateway (Invitrogen) cloning systems

Table 1. The Recent Developments of TAP in Plants
BCCD tag proved sufficient. As a result of their extensive efforts, RPS2, RIN4 and nine other proteins were discovered in at least three replicates and not in the non-biotinylated controls. Interestingly and similarly to the study by Liu et al. [17], other proteins known to interact with RPS2, such as NDR1 and RPM1, were not among the group of RPS2 interacting proteins indentified by AP-MS. Qi and Katagiri [20] also identified an ATPase in their AP-MS results, but theirs was not the same as the one identified by Liu et al. [17] and instead might function in metal ion transport. Further testing to verify any interactions with RPS2 or to affirm the importance of these protein partners in disease resistance has not been reported. However, these discoveries open up a number of new promising leads to investigate in the RPS2/RIN4 disease resistance protein-protein interaction network.

Another AP-MS project has revealed an extensive set of proteins under control of the defense signal salicylic acid (SA), a hormone that activates defenses locally and distally throughout a plant after infection (reviewed in [44]). One of the positive regulators of SA-dependent defense responses is NPR1; loss-of-function mutant npr1-1 gives enhanced susceptibility to *P. syringae* infection [45]. A genetic screen for suppressors of npr1-1 resulted in the identification of a gain in function mutation, suppressor of npr1-1, constitutive 1 (snc1) [46]. The snc1 mutant in turn was genetically screened for suppressor mutations named MODIFIER OF snc1, e.g., mos4 [47]. MOS4 was subsequently shown to encode a nuclear protein that interacts with the Myb-transcription factor AtCDC and the WD-40 protein PRL1 in a nuclear MOS4-Associated Complex (MAC) [47]. Each of these three proteins has homologues in yeast and humans that are components of a spliceosome-associated protein complex known as the Nineteen Complex (NTC) [48, 49]. Monaghan et al. [18] hypothesized that the *A. thaliana* MAC, like the NTC in yeast, could contain many more components than the three already identified and that these proteins might also have roles in pathogen defense.

To identify additional components in an *A. thaliana* MAC, Monaghan et al. [18] expressed an HA-tagged MOS4 fusion protein in a mos4-1 mutant background [18]. The fusion protein complemented all mutant phenotypes, which proved that the protein functioned properly. Since MOS4 is a nuclear protein, the authors then isolated nuclei and immunoprecipitated MOS4 from nuclear lysates using antibodies to the HA tag. Twenty-four co-purified proteins were identified by mass spectrometry, which included the MOS4 target protein and the previously identified MAC components AtCDC5 and PRL1. Eight of the other proteins had homology with yeast NTC proteins, another eight were presumed to have RNA-processing functions that were expected of a spliceosome-associated complex, and five had not been previously identified as part of an NTC and may have been contaminants. MAC3A and MAC3B were two of the new proteins homologous to proteins found in the yeast NTC. To determine if they played a role in pathogen defense the authors evaluated mutant knock-ourts for each of these proteins. Plants with a single mutation in either gene were unaffected when challenged with bacterial pathogens but the double mutants with mutations in both genes were more susceptible to bacterial infection, which indicates the proteins have redundant function in disease resistance. Moreover, the mac3a and mac3b double mutation, like the mos4-1 mutant, suppresses snc1. A role in disease response for the other MAC proteins identified remains to be tested but the results suggest that protein-protein interactions of the MAC are necessary for disease resistance responses routing through the nucleus.

*Oryza sativa* (rice) also exhibits many of the same defense responses as *A. thaliana*, even though it is susceptible to a different set of pathogens. For example, Rac1 is a GTPase in rice that protects against infection by the blast fungus *Magnaporthe grisea* by producing reactive oxygen species (ROS) at the site of infection, expressing pathogenesis-related protein (PR) genes, producing phytoalexins and promoting plant cell death [50-52]. Rac1 associates with RAR1 and HSP90, two proteins that contribute to pathogen defense through protein-protein interactions with SGT1, a ubiquitin ligase critical for proteolysis and disease resistance [53, 54]. To identify other proteins that may interact with Rac1, Nakashima et al. [19] also used AP-MS, albeit differently than in the previously mentioned projects. In this case, GST-Rac1 fusion protein was first expressed in and purified from *Escherichia coli* and then bound to glutathione-Sepharose 4B beads. Since GTPases take on different forms depending on binding with GTP or GDP, both forms were studied, and this was accomplished by incubating the GST-tagged Rac1 with GDP or GTP prior to immobilization. Protein extracts derived from rice cell cultures treated with a blast fungus sphingolipid elicitor were bound to the column matrix, washed and the interacting proteins were eluted. Twenty-one proteins were identified, including five with functional domains similar to canonical plant hypersensitive disease resistance R-genes and one homologous to RACK1, an adaptor protein that binds several proteins in various mammalian signaling pathways [55]. Using a Y2H assay, Nakashima et al. [19] validated the rice RACK1/Rac1 interaction. They also confirmed that the rice RACK1 regulates ROS production and defense gene expression and contributes to resistance to rice blast fungal infection. Hence, the AP-MS data extended the interaction network for Rac1 and suggested that rice RACK1, together with RAR1, SGT1 and HSP90, regulates plant immunity through active G-protein signaling that is linked to proteasome-activated degradation.

4. CONCLUSIONS AND PERSPECTIVES

A common thread to all the AP-MS studies discussed herein is that they benefited greatly from prior knowledge of specific protein-protein interactions found with other techniques, be it to hone method development, guide hypothesis formation or confirm findings. This does not mean that AP-MS is secondary to other discovery methods. Rather, this simply reflects the progressive development of the technology that has followed alongside greater accessibility to mass spectrometry. Certainly, AP-MS is complementary to other techniques for resolving protein-protein interactions, yet it can yield novel results, just as can the alternative methods.

The planning of an AP-MS experiment requires one to make several choices with regard to the mode of affinity purification, the type of affinity tag, the plant system and the appropriate cellular extraction method (Table 2). As discussed, one can purify a protein using a specific antibody
raised against it as in the study by Liu et al. [17], or one can engineer a fusion protein with a generic epitope tag that allows purification with an antibody to the tag as in the study by Qi and Katagiri [20]. With that in mind, it appears that with respect to the choice of tags, many are satisfactory for AP-MS in that a) they can allow for sufficient selectivity and b) the type and size of the tag are qualities that do not necessarily interfere with protein activity or protein-protein interactions [28].

As for the former tag choice, while the use of mammalian-protein derived tags in a plant system may help differentiate the tagged protein over other plant proteins that do not have the same epitope, other plant derived or generic tags work well enough, especially if the proper controls are put into place. Even biotin/streptavidin affinity methods work well if proper controls are used to reduce contamination with naturally biotinylated protein [15, 20]. As for the latter factor in the choice of tags, many are satisfactory for AP-MS in that a) they can allow for sufficient selectivity and b) the type and size of the tag are qualities that do not necessarily interfere with protein activity or protein-protein interactions [28].

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NDR1 = Nonrace-specific disease resistance
NPR1 = Non-expressor of pathogenesis related genes 1
NCT = Nineteen complex
PR = Pathogenesis-related
PRL1 = Pleiotropic regulator locus 1
R = Resistance
RACK1 = Receptor for activated C-kinase 1
RIN4 = RPM1 Interacting Protein 4
ROS = Reactive oxygen species
snc1 = Suppressor of npr1-1, constitutive 1
TAP = Tandem affinity purification
TEV = Tobacco etch virus
Y2H = Yeast-two-hybrid

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