# Protein Complexes Characterization in *Arabidopsis thaliana* by Tandem Affinity Purification Coupled to Mass Spectrometry Analysis

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## Abstract

Proteins are major elements participating in all the key functions of the cells. They rarely fulfill their physiological roles in an autonomous way but rather act as part of more complex cellular machines. Indeed they can bind different types of molecules (proteins, nucleic acids, metabolites, etc.), via stable or transient interactions, depending on their nature and functions. The identification of the molecular partners of a given protein is hence essential to better understand its roles, regulation, and mechanisms of action.

This chapter describes the use of a tandem affinity purification approach followed by mass spectrometry analysis to try to identify and characterize the proteins involved in protein complexes in *Arabidopsis thaliana* and decipher some mechanisms of regulation of the modules. Important elements to consider in such an approach are first extensively exposed in the introduction. This technique, in combination with complementary approaches like yeast two-hybrid and bimolecular fluorescence complementation, can be an interesting source of data to identify and characterize in vivo protein complexes.

**Key words** *Arabidopsis thaliana*, Protein complexes, Tandem affinity purification, Mass spectrometry, Posttranslational modifications

## 1 Introduction

Affinity-based purification methods were proven quite efficient for the enrichment and further analysis of protein complexes in several organisms, including plant species. These methods can be separated into two main groups: epitope-tagging (allowing single-step affinity purification) and tandem affinity purification (TAP) (two-step affinity purification). The TAP method was initially developed to isolate and identify protein complexes in the yeast *Saccharomyces cerevisiae* [1]. In the TAP method, a bait protein is fused to a combination of tags which are sequentially used to isolate a highly purified bait protein and its associated protein partners. In the first TAP version, three tags were used: two IgG-binding units of

protein A of *Staphylococcus aureus* (ProtA), a tobacco etch virus (TEV) protease cleavage site and a calmodulin-binding peptide (CBP). This method, applied in large-scale studies, led to numerous protein complex identifications in yeast [2, 3]. Due to its success TAP-tagging was rapidly implemented to protein interaction studies in other organisms including bacteria [4], mammalian cells [5], insect cells [6], and plants [7]. Adaptations of the technique mainly included the development and use of different combinations of tags as previously detailed [8].

In our work, a strategy based on TAP followed by mass spectrometry (MS) analysis was developed to identify in vivo protein partners of our proteins of interest. The stability of signaling complexes might be weaker as the interactions are expected to be transient; in this case, it makes the TAP approach rather challenging as this two-step affinity purification requires numerous incubations and washings. Alternatively, TAP protocols allow the purification of better purified protein complexes compared to single-step procedures; subsequent identifications of proteins usually have a higher confidence. Moreover, this strategy can allow the identification, of some posttranslational modifications (PTMs) of the bait and interacting proteins. This particularly interesting aspect will be discussed in the following sections of the chapter.

We designed a new combination of tags allowing a faster TAP protocol. This combination of tags, named PC2, is fused to the carboxy-terminal part of the protein. It is composed of a nine-Myc (EQKLISEEDL) peptide repeat followed by an eight-histidine (8×His) stretch and a Strep-tag II (WSHPQFEK) peptide (Fig. 1a). The relatively small size of the whole tag (about 19 kDa) reduces the risk of steric hindrance. To our knowledge this combination of tags has not been previously reported in the literature. As shown in Fig. 1b, the plant protein extract containing the TAP-tagged complexes is first bound on a resin bearing metal ions using the chelation interaction with the 8×His tag, like in an Immobilized Metal Affinity Chromatography (IMAC). After washes and elution using imidazole, the simplified protein extract is incubated with a Strep-Tactin resin. Strep-Tactin is a streptavidin mutant allowing enhanced interaction with the Strep-tag II, itself a variant of the original Strep-tag [9]. After washes and subsequent elution using desthiobiotin, proteins are precipitated and prepared for MS analysis. The 8×His motif, with an affinity constant for IMAC resin in the order of $10^{-13}$ M [10] allows for efficient capture of the diluted TAP-tagged protein in the initial protein extract. Oppositely, the Strep-tag II/Strep-Tactin interaction requires higher concentration of the bait but is much more specific than IMAC and represents an ideal second step in the TAP procedure. No protease cleavage, often the limiting step, is necessary in this purification process. The nine-Myc-peptide repeat can be used as an efficient epitope for western blotting. It can also serve as a step in the
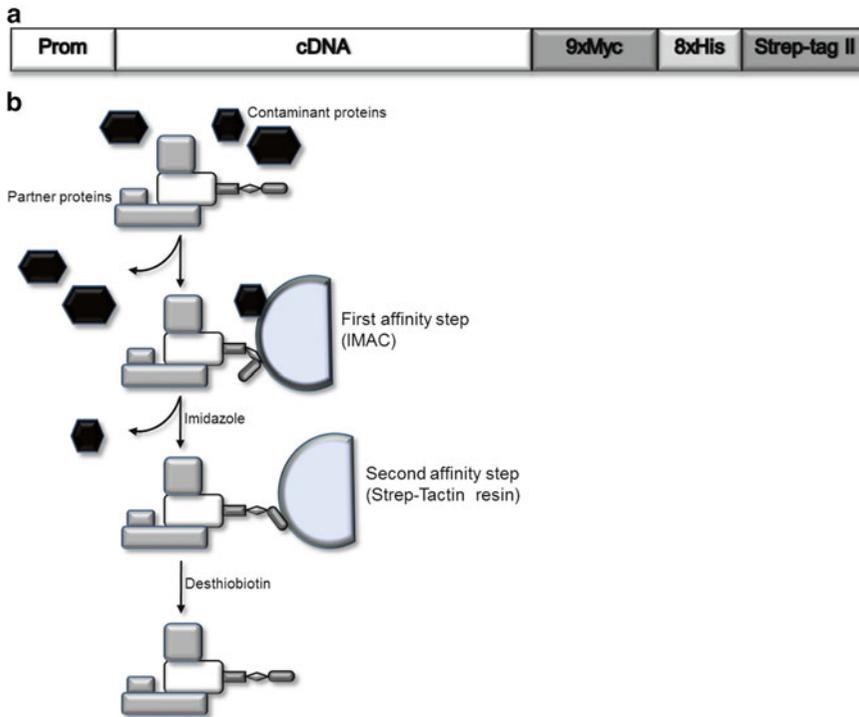
**Fig. 1** The gene of interest fused to the PC2 cassette and the TAP strategy. (**a**) Structure of the TAP PC2 cassette where the various tags are indicated. (**b**) Overview of the TAP strategy. *Grey boxes* represent partner proteins of the TAP-tagged bait protein and *black boxes* represent contaminant proteins; the *white box* and the three small *grey boxes* correspond to the TAP-tagged bait protein

purification process, preferentially as the last step because the corresponding elution necessitates stringent conditions, such as low pH or high concentration of detergents, which would disrupt non-covalent protein–protein interactions.

We originally designed a binary Gateway vector allowing the expression of the bait protein merged in frame with the PC2 tag under the control of the strong CaMV 35S promoter. This transgene did not always allow the generation of viable plant lines and is often silenced in the progeny. Even when the CaMV 35S promoter was replaced by an endogenous promoter, no stable expressing lines could be obtained. In contrast, expression of the entire genomic locus, including the promoter, exons, and introns and fused to a C-terminal PC2 cassette resulted in stable lines, in which the chimeric protein is supposed to be expressed as the endogenous protein. This construct was validated by complementation of the corresponding knockout T-DNA mutant. In conclusion, the full genomic locus seems to be of major importance for obtaining stable transgenic lines for critical genes of interest. Besides, the amount of expressed bait protein is a crucial factor in the success of the approach. From the literature, it can be stated, overall, that the

more a protein is expressed the higher is the amount of the purified bait protein. However, this rule, although often verified for the bait protein, does not apply to the purified protein partners in the complex(es) as plants usually do not adapt their expression and/or abundance to the level of the bait protein. In addition, if the physiological function of the bait protein necessitates a tight regulation, overexpression or underexpression will very probably lead to phenotypes different from the wild type. That is why the promoter for expression of the bait protein has to be chosen carefully. The locus promoter is probably the best choice. In addition, the genetic background needs to be considered, usually with two alternatives: a wild-type background or a bait protein mutant background. Working in a mutant background presents two major advantages in the TAP approach. First, the complementation of the mutant phenotype indicates with high confidence that the TAP-tagged protein is functional and that the protein complex(es) centered on it are functional as well. Second, it avoids the competition that would occur otherwise between the endogenous protein and the tagged protein for the formation of the complex(es).

To perform TAP purification, we use whole plantlets grown in liquid medium to obtain large amounts of material. Protein complexes are then purified from frozen material. Although cell cultures and transient in planta expression assays can theoretically be used, stable plant transformants are closer to physiological conditions. This is the reason why we chose this expression and culture system. Additionally, such strategy allows the use of mutant genotypes. Several biological replicates are performed for each TAP-tagged line, because some interaction partners are likely to be non-systematically identified by MS in a single purification experiment. Besides, even though the TAP provides rather clean samples, in which the number of proteins interacting nonspecifically with the affinity beads is limited, some contaminant proteins are still retrieved in the final sample. To distinguish between the true complex components and these contaminants, other transgenic lines expressing unrelated TAP-tagged proteins such as GFP (Green Fluorescent Protein) are used as controls. Purified proteins are digested by trypsin and the peptides analyzed by capillary liquid chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS). Proteins repeatedly identified in samples of interest but not in controls are considered to be likely components of the TAP-tagged complexes.

The TAP-MS approach also allows analyzing PTMs occurring on protein complexes without making a priori assumptions on their nature or localization. This requires of course that sufficient material of the bait proteins and its interaction partners be obtained by TAP, to reach significant protein sequence coverage by the MS

analysis of the proteolyzed protein complexes. Searching for PTMs has become more and more possible with the sensitivity and mass measurement accuracy of current mass spectrometers and with the PTM-related options in database search software. Indeed, database search options, like the "error tolerant" search function in Mascot, allow screening for any possible covalent modification in proteins of interest. The high sensitivity and dynamic range of current mass spectrometers makes it possible to detect and identify modified sequences of proteins even though the modification affects below 1 % of the biomolecules of same sequence (the dynamic range typically covers about four orders of magnitude). The high mass accuracy of current mass spectrometry instruments (below 5 ppm) contributes to accepting with confidence the proposed modified peptide sequences or can result in their prompt rejection. Phosphorylation events are of particular interest as they are key elements in protein regulation. It is also possible to identify other kinds of PTMs as far as these modifications are stable enough to withstand the physicochemical conditions of the whole analytical process or do not prevent the ionization and detection of the respectively modified peptides during the LC-MS/MS analysis. Whereas PTM characterization without a priori assumption can be successful on purified proteins, there are limitations, because modified sequences present in substoichiometric amounts in the complex digests are globally more likely to escape identification by MS than major abundance species. The identification of these species present in low amounts would require their enrichment. In large-scale studies of phosphorylation events, for example, phosphoproteins and/or phosphopeptides are usually first enriched by IMAC or metal oxide affinity chromatography (MOAC) before MS analysis [11, 12]. Yet there exists no enrichment technique compatible with the simultaneous purification of peptides carrying a variety of PTMs. Nevertheless, the approach to identify PTMs without a priori assumptions is quite easy to perform and can provide important clues on the underlying regulatory mechanisms.

In a second step, complementary approaches, like yeast two-hybrid or in planta bimolecular fluorescence complementation (BiFC), need to be used to validate the identified protein partners of bait proteins. Upon confirmation of the specific protein–protein interactions, further genetic, phenotypic, and biochemical studies can be carried out to gain further insight into the roles of these proteins.

The following experimental procedures describe the different steps of a standard study of a protein of interest by TAP PC2 coupled to MS analysis. Cloning steps to obtain a protein fused to the TAP PC2 cassette and selection of *Arabidopsis* stable lines expressing the TAP-tagged protein are not exposed here as they are beyond the frame of this chapter.

## 2    Materials

Remark 1: Use adapted protections when necessary (laboratory coat, nitrile gloves, eyeshields, respirator filter) as some chemicals used in the procedure are harmful.

Remark 2: Wash thoroughly reusable consumables and equipment.

### 2.1    Arabidopsis thaliana Plant Cultures

1. *Arabidopsis* culture medium (½ MS): 2.2 g/L MS including vitamins (e.g., Sigma), 10 g/L sucrose (e.g., Sigma), 0.5 g/L MES (e.g., Sigma), pH adjusted to 5.7 with KOH, autoclaved.

2. Small equipment and consumables: Petri dishes 145/20 mm, Parafilm, 50 mL Falcon tubes, liquid nitrogen.

3. Large equipment: sterile laminar flow hood, fridge, culture chamber [rotating platform, long day (16 h light–8 h dark), 24 °C].

### 2.2    Protein Extraction

1. Buffer 1: 50 mM Tris–HCl pH 7.5 with HCl, 150 mM NaCl, 10 % (v/v) glycerol, 0.1 % (v/v) NP40/IGEPAL CA-630. Prepare fresh Buffer 1 from sterilized or filtered reagent stock solutions.

2. Extraction Buffer: protease inhibitors (e.g., Complete EDTA-free, Roche, one tablet per 50 mL; and 1 mM PMSF), phosphatase inhibitors [1 mM NaF, 0.5 mM sodium orthovanadate ($Na_3VO_4$), 15 mM β-glycerophosphate, and 15 mM 4-nitrophenyl phosphate bis(tris) salt] in Buffer 1. Prepare fresh Extraction Buffer and let it on ice. Use stock solutions of protease/phosphatase inhibitors, stored according to the supplier recommendations.

3. Small equipment and consumables: mortars, pestles, 400 mL beakers, 200 mL glass bottles, spatulas, funnels, Miracloth, compatible centrifuge tubes, 50 mL Falcon tubes, liquid nitrogen.

4. Large equipment: centrifuge.

### 2.3    First Step of Purification

1. Ni Elution Buffer: 200 mM imidazole, protease inhibitors (Complete Mini EDTA-free, Roche, one tablet per 10 mL) and 1 mM PMSF, phosphatase inhibitors (1 mM NaF, 0.5 mM $Na_3VO_4$, 15 mM beta-glycerophosphate, and 15 mM 4-nitrophenyl phosphate bis(tris) salt) in Buffer 1. Prepare fresh Ni Elution Buffer and let it on ice. Use stock solutions of imidazole and protease/phosphatase inhibitors, stored according to the supplier recommendations.

2. Ni beads: Ni-NTA Agarose (e.g., Qiagen).

3. Small equipment and consumables: 15 mL Falcon tubes, 50 mL Falcon tubes, end-cut tips, rotating wheel for tubes, disposable plastic columns (e.g., Poly-Prep columns, Bio-Rad), retort stand tripod.

4. Large equipment: centrifuge.

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| *2.4  Second Step of Purification* | 1. Strep Elution Buffer: 10 mM D-desthiobiotin in Buffer 1. Prepare fresh Strep Elution Buffer and let it on ice. Use a stock solution of D-desthiobiotin, stored according to the supplier recommendations. |

2. Strep-Tactin beads: Strep-Tactin Sepharose (2-1201-010 IBA).

3. Small equipment and consumables: 15 mL Falcon tubes, 50 mL Falcon tubes, 1.5 mL Eppendorf tubes, end-cut tips, rotating wheel for tubes, 2 mL disposable plastic columns (e.g., Pierce), retort stand tripod.

4. Large equipment: centrifuge.

*2.5  Protein Precipitation*

1. Methanol Precipitation Solution: 0.1 M ammonium acetate, 1 % (v/v) β-mercaptoethanol, in absolute methanol. Prepare fresh Methanol Precipitation Solution under a fume hood and let it on ice. Add the 2-mercaptoethanol just before use.

2. 70 % Ethanol solution: 70 % (v/v) ethanol in water. Let at –20 °C or on ice.

3. Small equipment and consumables: 1.5 mL Eppendorf tubes, vortex.

4. Large equipment: –80 °C freezer, centrifuge.

*2.6  Protein Digestion*

1. Acetonitrile: HPLC grade.

2. Reducing agent: Tris-(2-carboxyethyl)phosphine (TCEP).

3. Alkylating agent: methyl methanethiosulfonate (MMTS).

4. Digestion enzyme: sequencing grade modified trypsin (e.g., Promega).

*2.7  MS Analysis*

1. HPLC solutions are prepared with HPLC grade solvents and Milli-Q water at 18.2 MΩ cm. Buffer A = $H_2O$–acetonitrile–formic acid, 98:2:0.1 (v/v/v); buffer B = $H_2O$–acetonitrile–formic acid, 20:80:0.1 (v/v/v).

2. Chromatographic system UltiMate 3000 (Dionex) equipped with a reversed-phase pre-column (Pepmap C18, 3 μm particle size, 100 Å porosity, 300 μm internal diameter, and 5 mm length) and a capillary column (Pepmap C18, same stationary phase characteristics as the pre-column, 75 μm internal diameter, and 15 cm length).

3. NanoESI-LTQ-Orbitrap XL instrument (Thermo electron), piloted with the software Xcalibur.

4. Metallized tips (e.g., New Objective).

5. Identification of peptide sequences from MS/MS spectra is provided by the software Mascot (www.matrixscience.com).

# 3   Methods

Remark 1: For all the following steps, it is strongly recommended to plan the experiment including the tagged line of interest together with a control line.

Remark 2: It is useful to keep aliquots corresponding to the key steps of the TAP purification and use them for a western-blot analysis with an anti-Myc antibody. This allows for the evaluation of the yield of tagged-protein purification and the marking of the steps that can be potentially improved.

## 3.1   Arabidopsis thaliana Plant Cultures

1. Under a sterile laminar flow hood, pour 55 mL of *Arabidopsis* culture medium in a 15 cm petri dish. Prepare ten such dishes.

2. Sow sterilized *Arabidopsis* seeds in equal amounts in the ten petri dishes and seal the plates with Parafilm.

3. Keep the petri dishes at 4 °C for 3–4 days for stratification of the seeds.

4. After stratification, transfer the petri dishes in the culture chamber and allow plants to grow for 17–18 days. This should allow the production of 5 g of plant material per dish.

5. Harvest plants: quickly dry the samples with several layers of absorbent paper and transfer them in 50 mL Falcon tubes. Freeze the tubes in liquid nitrogen (*see* **Note 1**). Use one 50 mL Falcon tube for two petri dishes.

## 3.2   Protein Extraction

1. Grind all the frozen plants in prechilled mortar and pestle to obtain a homogenous fine plant powder (*see* **Note 2**). Regularly add liquid nitrogen to keep the samples frozen.

2. Weight the ground plant powder so as to use the same amount of material for the TAP between the tagged-line of interest and the control line.

Remarks: All steps from here to Subheading 3.6 should be conducted on ice or at 4 °C. Cool consumables and equipment on ice or at 4 °C well before use.

3. In a 400 mL beaker, pour two volumes of cold Extraction Buffer (e.g., 80 mL for 40 g of plants).

4. Add the plant powder progressively in the beaker and homogenize gently with a spatula (*see* **Note 3**).

5. Keep on ice for about 10 min.

6. Filter the crude solution through two layers of Miracloth into a 200 mL glass bottle using a funnel.

7. Repeat filtration with one layer of Miracloth.

8. Distribute the filtered solution in centrifuge-compatible tubes, equilibrate them, and centrifuge at 4 °C for 20 min at $16,000 \times g$.

9. Keep the supernatant in new 50 mL-Falcon tubes (*see* **Note 4**).

*3.3* *First Step of Purification*

Remark: Prepare the Ni beads during the centrifugation of Subheading 3.2, **step 8**.

*3.3.1 Preparation of Ni Beads*

1. Disperse the Ni beads into homogeneous slurry and transfer 1 mL (500 µL beads) in a 15 mL Falcon tube using an end-cut tip.

2. Add 5 mL of Buffer 1 to the beads disperse gently and centrifuge at 4 °C for 2 min at $700 \times g$ to pellet the beads. Discard supernatant.

3. Repeat the previous step twice.

*3.3.2 Ni Purification*

1. Distribute equally the prepared Ni beads in three 50 mL Falcon tubes.

2. Add equal amounts of the protein extract from Subheading 3.2, **step 9** in these tubes.

3. Gently rotate tubes on a rotating wheel for 2 h at 4 °C (*see* **Note 5**).

4. Centrifuge the tubes at 4 °C for 3 min at $700 \times g$ to pellet the beads. Discard supernatant.

5. Add 2 mL of Buffer 1 to the beads homogenize gently and load them on a disposable plastic column using end-cut tips (*see* **Note 6**).

6. After the column has completely drained, add 2 mL of Buffer 1 to wash the beads.

7. Repeat the previous step.

8. Add 3 mL of Ni Elution Buffer to the column and collect the eluate in a new 15 mL Falcon tube.

*3.4* *Second Step of Purification*

Remark: Prepare Strep-Tactin bead suspension during the incubation on Ni beads of Subheading 3.3.2, **step 3**.

*3.4.1 Strep-Tactin Beads Preparation*

1. Gently resuspend the Strep-Tactin solution and transfer 200 µL of resin slurry (100 µL beads) in a 15 mL Falcon tube with an end-cut tip.

2. Add 3 mL of Buffer 1 to the bead slurry, resuspend gently, and centrifuge at 4 °C for 2 min at $700 \times g$ to pellet the beads. Discard supernatant.

3. Repeat the previous step.

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| *3.4.2 Strep-Tactin Purification* | 1. Add the 3 mL of Ni eluate from Subheading 3.3.2, **step 8** to the prepared Strep-Tactin beads. |

2. Gently rotate the tube on a rotating wheel for 1.5 h at 4 °C (*see* **Note 5**).

3. Centrifuge the tube at 4 °C for 2 min at $700 \times g$ to pellet the beads. Discard supernatant.

4. Add 300 μL of Buffer 1 to the beads, resuspend gently, and load them on a 2 mL-disposable plastic column using end-cut tips (*see* **Note 6**).

5. After the column has completely drained, add 300 μL of Buffer 1 to wash the beads.

6. Repeat the previous step.

7. Add 500 μL of Strep Elution Buffer to the column and collect the eluate in an Eppendorf tube.

8. After complete elution, reload the eluate to the column for a second elution cycle (*see* **Note 7**).

### 3.5 Protein Precipitation

*3.5.1 Mix with Methanol Precipitation Solution*

1. Split the Strep eluate in two Eppendorf tubes, adding ca. 250 μL per tube.

2. Add 5 volumes of cold Methanol Precipitation Buffer, ca. 1.25 mL per tube.

3. Vortex and let at –80 °C, overnight (*see* **Note 8**).

*3.5.2 Recovery of Precipitated Proteins*

1. Centrifuge the tubes at 4 °C for 30 min at $20,000 \times g$ and discard supernatant under a fume hood (*see* **Note 9**).

2. Add 1 mL of cold Methanol Precipitation Solution per tube, vortex, and centrifuge at 4 °C for 15 min at $20,000 \times g$. Discard the supernatant under a fume hood.

3. Repeat the previous step.

4. Add 1 mL of cold 70 % (v/v) Ethanol solution per tube, vortex, and centrifuge at 4 °C for 15 min at $20,000 \times g$. Discard the supernatant.

5. Repeat the previous step.

6. Open the tubes and let the pellet to air-dry at room temperature (RT) (*see* **Note 10**).

### 3.6 Protein Digestion

1. Protein pellets are resuspended in ACN/50 mM tri-ethylammonium bicarbonate (TEAB), 40/60 (v/v) (*see* **Note 11**). Complete solubilization of the pellets is achieved by 5–10 min sonication in an ultrasonic bath.

2. To facilitate trypsin digestion, proteins are heat-denatured at 95 °C for 5 min.

3. Disulfide bonds in proteins are reduced by addition of 5 mM TCEP and incubation at 60 °C for 1 h.

4. Resulting free sulfhydryl residues are alkylated by addition of 10 mM MMTS and incubation for 15 min on the bench.

5. Protein samples are finally digested by addition of 2 μL of trypsin at 0.4 μg/μL and overnight incubation at 37 °C (*see* **Note 12**).

*3.7* **MS Analysis**

1. Peptides are first loaded onto the pre-column, with a 20 μL/min flow rate of buffer A. Salts are eliminated by a 5 min wash with buffer A.

2. After valve switching, pre-column and column are brought in series.

3. Chromatographic separation of peptides is performed at about 300 nL/min, by running a gradient from 100 % of buffer A to 70 % of buffer B. The gradient is developed in 50 min, followed by a 10 min flush of the system with 100 % buffer B to efficiently elute possible larger peptides.

4. Create an acquisition method using Xcalibur that automatically alternates between detection in MS mode in the Orbitrap cell of the peptides eluted from the chromatographic column, and fragmentation in MS/MS mode in the linear ion trap of the five species giving the most intense signals in the previous MS scan. Acquisitions are limited to the mass range [400; 1,600] Da in MS mode, and span [100; 2,000] Da in MS/MS mode.

5. To identify peptide sequences from MS/MS spectra, perform searches using the Mascot search engine, while considering the TAIR database (*see* **Note 13**), a maximum of two missed tryptic cleavages, full modification of cysteine residues by MMTS (modification called Methylthio(Cys)), possible methionine oxidation, 5 ppm error on precursor ions (*see* **Note 14**), 0.6 Da error on fragments, and specifying the use of an "ESI-trap" instrument (*see* **Note 15**). The "decoy search" option can be selected to get an estimate of the False Discovery Rate, i.e., an estimate of the fraction of wrong peptide identifications.

6. The previous search allows identification of proteins present in the TAP samples based on non-modified sequences. One can try to identify additional modified peptide sequences without a priori hypothesis on the nature of the covalent modification while using the "error tolerant search" option. All the protein entries identified in the previous search step will be grouped to form a limited database. In the second pass search, the selected enzyme (here trypsin) becomes semi-specific (i.e., only one end of the theoretical peptide matched to the experimental spectrum needs to result from a tryptic cleavage); the full list of modifications is tested; for each protein, single-amino-acid substitutions that can arise from single-base

DNA substitutions are assessed (see matrixscience.com for further detail). In particular, one can detect phosphorylated peptides, semi-tryptic peptides (possibly coming from protein degradation during cell lysis, or protein truncation in vivo) and $N=>D$ modifications that can occur spontaneously in vitro. This search can allow for increasing the sequence coverage of the bait protein and some of its partners.

## 4   Notes

1. About 40 g of plant material are usually obtained with ten petri dishes of culture. Moreover, it is possible to store the harvested plants at –80 °C before continuing with post-processing.

2. It is easier to grind the plants "one tube after the other." Put back the ground plants to the tubes and keep them under liquid nitrogen until all material is ground and ready for subsequent step.

3. It can take some time to have a homogeneous solution because the frozen plant material decreases the temperature of the mix below 0 °C. It is possible to let the beaker at room temperature (RT) until complete homogenization, but control the temperature to avoid exceeding 4 °C.

4. The supernatant mainly corresponds to a cytosolic extract. However, some organelles, such as nuclei or chloroplasts, can be disrupted during the grinding step.

5. Do not rotate the tubes too fast. Use for example a tuning wheel at 10 rpm.

6. Use a retort stand tripod to fix the disposable plastic column and equilibrate the filter of the column with Buffer 1.

7. At the end, use a syringe or another kind of system to push air on top of the column so as to elute the maximum by "drying" the Strep-Tactin resin slurry.

8. Put several layers of Parafilm around the tubes as methanol is very "leaky."

9. Be careful not to discard the protein pellet which is sometimes difficult to see.

10. It is possible to store the precipitated proteins at –80 °C before continuing with the following step.

11. Proteolysis is performed in the presence of 40 % (v/v) acetonitrile, previously shown to accelerate the digestion of proteins by trypsin [13].

12. Digestion of protein samples by trypsin is usually advised to be performed with an enzyme to substrate mass ratio between 1/100 and 1/20. We did not estimate the amount of protein

material yielded by our TAP procedure applied to 40 g of plant material. However, given the signal detected in MS on digested TAP samples, we could estimate that 0.8 μg trypsin was sufficient.

13. We work with in-house installed Mascot software, allowing the downloading of any protein database of interest on site, and the updating of the database when relevant.

14. The LTQ-Orbitrap provides mass measurements with accuracy below 5 ppm when the Orbitrap analyzer is calibrated on a weekly basis. Better accuracy can be achieved when working with the "lock mass" option: during LC-M/MS analyses, the instrument takes as reference background ion(s) originating from ambient air to recalibrate all acquired MS spectra in real time [14].

15. The "Instrument" configuration specified for database search with Mascot determines the fragment ion types considered for interpreting MS/MS spectra. It is thus crucial to select the "Instrument" definition that best allows interpreting the experimental fragmentation spectra to obtain optimal identification results.

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