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Tandem Purification of His_6-3x FLAG Tagged Proteins for Mass Spectrometry from *Arabidopsis*

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[Abstract] Tandem affinity purification is a powerful method to identify protein complexes that function in association with a known gene of interest. This protocol describes a methodology to capture proteins tagged with His_6-3x FLAG explicitly for the purpose of on-bead digestion and identification by mass spectrometry. The high sensitivity and specificity of our methods allow for purification of proteins expressed at native levels from endogenous promoters to enable uncovering the functional roles of plant protein complexes.
Keywords: Tandem affinity purification, Mass spectrometry, *Arabidopsis thaliana*, Protein purification

[Background] Protein complexes function as signaling platforms, molecular machines, and scaffolds upon which cellular life is built. Identification of protein-protein interaction (PPI) or the composition of a protein complex is of enormous importance to provide insight into the biochemical function of genes. Therefore, facile and robust methods for discovering PPI are required to understand how genotype determines phenotype in plants.

Commonly used protein purification methods involve chromatography, such as size-exclusion chromatography (also known as gel filtration), ion exchange chromatography and affinity chromatography. Combining several different chromatographic approaches is often required to reach sufficient purity to identify relevant complexes. Recently, affinity purification coupled with mass spectrometry (AP-MS) has emerged as a powerful biochemical approach of systematically identifying *in vivo* protein-protein associations. By fusing one (for one-step AP-MS) or two (for tandem AP-MS) affinity tags to a protein that serves as a bait, one can simultaneously isolate the bait protein and co-purify any proteins directly or indirectly associated with the bait from crude protein extracts. One-step AP-MS method can suffer a high false positive rate because of many sticky or highly abundant contaminating proteins. The tandem AP-MS method leverages an additional affinity purification step to increase selectivity, further filter out contaminants, and simultaneously enrich for captured complexes. Several affinity tags have been widely used, such as the FLAG peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) and polyhistidine (*e.g.*, His_6), each of which has different binding capacity and methods of elution, resulting in different yields and purity (Lichty *et al.,* 2005). It is noteworthy that AP-MS has its limitations, such as different tags or combinations may lead to distinct group of non-specific interactors (false positives), long protocols may lead to loss of weakly associate partners (false negatives), and AP-MS cannot distinguish direct from indirect interaction. For more information on comparing different PPI techniques, as well as challenges or limits of those approaches, we would like to point to several in-depth reviews (Van Leene *et al.*, 2008; Fukao, 2012; Braun *et al.*, 2013; LaCava *et al.*, 2015).

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We have developed a methodology for tandem-affinity purification for use in plants that is designed for the specific identification of protein complexes by mass spectrometry (Huang *et al.*, 2016a and 2016b). We fused a His_6-3x FLAG tandem affinity tag to the C-terminus of the bait protein, transformed the construct expressing the fusion protein into the model plant *Arabidopsis thaliana* by the floral dipping method (Zhang *et al.*, 2006), and conducted tandem AP-MS to identify associated proteins using this methodology. The pB7HFC3.0 construct we used for cloning and expressing the fusion protein has been described (Huang *et al.*, 2016a; 2016b; 2016c). We choose the FLAG purification over other affinity purification methods as the first purification step. This is because FLAG antibodies have low background in *Arabidopsis thaliana,* and by applying excessive free FLAG peptides, we can elute off FLAG-tagged proteins from beads in gentle, non-denaturing conditions. We then used Cobalt beads to bind His_6-tagged proteins present in the FLAG eluates since the high binding capacity of Cobalt beads facilitates the enrichment, FLAG elution peptide removal, and further cleanup of all His_6-tagged proteins from the first purification step. The tandem AP-MS protocol using the His_6-3x FLAG tag was previously shown able to be sensitive and selective enough to identify protein complexes expressed at near native levels in *Arabidopsis thaliana* seedlings (Huang *et al.*, 2016a).

Materials and Reagents

1. 125 mm Qualitative Whatman paper (GE Healthcare, catalog number: 1001125)
2. 2 ml Seal-Rite microcentrifuge tubes (USA Scientific, catalog number: 1620-2700)
3. P1000 pipet tip
4. 50 ml centrifuge tubes (VWR, catalog number: 89039-658)
5. Oak Ridge centrifuge tubes, 50 ml (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 3118-0050)
6. 15 ml conical tubes (VWR, catalog number: 89039-666)
7. 0.45 µm PVDF syringe filters with luer lock (EMD Millipore, catalog number: SLHVM33RS)
8. Serological pipets
9. 1.5 ml tube
10. 1.7 ml Low Retention microtubes (PHENIX Research Products, catalog number: MAX-715L)
11. 0.22 µm PVDF syringe filters with luer lock (EMD Millipore, catalog number: SLGV033RS)
12. 15 cm plates (VWR, catalog number: 25384-326)
13. 30 ml syringe (BD, Luer-Lok™, catalog number: 302832)
14. 5 ml syringe (BD, catalog number: 305855)
15. 3.2 mm stainless steel balls (Bio Spec Products, catalog number: 11079132ss)
16. *Arabidopsis* seedlings (Col-0 ecotype) containing transgenes that express His_6-3x FLAG epitope tagged proteins (Huang *et al.*, 2016a; 2016b; 2016c)
17. Liquid N_2 for flash freezing samples
18. Dry Ice to maintaining samples cold
19. Phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, catalog number: 10837091001)

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20. Isopropanol
21. Protease inhibitor tablets, EDTA-free (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 88266)
22. Phosphatase inhibitor cocktail 2 (Sigma-Aldrich, catalog number: P5726)
23. MG-132 (Peptides International, catalog number: IZL-3175-v)
24. Phosphatase inhibitor 3 (Sigma-Aldrich, catalog number: P0044)
25. Dynal Talon Magnetic beads (Thermo Fisher Scientific, Noves™, catalog number: 10104D)
26. M2 anti-FLAG antibody (Sigma-Aldrich, catalog number: F1804)
27. Dynal Protein G Magnetic beads (Thermo Fisher Scientific, Noves™, catalog number: 10003D)
28. 3x FLAG elution buffer
29. 3x FLAG peptide (Sigma-Aldrich, catalog number: F4799)
30. Murashige and Skoog medium (MP BIOMEDICALS, catalog number: 092610024)
31. Agar (Sigma-Aldrich, catalog number: A1296)
32. Sodium phosphate monobasic dehydrate ($NaH_2PO_4 \cdot H_2O$) (Sigma-Aldrich, catalog number: 71505)
33. Sodium phosphate dibasic dehydrate ($Na_2HPO_4 \cdot H_2O$) (Sigma-Aldrich, catalog number: 71662)
34. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S7653)
35. EDTA (Sigma-Aldrich, catalog number: EDS)
36. EGTA (Sigma-Aldrich, catalog number: E3889)
37. Triton X-100 (Sigma-Aldrich, catalog number: T9284)
38. Ammonium bicarbonate (Sigma-Aldrich, catalog number: A6141)
39. ddH_2O
40. ½x MS-agar media (see Recipes)
41. SII buffer (store at 4 °C) (see Recipes)
42. SII+ buffer (make fresh) (see Recipes)
43. FLAG to His buffer (store at 4 °C) (see Recipes)
44. Ammonium bicarbonate buffer (make fresh) (see Recipes)
45. 3x FLAG peptide (store at -80 °C) (see Recipes)

Equipment

1. Autoclave
2. Retsch 400 mixer mill (Retsch, model: 400 Mixer Mill)
3. Retsch mixer mill adapter racks for single use tubes (Retsch, catalog number: 22.008.0008)
4. 35 ml grinding jars with 20 mm stainless steel balls (referred as ‘ball mill’ hereafter) (Retsch, catalog numbers: 01.462.0214, 05.368.0062)
5. Growth chamber for seedlings (Geneva Scientific, catalog number: CU-36L6)
6. Probe sonicator with microtip attachment (Thermo Fisher Scientific, Fisher Scientific, model: 505)

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7. High speed centrifuge (≥ 20,000 *x g*) (Beckman Coulter, model: any Avanti J series)
8. High speed rotor (Beckman Coulter, model: JA-20)
9. Microcentrifuge (Eppendorf, model: 5424)
10. Swinging bucket centrifuge (Eppendorf, model: 5810 R)
11. Magnetic stand to capture beads, Dynamag-15 (Thermo Fisher Scientific, catalog number: 12301D)
12. Magnetic stand to capture beads, Dynamag-2 (Thermo Fisher Scientific, catalog number: 12321D)
13. Heating mixer (Eppendorf, model: 5355)

Procedure

A. Harvest tissue
   1. Grow *Arabidopsis* seedlings expressing His_6-3x FLAG tagged proteins on sterilized Whatman on top of ½x MS 15 cm plates for 10-12 days under specific light conditions.
   2. For small scale experiments (approximately 0.5 g tissue per sample), collect tissue into 2~5 2 ml bullet tubes containing three 3.2 mm stainless steel balls. For large scale affinity purification/mass spectrometry (AP/MS), harvest 5 g tissue per sample in foil and harvest 3 packages (5 g) as replicates.
   3. Label with date, name of tissue, weight, growth conditions (constant light, 12 h light:12 h dark, Short Days, Long Days, constant dark, constant blue, constant red, *etc.*), and Zeitgeber time.
   4. Freeze in Liquid N_2 and store at -80 °C.

   Note: Growing seedlings on Whatman facilitates seedling removal while minimizing transfer of growth media. For large scale affinity purification and mass spectrometry (AP/MS), put tissues in a corner of the foil and form a ball, so that it is easy to be broken up and poured into the metal ball mill.

B. Grind tissue using the Retsch 400 mixer mill
   1. Put Retsch mixer mill adapter racks (for 2 ml tubes) or the 35 ml grinding Jar which contains one 20 mm stainless steel ball (ball mill) into Liquid N_2 to cool before use. Carefully put 5 g frozen tissue into the 35 ml grinding Jars using a pre-cooled spatula.
   2. Break up tissue by gently inverting the ball mill (large scale) or by using pre-cooled P1000 tip to crunch tissue in bullet tubes (small scale).
   3. For small scale, grind samples either 4 times at 30 Hz for 45 sec for bullet tubes. For large scale using the ball mill, first grind at 25 Hz for 45 sec to create cushion of tissue in ball mill to prevent damage, then 4 times at 30 Hz for 45 sec.
   4. Cool the ball mill in Liquid N_2 between grinding. For 2 ml bullet tubes, open caps once to release pressure inside the tube between each grinding. Also scrape compacted sample off lid with pre-

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cooled pipet tip to dislodge. During handling, keep grinding jars, adapter racks, holders and tubes in Liquid N_2 and work quickly to prevent samples from thawing.

5. When finished, transfer bullet tubes to dry ice (open once to release pressure), for ball mill transfer powder to 50 ml conical on dry ice.
6. Continue to the purification steps, or store at -80 °C for up to one month.

Notes:

a. *When handling samples: Use Liquid N_2 and dry ice to keep everything cool. Label 50 ml Falcon conical tubes (on both cap and side) for the ground powder (large scale) and put them on dry ice. The grinding process takes ~1 h with clean up, so we grind multiple samples one day before the IP.*
b. *If you will go directly from grinding samples into the prep, before grinding preps you should make fresh 100 mM phenylmethylsulfonyl fluoride (PMSF) in isopropanol, add protease inhibitor tablet to buffer SII and thaw the phosphatase inhibitor 2 and MG-132 at RT, (at 4 °C it will freeze again). Do not add into the buffer until you grind tissue into powders. Put all 15 ml, 50 ml Falcon conical tubes, 50 ml centrifugation tubes, long pipet tips, tips with filter into the cold room to cool before use. Put the cross-linked anti-FLAG beads on a roller to fully resuspend (use the magnetic stand to help get beads off the bottom, or just tapping the tube bottom with fingers).*

C. Resuspend ground tissue

1. Make up SII+ buffer with protease inhibitor tablet (1 mini tablet for 10 ml or 1 tablet up to 50 ml), 1 mM PMSF (100x stock freshly made in isopropanol), 1x phosphatase inhibitors 2&3 (from 100x stocks), 50 µM MG-132 (from 200x,10 mM stock). 30 ml is enough for two packages of 5 g tissue.
2. Add 1 packed tissue volume of SII+ (typically 500~800 µl for 0.5 g of tissue for small scale or ~12-14 ml for 5 g of tissue) to ground tissue and rotate at 4 °C for 10 min. Do not vortex (you can put a rotator onto a shaker, so that it is resuspended gently).
   Note: During this time, label 1.5 ml tubes for quality control steps: Input before FLAG IP/total extraction, FLAG IP flow through, FLAG beads, FLAG elution 1-4 (E1-E4), FLAG elution combined/His IP input, His IP flow through, His beads/Talon beads as well as washes if you want to save. Three low retention tubes are needed for each sample, and they are for: FLAG IP transfer, FLAG IP combined elutes, Talon Dynal beads (His beads, final tube, label well).

D. Sonicate

1. Sonicate resuspension twice at 40% amplitude (power) for 20 sec, with 1 sec on/off pulse. Keep the sample tubes on ice between each sonication. For small scale preparations, move resuspended tissue to a new tube to avoid damaging the sonicator tip with the stainless steel beads used for tissue disruption in Liquid N_2.

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Note: Wear earplugs to protect your ears. Let the sample sit on ice to cool before moving on to the next sonication. Move the 50 ml tubes up and down while sonicating so that the microtip will thoroughly break up any chunks. Also, precool the centrifuge and rotor to 4 °C at this time.

E. Clarify extract
1. Spin clarify the samples at ≥ 20,000 *x g*, 10 min, 4 °C, twice.
2. Filter the clarified supernatant to remove any chunks with a 0.45 µm filter attached to syringe of adequate size (*e.g.*, 30 ml syringe). The filtered extract goes into a 15 ml conical tube. Note volume.
3. Save 90 µl for input control.
4. Measure protein concentration. Usually for 5 g 10-day-old *Arabidopsis* seedlings, we had a concentration of 5-10 mg/ml. So for large scale, we use a total protein of 75-150 mg.

F. Pre-wash the crosslinked anti-FLAG Dynal beads
1. During the second centrifugation, add 900 µl SII buffer without supplements in a tube.
2. Add 250 µl of crosslinked anti-FLAG Dynal beads (we calculated ~5 µl beads/1 mg of extract [*e.g.,* 250 µl for 5 g tissues]) into the liquid, pipet several times to wash the tip so that all beads go into the solution.
3. Spin tubes for 1 min at ≥ 1,000 *x g* to collect solution from caps.
4. Put beads on magnetic stand Dynamag-2, and wait 1 min for beads being separated from supernatant.
5. Remove supernatant.
6. Wash once more with 900 µl SII buffer without supplements, then remove supernatant and add 400 µl SII+ buffer. Keep pre-washed beads sit on ice.

Note: Crosslink 2 µg of M2-FLAG antibody per 60 µl of Protein G beads following instructions in the manual for Dynal Protein G beads.

G. Begin incubation with anti-FLAG Dynal beads
1. When ready to incubate beads with extract, put beads on magnetic stand and wait 1 min.
2. Remove supernatant.
3. Pipet ~500 µl of extract onto the beads, resuspend, and pipet back all the beads into the 15 ml conical tube, repeat 2 more times to transfer all the beads.
4. Start the immunoprecipitation (IP) on a rotator for 30 min-1 h, 4 °C.

Note: Preparation during FLAG-IP: prep the 3x FLAG elution buffer. To make 500 µg/ml 3x FLAG peptide in the FLAG to His buffer, pipet 54.5 µl of 33 mg/ml 3x FLAG stock solution into 3.6 ml buffer (3.6 ml gives a little extra volume for two samples' elution).

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H. Bead capture, washes, and transfer to 1.5 ml low retention tube
1. Spin tubes for 1 min at ≥ 1,000 *x g* to collect solution from caps.
2. Place tubes on magnetic rack Dynamag-15. Wait 2 min.
3. Remove flow through without disturbing beads. Stick a P1000 pipet tip on end of 14 ml serological pipet to control flow for large scale capture.
4. Save the flow through for controls.
5. Wash beads in 10 ml SII buffer (no supplements needed) (≥ 20 bead volumes, so at least 5 ml for large scale or 1 ml for small scale).
6. Rotate for 5 min.
7. Spin tubes for 1 min at ≥ 1,000 *x g* to collect solution from caps.
8. Place tubes on magnetic rack Dynamag-15. Wait 2 min.
9. Remove Wash without disturbing beads. Save for controls.
10. Wash beads in 10 ml SII buffer (no supplements needed).
11. Rotate for 1 min.
12. Spin tubes for 1 min at ≥ 1,000 *x g* to collect solution from caps.
13. Place tubes on magnetic rack. Wait 2 min.
14. Remove Wash without disturbing beads. Save for controls.
15. On the third wash, wash beads off wall with 900 µl FLAG to His buffer.
16. Transfer beads to a 1.5 ml low retention tube labeled as FLAG IP transfer.
17. Place tubes on magnetic stand Dynamag-2. Wait 1 min.
18. Remove Wash without disturbing beads.
19. Transfer any remaining beads from 15 ml conical to 1.5 ml tube in 900 µl FLAG to His buffer.
20. Place tubes on magnetic stand Dynamag-2. Wait 1 min.
21. Remove Wash without disturbing beads.
22. Repeat washing with 900 µl FLAG to His buffer two more times.

Note: On the third wash, control volume so that you can transfer all beads from 15 ml conical tube to 1.5 ml low retention tube to do the following washes. The FLAG to His buffer has no EDTA/EGTA and less detergent, so it is compatible with Cobalt/Nickel NTA beads.

I. Elution off the beads
1. Remove supernatant off beads.
2. Add 400 µl elution buffer made earlier (FLAG to His buffer + 500 µg/ml [ng/µl] 3x FLAG peptide).
3. Rotate beads for 15 min at 4 °C.
4. Spin tubes for 1 min at ≥ 1,000 *x g* to collect solution from caps.
5. Place tubes on magnetic stand Dynamag-2. Wait 1 min.
6. Remove elution without disturbing beads, transfer 1/10 vol to elution 1 (E1) sample tubes, rest to low retention protein tube labeled FLAG IP combined elutes.

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7. Repeat elution at 4 °C for 15 min, save the same amount from the second elution for quality control and put the rest of the 2nd elution to low retention protein tube labeled FLAG IP combined elutes.
8. Elute another two times with 400 µl elution, rotate beads at 30 °C for 15 min. Save 1/10 of each elution for quality controls and combine the rest of the 3rd and 4th elution to the low retention protein binding tube.
9. Mix combined elution tube. Remove 1/20 volume to tube for analysis.

Note: During the 2nd~3rd elution, pre-wash Talon Dynal beads (His beads) with FLAG to His buffer (No EDTA or EGTA, since it will strip cobalt off the resin). Use at least ~1/5 volume of beads that you used in FLAG IP, but can go higher if you observe protein in Talon bead flow through. Also, make ≥ 5 ml 25 mM ammonium bicarbonate buffer, prepared fresh. Filter through a 0.22 µm filter.

J. His IP

1. Transfer combined eluates to low protein binding tube with washed Talon beads.
2. Incubate for 20 min, at 4 °C with rotation.
3. Spin tubes for 1 min at ≥ 1,000 *x g* to collect solution from caps.
4. Place tubes on magnetic stand Dynamag-2. Wait 1 min.
5. Remove Wash without disturbing beads.
6. Wash with 900 µl FLAG to His buffer by mixing by gently inverting the tube.
7. Spin tubes for 1 min at ≥ 1,000 *x g* to collect solution from caps. Then repeat the wash one more time.
8. Remove the 2nd FLAG to His wash without disturbing beads. Then wash beads with 900 µl 25 mM ammonium bicarbonate buffer and gently invert the tube. Repeat twice as previous washes.
9. During the last wash with 900 µl 25 mM ammonium bicarbonate buffer, once the beads are completely resuspended by gently inverting the tube, remove 1/10 (90 µl) volume to new tube for quality control.
10. Spin tubes for 1 min at ≥ 1,000 *x g* to collect solution from caps.
11. Place tubes on magnetic stand Dynamag-2. Wait 1 min and then remove all of wash without disturbing beads.
12. Flash freeze beads in Liquid N_2, store in -80 °C.
13. After His IP, running a Western blot or silver staining to check the quality of the affinity purification. For example, load on 10% SDS-PAGE gel of combined FLAG eluates (serves as an input for His purification), 10% of flow-through/unbound for the His purification, and 10% of His beads after binding. This is to test if the bait protein is well enriched after His purification. A good practice is to also compare protein purifications of your protein of interest to control purifications (using a His_6-3x FLAG tagged control protein such as Green Fluorescent Protein) by silver stain (Chevallet *et al*., 2006) to identify unique bands associated with your protein of interest. Submit protein/beads complex for digestion and sequencing at mass spectrometry facility.

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Data analysis

Two to four independent biological replicate affinity purifications should be done to determine reproducibility of mass spectrometry identifications from purifications, as mentioned previously (Huang *et al.*, 2016a and 2016b). All epitope-tagged lines should be checked for functionality prior to use, preferably by complementing characterized mutants (Huang *et al.*, 2016a).

Notes

1. Wear and change gloves often. The top identified contaminating proteins are keratin and collagen from humans. Do not touch clothes with gloved hands. All the buffers, tips, filters, syringes are kept in cold room and separated from others, and for MS-use only.
2. Other than when specified, all the work should be done in the cold room with ice bucket.
3. The use of non-carbohydrate based resins, such as the polystyrene paramagnetic Dynal beads, will reduce background from plant tissues.
4. Refer to the resin manufactures specifications for information about buffer, salt and detergent compatibility.

Recipes

1. ½x MS-agar media (1 L)
   2.205 g Murashige and Skoog medium
   7 g agar
   Autoclave, and dispense ~40 ml per 15 cm dish
2. SII buffer, store at 4 °C
   100 mM Na-Phosphate, pH 8.0
   150 mM NaCl
   5 mM EDTA
   5 mM EGTA
   0.1% Triton X-100
   Filter through a 0.22 µm filter to sterilize
3. SII+ buffer (make fresh)
   Note: Add following supplements to SII buffer above just before use.
   1 mM phenylmethylsulfonyl fluoride
   1x protease inhibitor mix
   1x phosphatase inhibitor II
   1x phosphatase inhibitor III
   50 µM MG-132

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4. FLAG to His buffer (store at 4 °C)
   100 mM Na-Phosphate, pH 8.0
   150 mM NaCl
   0.05% Triton X-100
   Filter through a 0.22 µm filter to sterilize
5. Ammonium bicarbonate buffer (make fresh)
   25 mM ammonium bicarbonate in ddH_2O, made fresh, then filter through a 0.22 µm filter to sterilize
6. 3x FLAG peptide (store at -80 °C)
   ≥ 33 mg/ml MDYKDHDGDYKDHDIDYKDDDDK resuspended in phosphate buffer, pH 8.0

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References

1. Braun, P., Aubourg, S., Van Leene, J., De Jaeger, G. and Lurin, C. (2013). Plant protein interactomes. *Annu Rev Plant Biol* 64: 161-187.
2. Chevallet, M., Luche, S. and Rabilloud, T. (2006). Silver staining of proteins in polyacrylamide gels. *Nat Protoc* 1(4): 1852-1858.
3. Fukao, Y. (2012). Protein-protein interactions in plants. *Plant Cell Physiol* 53(4): 617-625.
4. Huang, H., Alvarez, S., Bindbeutel, R., Shen, Z., Naldrett, M. J., Evans, B. S., Briggs, S. P., Hicks, L. M., Kay, S. A. and Nusinow, D. A. (2016a). Identification of evening complex associated proteins in *Arabidopsis* by affinity purification and mass spectrometry. *Mol Cell Proteomics* 15(1): 201-217.
5. Huang, H., Alvarez, S. and Nusinow, D. A. (2016b). Data on the identification of protein interactors with the Evening Complex and PCH1 in *Arabidopsis* using tandem affinity purification and mass spectrometry (TAP-MS). *Data Brief* 8: 56-60.
6. Huang, H., Yoo, C. Y., Bindbeutel, R., Goldsworthy, J., Tielking, A., Alvarez, S., Naldrett, M. J., Evans, B. S., Chen, M. and Nusinow, D. A. (2016c). PCH1 integrates circadian and light-signaling pathways to control photoperiod-responsive growth in *Arabidopsis*. *Elife* 5: e13292.
7. LaCava, J., Molloy, K. R., Taylor, M. S., Domanski, M., Chait, B. T. and Rout, M. P. (2015). Affinity proteomics to study endogenous protein complexes: pointers, pitfalls, preferences and perspectives. *Biotechniques* 58(3): 103-119.
8. Lichty, J. J., Malecki, J. L., Agnew, H. D., Michelson-Horowitz, D. J. and Tan, S. (2005). Comparison of affinity tags for protein purification. *Protein Expr Purif* 41(1): 98-105.

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9. Van Leene, J., Witters, E., Inze, D. and De Jaeger, G. (2008). Boosting tandem affinity purification of plant protein complexes. *Trends Plant Sci* 13(10): 517-520.
10. Zhang, X., Henriques, R., Lin, S. S., Niu, Q. W. and Chua, N. H. (2006). Agrobacterium-mediated transformation of *Arabidopsis thaliana* using the floral dip method. *Nat Protoc* 1(2): 641-646.