Chapter 21

Phosphoprotein Enrichment from Tobacco Mature Pollen Crude Protein Extract

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Abstract

Protein phosphorylation was repeatedly shown to be the most dynamic post-translational modification mediated by a huge orchestra of protein kinases and phosphatases. Upon landing on a stigma, pollen grain dehydration and activation are accompanied by changes in protein phosphorylation together with the translation activation of stored mRNAs. To enable studies of the total phosphoproteome, it is usually necessary to apply various enrichment techniques. In this chapter, one of these protocols that worked previously well on tobacco mature pollen is presented in more detail. The method comprises of three basic steps: (1) picking flowers from the flowering tobacco plants (*Nicotiana tabacum* cv. Samsun), and collection of the shed pollen grains; (2) extraction of total proteins by TCA/acetone; (3) phosphoprotein enrichment by MOAC with aluminum hydroxide matrix. Taken together this protocol describes how to isolate phosphoproteins out of tobacco mature pollen.

Key words Phosphoprotein enrichment, Metal-oxide/hydroxide affinity chromatography, MOAC, Male gametophyte, Pollen grain, Aluminum hydroxide, TCA/acetone protein extraction

1 Introduction

Angiosperm mature pollen has an extremely desiccated cytoplasm, which is surrounded by a tough cell wall. After reaching the papillary cells of a stigma, pollen cytoplasm becomes re-hydrated, the metabolism is activated, and one particular pollen aperture is used for pollen tube growth (reviewed in [1]). Such change from a quiescent to an activated structure during progamic phase is accompanied by the translation activation of mRNAs stored in EDTA/puromycine-resistant particles (EPPs; [2, 3]), and also post-translational modifications of the existing proteins, phosphorylation in particular [4, 5]. Such re-hydration-related phosphorylation was described also in the xerophyte *Craterostigma plantagineum* [6], and in the leaf growth zone in maize (*Zea mays*) [7].

Protein phosphorylation represents one of the most dynamic post-translational modifications that plays a crucial role in plenty of

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cellular processes and structures (e.g., [8–11]). Protein phosphorylation is mediated by protein kinases, whereas the phosphate removal is performed by protein phosphatases. The studies of protein phosphorylation usually apply one of the available enrichment protocols [12]. The enrichment techniques are mostly essential since (1) only part of the phosphoproteins is phosphorylated at a time in a cell, (2) the phosphorylated protein can co-exist with the non-phosphorylated isoform of the same species, (3) the technical aspects of mass spectrometry make the identification of phosphorylated peptides in the mixture where non-phosphorylated species dominate very unlikely.

The enrichment step can be performed either at the level of intact phosphoproteins or at the level of peptides that were acquired by proteolytic digest of the whole proteins (reviewed in [12]). Both of these approaches showed their advantages as well as limitations. In this chapter, we focus on phosphoprotein enrichment by metal oxide/hydroxide affinity chromatography (MOAC) with aluminum hydroxide matrix [13]. The presented protocol starts with the collection of pollen grains from flowering tobacco (*Nicotiana tabacum* cv. Samsun) plants [14]. After the pollen grains are collected, the extraction of total proteins is carried out. In tobacco pollen, it was shown that the proper homogenization approach together with the selection of extraction protocol is vitally important [15]. In this chapter, the TCA/acetone protein extraction protocol [16] was adopted for mature pollen since it was the optimal one prior to the phosphoprotein enrichment. The obtained total protein extract was subjected to phosphoprotein enrichment by MOAC with aluminum hydroxide matrix [13]. Further separation steps, such as SDS-PAGE or 2D IEF-PAGE, were not subjected to a detailed description in this chapter. Taken together, this protocol comprises three parts: (1) flower collection, and pollen grain isolation, (2) extraction of total proteins, and (3) MOAC phosphoprotein enrichment (for a scheme, *see* Fig. 1).

2 Materials

2.1 Pollen Grain Collection

Prepare the following items to collect pollen grains:

1. Grown flowering tobacco plants (*Nicotiana tabacum* cv. Samsun).
2. Petri dish, 15–20 cm in diameter.
3. Roundly shaped filtration paper of the size matching inside the Petri dish.
4. Sieve from a stocking fixed onto a glass roll (*see* **Note 1**).
5. Laboratory scales.
6. 1.5 ml microcentrifuge tubes.

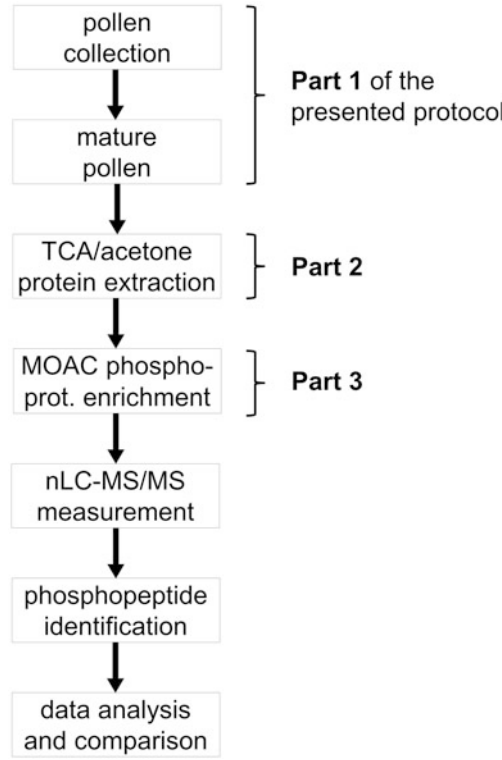

Fig. 1 Working scheme of a phosphoproteomics experiment. The parts presented in this manuscript are highlighted

2.2 Protein Extraction by TCA/Acetone

Prepare the following solutions freshly before each extraction together with the following laboratory equipment:

1. 500 mg tobacco mature pollen (from Subheading 3.1) (*see* **Note 2**).
2. Extraction buffer 10% TCA, and 1% DTT in acetone: weigh 1 g trichloroacetic acid (TCA), and 100 mg dithiothreitol (DTT) on laboratory scales; transfer the weighted chemicals into a 50 ml Falcon tube and top up to 10 ml by acetone (*see* **Note 3**).
3. Washing buffer 1% DTT in acetone: weigh 150 mg dithiothreitol (DTT), put it to a 50 ml Falcon tube and fill in with acetone to 15 ml (*see* **Note 4**).
4. Pestle and mortar.
5. 2 ml microcentrifuge tubes.
6. A 15 ml Falcon tube.
7. Cooled centrifuge (4 °C).
8. Freezer −20 °C.
9. Ultrasonic bath.

10. Liquid nitrogen.
11. Glass rod.
12. Vortex.
13. Vacuum pump.
14. Injection needle (*see* **Note 5**).

2.3 Phosphoprotein Enrichment by MOAC

Prepare the following solutions and laboratory equipment prior to the enrichment. The stock solutions can be stored for a longer period whilst the working solutions should be prepared freshly before each use and any rest of them should be discarded afterward.

1. Incubation buffer stock solution: 30 mM MES, 20 mM imidazole, 0.2 M L-aspartic acid potassium salt, 0.2 M L-glutamic acid sodium salt (*see* **Note 6**), 8 M urea, pH 6.1. Dissolve 120.2 g urea in 100 ml distilled water (*see* **Note 7**). After urea becomes completely solubilized, add 1.599 g MES, 8.56 g L-aspartic acid potassium salt, 8.455 g L-glutamic acid sodium salt, and 0.3404 g imidazole. Every time, wait with the addition of the subsequent substance until the previous one is fully dissolved. Finally, set the pH of the buffer to 6.1 with hydrochloric acid.
2. Incubation buffer solution ready to use: 2.5% CHAPS, phosphatase inhibitors in incubation buffer stock solution. Prepare 30 ml buffer per enriched sample: dissolve 0.75 g CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) together with 0.3 ml phosphatase inhibitor cocktail to a final volume of 30 ml incubation buffer stock solution.
3. Elution buffer stock solution A: 100 mM KH_2PO_4. Dissolve 0.6805 g KH_2PO_4 in a final volume of 50 ml distilled water.
4. Elution buffer stock solution B: 100 mM K_2HPO_4. Dissolve 4.355 g in a final volume of 250 ml distilled water.
5. Elution buffer stock solution AB: 8 M urea in elution buffer stock solution B (100 mM K_2HPO_4), pH 9.0. Dissolve 48.08 g urea in 50 ml elution buffer stock solution B (100 mM K_2HPO_4) (*see* **Note 7**), top the volume up to 100 ml elution buffer stock solution B, and set the pH of the final solution to 9.0 with buffer stock solution A (100 mM KH_2PO_4).
6. Elution buffer ready to use: stock solution AB plus phosphatase inhibitors. Prepare freshly before each use 2 ml elution buffer per enriched sample: add 20 µl phosphatase inhibitor cocktail into 2 ml elution buffer stock solution AB and mix well.
7. 160 mg aluminum hydroxide in a 15 ml Falcon tube (per enriched sample).
8. 15 ml Falcon tubes (*see* **Note 8**).

9. 1.5 ml microtubes.
10. Thermoblock (set to 37 °C).
11. Vortex.
12. Ultrasonic bath.
13. Cooled centrifuge suitable for Falcon tubes.
14. Rotator in a cold place.

3 Methods

3.1 Pollen Grain Collection

1. Grow the tobacco plants until they start flowering. Cut the overgrown flowers and also whole old branches almost without any flowers to encourage the tobacco plants in setting new flower buds.
2. From the flowering tobacco plants, pick the flowers 1 day before anthesis (*see* **Note 9**) (Fig. 2).
3. Remove the anthers from the flowers and put them onto a filtration paper in a Petri dish. Do not close the Petri dish.
4. Let the anthers dehisce overnight at room temperature (*see* **Note 10**), they will open and pollen grains will be shed out of them.
5. Separate pollen grains from the debris, represented mainly by the green parts of the anthers, by a home-made sieve prepared from a stocking fixed onto a glass roll.
6. Transfer the filtered pollen grains (*see* **Note 11**) into 1.5 ml microcentrifuge tubes, weigh them and store them at −20 °C (*see* **Note 12**).

Fig. 2 Flowering tobacco plant (*Nicotiana tabacum* cv. Samsun). The anther 1 day before anthesis for the collection is labeled

3.2 Protein Extraction by TCA/Acetone

Before the extraction, cool down the centrifuge to 4 °C since all centrifugation steps will be carried out at 4 °C in this part of the protocol.

1. Fill in the mortar with liquid nitrogen and let it evaporate, and also cool down the pestle in liquid nitrogen.
2. Fill in the mortar again with liquid nitrogen and add 500 mg pollen grains (*see* **Note 13**) into it.
3. Grind the pollen grains under liquid nitrogen and transfer the homogenized pollen into a 15 ml Falcon tube.
4. Resuspend the transferred sample with tenfold volume (i.e., 5 ml per 500 mg pollen grains) extraction buffer (10% TCA + 1% DTT in acetone), and vortex thoroughly.
5. Transfer the resuspended sample (*see* **Note 14**) by a 1 ml pipetman with a cut tip to four 2 ml microtubes (1.25 ml sample per each microcentrifuge tube).
6. Snap freeze the samples in liquid nitrogen.
7. Incubate the samples at −20 °C for 45 min. Mix the suspension by turning the tubes upside down several times after 5, 10, and 15 min of incubation.
8. Centrifuge the samples (15 min, 25,000 × *g*, 4 °C), and immediately after the centrifugation, discard the supernatant by using an injection needle (*see* **Note 15**) connected to the vacuum pump.
9. Resuspend the pellet by 1.5 ml washing buffer (1% DTT in acetone), and incubate for 5 min in an ultrasonic bath.
10. In case the pellet is still compact, disturb it by a glass rod.
11. Snap freeze the samples in liquid nitrogen.
12. Centrifuge the samples (10 min, 25,000 × *g*, 4 °C), and immediately after the centrifugation, discard the supernatant by an injection needle (*see* **Note 15**) connected to the vacuum pump.
13. Resuspend the pellet in 1.5 ml washing buffer (1% DTT in acetone), and incubate for 5 min in an ultrasonic bath.
14. In case the pellet is still compact, disturb it by a glass rod.
15. Snap freeze the samples in liquid nitrogen.
16. Centrifuge the samples (10 min, 25,000 × *g*, 4 °C), and immediately after the centrifugation discard the supernatant by an injection needle (*see* **Note 15**) connected to the vacuum pump.
17. Dry the pellet in vacuum, and store the dried pellet at −20 °C for a short-term storage, and at −80 °C for a long-term storage.

3.3 Phosphoprotein Enrichment by MOAC

Prior to the enrichment, warm the thermoblock to 37 °C, and place the rotator to the cold room.

1. Add 50 μl incubation buffer per 1 mg TCA/acetone precipitate from **step 2** (*see* **Note 16**).
2. Vortex the sample briefly, and incubate for 5 min in an ultrasonic bath.
3. Put the sample onto the thermoblock (1 h, 37 °C).
4. Centrifuge the sample (25,000 × *g*, 15 min, 22 °C), and immediately transfer the supernatant into a fresh tube (*see* **Note 17**).
5. Estimate protein quantity (*see* **Note 18**).
6. Dilute the samples in the incubation buffer to have 3 ml sample of 0.66 mg ml^{-1} concentration for the subsequent steps. The rest of the sample can be stored at −20 °C.
7. Prepare 160 mg aluminum hydroxide into a 15 ml Falcon tube.
8. Add 2 ml incubation buffer, and vortex vigorously to moisten the aluminum hydroxide powder (*see* **Note 19**).
9. Spin the aluminum hydroxide particles down (9000 × *g*, 2 min, 4 °C), and discard supernatant.
10. Put 3 ml of the diluted sample to the aluminum hydroxide powder, and vortex briefly to get a homogenous suspension.
11. Incubate 30 min in the cold room on a rotator (*see* **Note 20**).
12. Centrifuge the samples (9000 × *g*, 2 min, 4 °C) to pellet the aluminum hydroxide particles (*see* **Note 21**), and transfer the supernatant to a fresh tube (*see* **Note 22**).
13. Wash the pellet with 3.2 ml incubation buffer, and vortex thoroughly for 1 min.
14. Centrifuge the tube (9000 × *g*, 2 min, 4 °C) to pellet the aluminum hydroxide particles and keep supernatant (*see* **Note 23**).
15. Repeat **steps 13** and **14** four times.
16. Wash the pellet with 1.6 ml incubation buffer, and vortex thoroughly for 1 min.
17. Centrifuge the tube (9000 × *g*, 2 min, 4 °C) to pellet the aluminum hydroxide particles and keep supernatant.
18. Add 1.6 ml elution buffer to the pellet, and thoroughly vortex for 1 min.
19. Incubate 20 min in the cold room on a rotator (*see* **Note 20**).
20. Spin the aluminum hydroxide particles down (9000 × *g*, 2 min, 4 °C).

21. Keep the supernatant containing phosphoprotein-enriched fraction, and discard the pellet with aluminum hydroxide particles.

4 Notes

1. This type of sieve is used in our laboratory. However, it can be replaced by any other sieve that will efficiently separate debris from mature pollen grains.
2. The amount of used pollen grains does not have to be exactly 500 mg but can be slightly higher or lower since TCA/acetone volume can be adjusted accordingly.
3. Be careful while manipulating with solid TCA; wear gloves since it is a corrosive compound.
4. The final volume of washing buffer was calculated for four microcentrifuge tubes containing aliquots of protein extract. In case of planning more aliquots due to higher amount of starting material used for protein extraction, increase the volume of prepared washing buffer by 3 ml per additional microcentrifuge tube.
5. It is important to have a thin enough needle that will enable sucking the supernatant on one hand but will not remove any parts of the pellet on the other hand.
6. The concentrations of L-aspartic acid potassium salt and L-glutamic acid sodium salt can be optimized since too low concentration causes a higher non-specificity of the enrichment (i.e., lower selectivity) whereas too high concentration reduces the sensitivity of the method [6].
7. Warm up the urea solution until urea crystals become completely dissolved.
8. Falcon tubes can be replaced by any other tubes suitable for centrifugation. In case Falcon tubes are used, check their suitability for centrifugation, otherwise Falcon tube cracking might occur.
9. It is necessary to pick the flowers exactly 1 day before anthesis, and not the younger ones since their anthers will be less developed and will not open until the following day. On the other hand, the older flowers do not contain much pollen grains since they were already shed.
10. The temperature in the room has to be above 20 °C. A warmer temperature up to 30 °C causes no harm to the drying anthers. However, a temperature lower than 20 °C is not warm enough to enable anther dehiscence and shedding of the pollen grains.

11. It is critical that all remnants of the anthers will be removed from the pollen grains. Repeat the filtration step in case several anther pieces remained in the pollen sample.
12. Do not store the pollen grains under other temperatures. The temperature of −80 °C would diminish pollen grain germination rate notably. Moreover, avoid the stored samples from multiple freeze–thaw cycles and aliquot the pollen grains per several 1.5 ml microcentrifuge tubes, of which every tube will be dedicated for a single use only.
13. Avoid re-freezing of the pollen grains. Usually the whole amount of pollen grains inside one microtube is used.
14. The resuspended sample has to be really homogenous; ensure sample homogeneity by mixing the sample thoroughly before pipetting every aliquot.
15. It is important to use a thin injection needle to prevent any losses of the homogenized pollen grains.
16. The TCA/acetone pellet should be weighted before resuspension, usually the amount of approximately 40–50 mg should be sufficient for one round of phosphoprotein enrichment.
17. The centrifuge should not be cooled during this step in order to avoid any precipitation of salts in the incubation buffer.
18. We typically apply the 2D Quant kit (GE Healthcare) using the manufacturer's instructions for the estimation of protein concentration, but in principle any method that tolerates 8 M urea in the incubation buffer should be feasible.
19. It is of vital importance to make the whole volume of the aluminum hydroxide particles wet.
20. Other ways of keeping the aluminum hydroxide and the proteins in a homogenous mixture can be applied. In any case it is important not to have the samples in warmer temperatures than 8 °C.
21. The phosphoproteins should be bound to the aluminum hydroxide particles whereas non-phosphorylated species should be in the supernatant.
22. The supernatant should contain non-phosphorylated proteins. Nevertheless, it should be kept to enable its analysis by 1D SDS-PAGE in order to compare protein spectra from the supernatant and the phosphoprotein-enriched fraction.
23. This supernatant together with all following supernatants containing wash-out proteins should also be kept to enable their analysis by 1D SDS-PAGE.

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