CHAPTER SIX

# Three-in-one method for high throughput plant multi-omics

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## Abstract

Multi-omics has gained momentum over the past few years especially in plant single cell-type analysis as they aim to understand cellular molecular networks across different levels of genetic information flow. For multi-omics sample preparation, molecular extractions performed non-simultaneously create rooms for variation, inaccurate data, waste of limited samples, resources and labor. Here we optimized a protocol for 3-in-1 simultaneous extraction of RNA, metabolites, and proteins from the same single cell-type sample. We adapted a commercially available RNA kit with a few modifications to obtain high quality starting materials for sequencing and LC-MS/MS-based metabolomics and proteomics. RNAs are bound to the column, metabolites were extracted in a polar solvent and proteins are precipitated using acetone. This creates an all-in-one workflow using a standard RNA kit. Little training is required to carry out this protocol as it is simple and easy to use. It may be used with a wide range of plant species and different amounts of starting materials, including single cells.

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## 1. Introduction

*Arabidopsis thaliana* was the first plant species to be fully sequenced and it has been used as a reference species for plant biology research. To date, most of the multi-omics studies use different plant tissues like whole leaves, roots, or root hairs (Becker, Takeda, Borges, Dolan, & Feijó, 2014; Wang, Lan, & Shen, 2016), seeds (Picard, Povilus, Williams, & Gehring, 2020), silique, flowers (Mergner et al., 2020), seed coat (Zhang et al., 2022), or pollen tube (Costa, Silva, & Coimbra, 2020). There have been papers published that describe 3-in-1 (e.g., DNA, RNA, and proteins (Valledor et al., 2014), or metabolites, lipids and proteins (Kang, David, Li, Cang, & Chen, 2021)), 4-in-1 (Vorreiter et al., 2016), and 5-in-1 method (metabolites, lipids, proteins, starch, and cell wall polymers (Salem, Jüppner, Bajdzienko, & Giavalisco, 2016)). The disadvantages of these methods are that they are time-consuming and require large amounts of starting material to get high-quality samples. The tissue types used are available in abundance and are well-studied compared to single cell-type samples like guard cells (Zhao, Zhang, Stanley, & Assmann, 2008; Zhu & Assmann, 2017; Zhu et al., 2014), mesophyll cells (Jin et al., 2013), trichomes (Frerigmann, Böttcher, Baatout, & Gigolashvili, 2012; Yang & Ye, 2013), vasculature and epidermal cells (Bruex et al., 2012). Studying single cells can provide deeper insight on molecular processes in different types of cells. Importantly, it solves the problem of averaging molecular changes from different types of cells when using whole tissue samples. Furthermore, for complete understanding of the functions of plant cells, one type of omics is inadequate (Larkin, 2007). For example, protein levels may not be closely correlated with their corresponding RNA levels (Shaw, Tian, & Xu, 2021; Walley et al., 2016). Furthermore, the function of proteins and/or metabolites may depend on individual cellular and subcellular localization. Therefore, multi-omics approaches enable a deeper understanding of plant biology (Libault, Pingault, Zogli, & Schiefelbein, 2017). The method described here was developed as a proof-of-concept study where the material is not limited to single cells but can be applied to any tissue or organ. The optimization comprises the addition of acetone to obtain both metabolites and proteins from the commercially available Qiagen RNA kit. The results from this method can be used effectively for systems integration of the multi-level molecular data. To our best knowledge, this is the first 3-in-1

extraction protocol developed for plant single cell-types like guard cells and mesophyll cell protoplasts for simultaneous analysis of the RNA, proteins, and metabolites.

## 2. Methods

Plant samples are difficult to work with when extracting sequencing grade high quality RNA because they contain polysaccharides and phenolic compounds that are known to reduce the quality of RNA (Leh, Yong, Nulit, & Abdullah, 2019; Umesh, Ansari, & Sridevi, 2017). It is imperative to remove these contaminants through downstream treatments. In recent years, a few papers have been published for high throughput plant single cell-type and single-cell extractions (Jin et al., 2013; Misra, Tong, & Chen, 2015). The multi-omics extraction for plant single cells is important as it provides different types of molecules from the same sample to avoid any bias or averaging effects and can help to reduce the variations between replicate samples.

### 2.1 Plant growth conditions

*A. thaliana* plants grown from seeds in short day conditions (22 °C in light for 8 h and 20 °C in dark for 16 h) with a light intensity of 160 μmol photons $m^{-2}$ $s^{-1}$ in a growth chamber using potting mixture (Sungro Horticulture Propagation Mix). Plants were fertilized once a week by fertilizer: 20-10-20 Peat-Lite according to manufacturer instructions, and 5-week-old plants were used for the preparation of single cells.

### 2.2 Single cell-type sample preparation

#### *2.2.1 Preparation of guard cell enriched sample on tape*

**Equipment and consumables**

pH meter
Rocking shaker
Forceps
Petri plates
Scotch tape
Degasser
Falcon tubes
Liquid nitrogen

**Reagents and solvents**

1. Opening Buffer: 1 L

| 50 mM KCl | 3.7 g |
|---|---|
| 10 mM MES | 1.95 g |

Adjust pH to 6.2 with KOH. Degas for 5 min. Store at RT (room temperature). Whenever possible make fresh.

2. Guard cell enzyme in 50 mL opening buffer

| 0.7% Calbiochem cellulysin R10 | 0.35 g |
|---|---|
| 0.025% Macerozyme R10 | 0.0125 g |
| 0.25% BSA | 0.125 g |
| Y23 Pectolyase | 0.1 g |

Dissolve all the above components together in a 50 mL falcon tube and keep it on the rocker at 10 rocking motions per minute for 12–15 min**. DO NOT VOTEX** otherwise it will create lot of bubbles. No filtration is required, although it is preferred.

**Procedure for guard cell isolation by a tape-peel method**

1. Cut the fully expanded leaves from 5-week-old *Arabidopsis* plants and adhere the lower epidermis to the scotch tape of each leaf.
2. Take another scotch tape and put it on the top. The leaves are now sandwiched between two layers of scotch tape.
3. With the help of a tweezer, pull apart the two scotch tapes like in Fig. 1. NOTE: If there are some green mesophyll cells still stuck on the guard cell side, double tape near the green part to remove it to avoid cell to cell contamination.
4. Place the guard cell peels in opening buffer (with the guard cells side touching the opening buffer) under light for 2 h. Use a big Petri-plate and make sure that the peels are not overlapping or sticking with each other.
5. Place the mesophyll tape-peels in the mesophyll cell digestion enzyme (with the mesophyll cells side touching the enzyme solution) under dark

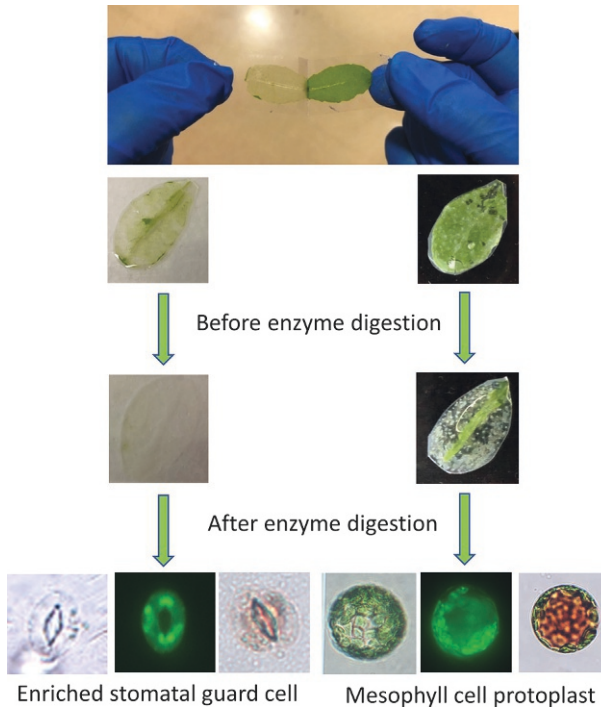


**Fig. 1** Scotch tape-peel preparation of guard cells (left) and mesophyll cell protoplasts (right) before and after enzyme treatment. The cells were tested for viability by fluorescein diacetate (FDA) (green in color) and neutral red viability staining. From the lighter side, guard cells were prepared, and from the darker side mesophyll cell protoplasts were isolated. A part of this procedure with video has been published earlier (Lawrence, Pang, Kong, & Chen, 2018).

for 3 h on a slow speed rocker. Follow the steps in the next section for mesophyll cell protoplast preparation.

6. After 2 h, put the guard cell peels in the enzyme solution (in small Petri-dish) for 7 min on shaker (25 mL enzyme for 50 peels). NOTE: **Do not over-digest.** Keep checking the tape peel under the microscope to prevent over digestion (loss of cells) and optimize the time according to your batch of enzymes.
7. Wash the peels with opening buffer (in big Petri-dish) for 5 min on a shaker (twice).
8. Transfer the peels back under light for 1 h in the opening buffer (big Petri dish) to allow recovery after the enzyme shock.
9. After 1 h, freeze the tape peels in liquid nitrogen. Store in −80 °C for further use.

### 2.2.2 Mesophyll cell protoplast preparation

This protocol for is adapted from Zhu, Dai, McClung, Yan, and Chen (2009) with minor modifications.

**Equipment and consumables**

Petri plates
15 mL centrifuge tube
Nylon mesh of 35–75 μm pore size
1.5 mL Eppendorf tube
Round bottom 50 mL tube
Aspirator
Cold centrifuge
pH meter

**Reagents and solvents**

**Solution 1#**

10 mM MES-KOH (pH 5.5)
0.6 M sucrose

**Basic solution: (Solution 2#)**

0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 μM $KH_2PO_4$ in 10 mM MES-KOH (pH 5.5)

| | 1 L | 500 mL | 100 mL |
|---|---|---|---|
| MES | 1.952 g | 0.976 g | 0.1952 g |
| 0.6 M Sorbitol | 109.32 g | 54.66 g | 10.932 g |

Adjust the pH to 5.5 with KOH and make up the volume.

**Enzyme solution**: (**Solution 3**)

| | |
|---|---|
| 0.5% (w/v) macerozyme R-10 (Yakult Honsha) | 0.25 g |
| 1% (w/v) Onozuka RS cellulose (Yakult Honsha) | 0.5 g |
| 0.01% (w/v) pectolyase Y-23(Seishin pharmaceutical) | 0.005 g |
| 0.2% (w/v) BSA (sigma) | 0.1 g |
| 0.1% (w/v) PVP-40 (sigma) | 0.05 g |

Put to 50 mL basic solution the day before making the protoplasts. No need to filter.

**Procedure**

1. Place the mesophyll peels in the enzyme solution (15 mL for 50 peels) and digest the leaves at RT degree for 3 h with gentle agitation. Cover the plate with aluminum foil.
2. Pull out the suspension and after passing it through a nylon mesh of 35–75 μm pore size transfer to 15 mL centrifuge tube and centrifuge at 150 × *g* for 10 min.
3. Pellet obtained was resuspended in 5 mL of solution 1#, first put 1 mL and carefully resuspend the pellet and then carefully add the remaining 4 mL of solution 1#.
4. Carefully add another 5 mL of solution 2#, which are layered on the top of solution 1#.
5. Centrifuge for 7 min at 150 × *g*, and the interface containing mesophyll protoplasts is transferred to a new tube (round bottom), and then resuspended in 5 mL of solution 2 (Basic solution).
6. The suspension was then centrifuged at 150 × *g* for 3 min and the final pellet was gently resuspended in 10 mL of solution 2 (put the first mL very carefully).
7. Keep the solution in the dark for 1 h and then centrifuge for 5 min at 150 × *g* to pellet the mesophyll cell protoplasts.
8. Remove the supernatant and flash freeze the pellet. Store in −80 °C until further use.

## 2.3 3-in-1 method for simultaneous RNA, metabolite and protein extraction

### *2.3.1 RNA extraction*

**Equipment and consumables.**

Qiagen RNeasy Plant mini kit (Cat. No. 74904)
On-column DNase digestion using the RNase-Free DNase Set
Centrifuge (Eppendorf 5417R)
RNase free Filter tips, sterile
Pestle mortar
Liquid nitrogen
Kim wipes
Disposable gloves
Nanodrop

**Reagents and solvents.**

Actinomycin
Cordycepin
β-Mercaptoethanol (β-ME)
RNA Zap
Ethanol
Acetone
PMSF (Phenylmethylsulfonyl fluoride)

**Procedure**

Detailed protocol is available on Qiagen website for RNA extraction (https://www.qiagen.com/us/resources/resourcedetail?id=14e7cf6e-521a-4cf7-8cbc-bf9f6fa33e24&lang=en). Fig. 2 shows details of step-by-step the optimization and modifications to the kit that we use to perform the 3-in-1 extraction.

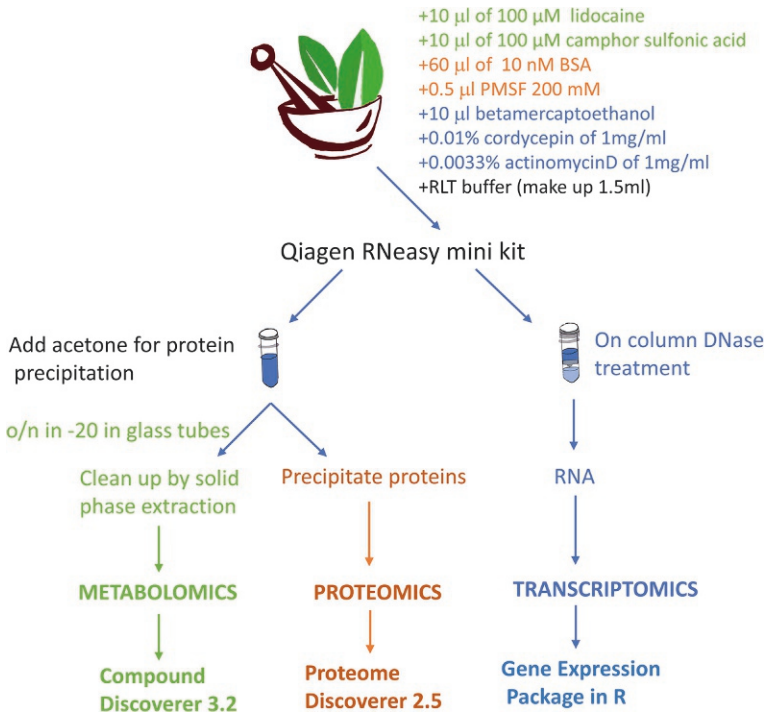


**Fig. 2** Detailed flowchart of 3-in-1 extraction of RNA, metabolites, and proteins with minor modifications to the Qiagen RNA kit using Arabidopsis guard cells and mesophyll cell protoplasts. Instead of discarding the flow through from the RNA kit, this method uses it for protein and metabolite extractions. BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; SPE, solid phase extraction.

1. To 1 mL of RLT buffer from the kit add 10 µL of 100 µM lidocaine (positive mode internal standard), 10 µL of 100 µM camphorsulfonic acid (negative mode internal standard), 60 µL of 10 nM BSA (protein internal standard), 0.5 µL 200 mM phenylmethylsulfonyl fluoride (PMSF, a serine protease inhibitor), 10 µL β-ME, 0.01% cordycepin of 1 mg/mL stock (in water), 0.0033% (v/v) actinomycin D of 1 mg/mL stock (in DMSO). Make up the volume to 1.5 mL using RLT buffer for 1 sample.
2. Grind stomata tape-peel samples in the pestle and mortar with liquid nitrogen and then add the above 1.5 mL solution. To the mesophyll cell protoplast add directly the above 1.5 mL solution and vortex.
3. Put the ground samples in RNase-free, liquid-nitrogen–cooled, 2 mL microcentrifuge tube. Vortex vigorously.
4. Transfer the sample to a QIAshredder spin column (lilac) placed in a 2 mL collection tube. Centrifuge at 17,900 × *g* for 2 min.
5. The flow-through from the collection tube is transferred to a new 2 mL microcentrifuge tube. (NOTE: Do not transfer the cell-debris pellet).
6. Add 0.5 volume of 100% ethanol and mix completely. Do not centrifuge.
7. Transfer the supernatant (with precipitate) to RNeasy Mini spin column (pink) in a 2 mL collection. Centrifuge for 15 s 10,600 × *g*.
8. The column has the RNA and from the flow-through extract the metabolites and proteins.
9. **Transfer the flow-through to a glass tube and add four volume of ice cold 100% acetone Keep in -20 °C overnight for protein precipitation (follow next two sections for metabolite and protein extractions).**
10. To the column (containing RNA) add 350 µL RW1 buffer to prepare for on column DNase treatment. Centrifuge for 15 s at 10,600 × *g*. Discard the flow-through.
11. For 1 sample add 80 µL of DNase I incubation mix (10 µL DNase I stock solution +70 µL Buffer RDD) on the centre of the column. **NOTE: Invert mix, DO NOT VOTEX the incubation mix.** Keep this for 30 min at room temperature.
12. Add 350 µL Buffer RW1 and centrifuge for 15 s at 10,600 × *g*. Discard the flow-through.
13. Add 500 µL Buffer RPE to the RNeasy spin column. Centrifuge for 15 s at 10,600 × *g*. Discard the flow-through.

14. Add 500 µL Buffer RPE centrifuge for 2 min at 10,600 × *g*. Discard the flow-through.
15. Do a dry spin at 10,600 × *g* for 1 min.
16. Place the column in a new 1.5 mL tube. Add 35 µL of RNase-free water directly to the center of column membrane. Keep at RT for 10 min.
17. Centrifuge at 10,600 × *g* for 1 min to elute the RNA.
18. Preform nanodrop to check RNA concentration and integrity. The A260/A280 ratio of 1.9–2.1
19. Run a 1% agarose gel to check the two RNA bands as shown in Fig. 3A

### *2.3.2 Metabolite extraction*

**Equipment and consumables**

Nitrogen blower

SPE clean up using vacuum: Oasis HLB 3CC (60 mg) extraction cartridges (part no. WAT094226).

Supelco Visiprep ™ 24

Pipette

Savant SPD1010 SpeedVac Concentrator, Thermo Fisher Scientific

Laminar

Centrifuge

**Reagents and solvents**

Bradford reagent (Sigma Aldrich B6916-500ML)

Promega sequencing grade modified trypsin (V5111)

Methanol

0.1% FA (formic acid) (v/v) water

Acetone.

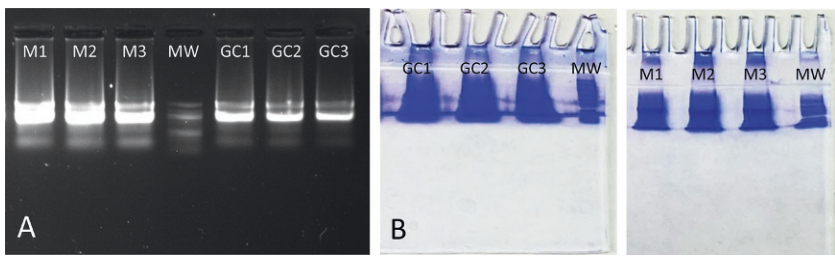


**Fig. 3** Electrophoresis of RNA and protein samples. (A) RNA (1 µg) loaded on 1% agarose gel, and (B) protein (20 µg) loaded on 10% SDS-PAGE gel. M, mesophyll cell protoplasts; GC, guard cells; MW, molecular weight marker.

**Procedure**

1. After addition of four volumes of ice-cold acetone to the flow-through and incubate overnight at −20 °C, centrifuge at 15,300 × *g* for 30 min.
2. Collect the supernatant in a new glass tube for metabolite extraction and the pellet (for protein extraction).
3. Use nitrogen blower to evaporate all the acetone to get the metabolite.
4. Add 500 µL of 0.1% FA water to the glass tube.
5. Place the oasis HLB cartridge on the Supelco Visiprep™ 24 vacuum manifold. Do not turn on the vacuum.
6. Condition the HLB cartridge with 2 mL methanol. Let it flow by gravity.
7. Equilibrate the HLB cartridge with 2 mL 0.1% FA water. Set the vacuum to 5″ Hg.
8. Switch off the vacuum before loading the 500 µL sample on the HLB cartridge. Set the vacuum at lowest pressure and let the sample come down drop-by-drop.
9. Slowly increase the pressure and let the entire sample come down in the collection tube.
10. Stop the vacuum and apply 2 mL 0.1% FA water. Apply vacuum again at 5″ Hg.
11. Continue applying vacuum to remove any residual wash solvents. Switch off the vacuum.
12. Replace the collection tube with 2 mL Eppendorf tube.
13. Apply 2 mL of 100% methanol to the HLB cartridge and let it flow through by gravity.
14. Switch on the vacuum pump and collect the elution solvent.
15. Speed vac the eluted sample and store in −20 °C.
16. Resuspend in 25 µL of 0.1% FA water.
17. Sonicate 5 min, vortex 30 min at 4 °C and then centrifuge at 15,300 × *g* for 30 min.
18. Send 13 µL for MS analysis and store the remaining 12 µL at −20 °C as backup sample.

### *2.3.3 Protein extraction*

**Equipment and consumables.**

Millipore Zip Tip (C18, REF-ZTC18S960)

**Reagents and solvents.**

100% acetonitrile (ACN)

50 and 25 mM Ammonium bicarbonate (ABC)

| Water | 10 mL | Final Con. |
|---|---|---|
| Ammonium bicarbonate | 79 mg | 100 mM |

10 mM DTT in 100 mM ABC (10 µL 1 M DTT + 990 µL 100 mM ABC)
55 mM fresh chloroacetamide in 100 mM ABC

| 100 mM ABC | 1 mL | Final Con. |
|---|---|---|
| chloroacetamide | 5.14 mg | 55 mM |

25 mM ABC
1% formic acid (FA) (v/v) water

**Procedure for Acetone Precipitation of Protein**

19. After adding four volumes of ice-cold acetone to the flow-through and incubate overnight at −20 °C, centrifuge at 15,300 × *g* for 30 min.
20. Collect the supernatant in a new glass tube (for metabolite extraction) wash the pellet with 100 µL ice-cold ethanol.
21. Air-dry the pellet till the smell of ethanol goes away. Over drying will make the pellet difficult to dissolve in urea buffer.
22. After performing Bradford assay for determining protein concentration run SDS-PAGE gel 20 µg protein for each sample was loaded in each well and ran till 1 cm. Stain and de-stain the gel overnight (Fig. 3B).
23. Wash the gel with water 2 × and take a photo for your record.
24. Cut the gel bands into small pieces on a clean glass plate using a scalpel and transfer to a 1.5 mL tube
25. Store these cut gel bands in −80 °C until use.

**In-gel trypsin digestion**

1. Add Solution 1 (25 mL ACN + 25 mL 50 mM ABC) to the gel pieces
2. Shake for 2–3 h, till the blue color is completely gone. Remove solution1
3. Add 200 µL of 100% ACN
4. Keep on the shaker for 10 min. Discard the supernatant
5. Add 200 µL of 100% ACN

6. Keep on the shaker for 5 min, remove supernatant. Speed vac 1 min (optional)
7. Reduce by adding100 µL of 10 mM dithiothreitol (DTT) and keep for 45 min at RT
8. Alkylate with 100 µL of 55 mM chloroacetamide in darkness at room temperature for 1 h
9. Discard the supernatant. Add 500 µL of 100%ACN. After 5 min on the shaker, discard the supernatant
10. Speed vac for 5 min
11. Add trypsin (Sequencing grade, Promega, Madison) to a trypsin: protein ratio of 1:50 (w/w) and incubate 37 °C overnight
12. Remove supernatant in new 1.5 mL tube
13. Add 100 µL 1% FA and 2% ACN to the gel pieces
14. Remove the supernatant and collect in same 1.5 mL supernatant tube
15. To the gel pieces add 50 µL of 80 ACN:20 water with 0.1%FA
16. Vortex for 30 min
17. Transfer the buffer from gel tube and add to the supernatant tube
18. Add 50 µL of 60% (v/v) ACN
19. Mix all supernatant and speed vac. Sample can be stored at −20 °C

**Zip tip clean up (optional)**

(1) After digestion the dried peptides were dissolved in 20 µL 0.1% FA water
(2) Sonicate 5 min, then vortex 30 min
(3) The zip tip pipette tip was equilibrated with 10 µL of 100% ACN (5 ×) and 10 µL of 0.1% FA water (5 ×).
(4) The peptides mixture (samples) was loaded into the tip by taking 25 µL aliquots, pipetted up and down slowly 20 times
(5) Elute the sample with 5 µL elution buffer (80% can + 0.1% FA) with pipetting up and down 10 times. Repeat it one more time
(6) Repeat steps 3–5
(7) Retain the flow-through until your mass spec proteomic data is confirmed
(8) The enriched samples were dried down in speed vac and kept at −20 °C until use
(9) When mass spec is ready dissolved the dried peptides in 25 µL 0.1% FA solution. Sonicate 5 min, vortex 30 min at 4 °C, and then centrifuge at 15,300 × *g* for 30 min
(10) Send 13 µL for MS analysis and store 12 µL in −80 °C as backup

## 2.4 Sample processing and data analysis

RNA sample concentration and integrity are measured using a nanodrop instrument. The A260/A280 ratio of pure RNA samples should be between 1.9 and 2.1. To visually check if RNA is intact, analyze the sample using a 1% agarose gel. The presence of two RNA bands (28S and 18S) is a clear indicator of intact RNA. For untargeted mass spectrometry-based analysis of metabolites, the dried samples were resuspended in 25 μL of 0.1% FA water. Analyzed on an Thermo Fisher Scientific. Separation was obtained on an Accucore™ C18 HPLC Columns (Catalog number: 17126-102130) with particle size of 2.6 μm, diameter of 2.1 mm, length 100 mm (Thermo Fisher Scientific). Mobile phase A was water with 0.1% FA and Mobile phase B was acetonitrile with 0.1% FA with a flow rate of 350 μL/min. A 40 min gradient was used for separation in both positive and negative mode. Q-Exactive mass spectrometer and liquid chromatography Vanquish-Flex (Thermo Fisher Scientific, San Jose, CA, USA). The LC gradient is set to 22 min: 30% of B, 30 min: 98% of B, 31 min: 98% of B, 32 min: 98% of B, 8 min stop run. Full MS1 used the Orbitrap mass analyzer with a resolution of 70,000, scan range ($m/z$) of 200–1000 m/z and normalized collision energy 0f 30. Data analysis was performed using Compound Discoverer 3.1 (Thermo Fisher Scientific, San Jose, USA). The results showed 1434 and 1200 metabolites were identified in negative and positive ion modes, respectively. The data was cleaned by filtering out the metabolites without MS/MS and where the name of metabolite does not appear in spectral libraries. For removing the duplicates, we made sure that there are good-shaped peaks and kept the ones with high FISH score. Manual inspection was done to remove non-plant metabolites and contaminants. Venn diagram in Fig. 4A indicates a total of 118 metabolites were obtained from just guard cells and 190 from mesophyll cell protoplasts, with 50 metabolites being identified in both cell types.

The generated peptides were solubilized in 25 μL 0.1% FA in water and analyzed on Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Liquid chromatography was carried out using a 180 min gradient with 0.1% FA in 100% water (solvent A) and 100% ACN (solvent B). Peptides were separated on Acclaim PepMapTM 100 pre-column (75 μm × 2 cm, nanoViper C18, 3 μm, 100 A) combined with an Acclaim PepMapTM RSLC (75 μm × 25 cm, nanoViper C18, 2 μm, 100 A). The following gradient of solvent B in solvent A: starting at 2%, for 140 min, 35% at 160 min, 95% 165 min, 95% at 170 min, 2% at 176 min, and 2% at 180 min with a flow rate of 350 nL/min. Full-scan

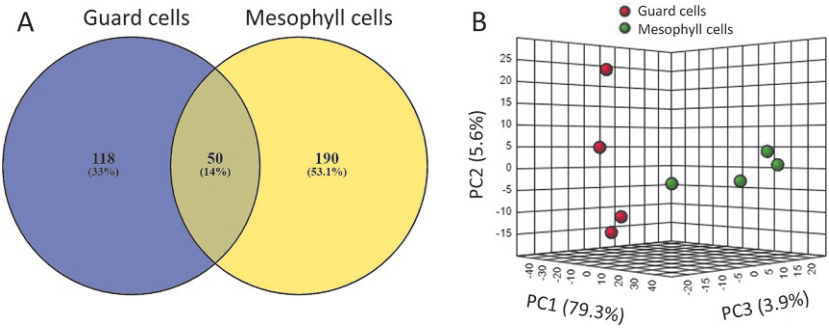


**Fig. 4** Venn diagram (for metabolites, A) and PCA plot (for proteins, B) of guard cells and mesophyll cells showing cell type differences.

MS spectra were acquired in a positive ion mode in Orbitrap MS with scan range of 400–2000 m/z and a resolution of 70,000. The normalized collision energy was 35. Data analysis was performed on Proteome Discoverer 2.5 (Thermo Fisher Scientific, San Jose, USA) using the following parameters for the identification and quantification of proteins: TAIR10 database with mass tolerance of 10 ppm and fragment mass tolerance of 0.02 Da. LC-MS/MS Proteomics data processing was performed on Q-Exactive Plus using label-free quantitative proteomics. After applying the filter of FDR (false discovery rate) of less than 1%, unique peptides numbered greater than or equal to 2, In Proteome Discoverer2.5, the number of proteins identified with high confidence were 2599 out of 3943 total proteins. The peptide groups identified were 22,514. Median data normalization was done using R-statistics in METABOANALYST. The cell type differences were obvious in the 3D PCA plot (Fig. 4B). Guard cells seem to have completely different profile than mesophyll cells. A twofold change cut-off along with a *P*-value of less than 0.05 were used to determine differences in single-cell type, where a total of 789 proteins were increased and 994 were found to be decreased in guard cell by mesophyll cell ratio.

## 2.5 Safety considerations and standards

Please follow the safety considerations, standards, and regulations mentioned on all chemical label. Use nitrile gloves, masks, and safety goggles. While working with proteins special care should be taken to avoid keratin contamination especially from skin and hair. Follow your lab guideline for proper hazardous and bio-hazardous waste separation and disposition. When working with RNA make sure you autoclave and treat pestle and motor with

RNA zap. Filter tips should be used when you are dealing with RNA. Make sure to use clean glass wear for metabolomics experiment. Do not use plastic when dealing with acetone. Always use glass. Dispose the glass waste in the proper glass bin.

## 2.6 Troubleshooting & Optimization

| Problem | Solution |
|---|---|
| Reproducibility issues | Between the biological replicates keep the weight of sample same. If using peels, use the exact same number of peels |
| Some molecules are liable to degradation and changes and therefore need to preserve integrity of the sample | Make sure the storage conditions are maintained properly. Light and cold sensitive chemicals should be appropriately handled and stored |
| Over-digesting of protein samples | Make sure you note the timing of trypsin addition. Do not exceed more than 17 h for overnight trypsin digestion. Use the same amount of trypsin for all samples |
| Glass tubes breaking in centrifuge | Make sure the glass tubes are centrifuge compatible and check the manufacturer label up to what speed they can tolerate |

## 3. Summary

The method outlined above is simple, cost-effective, and reproducible for users of all levels. Using a commercially available kit with some modifications provides efficient separation of 3 cellular components from 1 sample. A major merit of using this 3-in-1 multi-omics method lies in that it can use limited amount of starting material to provide maximum information across omics platforms and is applicable to various plant materials, such as organs, tissues, and cells.

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