A cell-sorting-based nano-scale pipeline for cell-type specific proteomics in plants

Hongliang Wang[1], Rongxia Li[1], Weiwei Ren[2], Jingkai Rong[1,3], Tian Sang[1,3], Chanhong Kim[1], Chun-Peng Song[4], Shaojun Dai[2], Chuan-Chih Hsu[5], Pengcheng Wang[1]

[1]Shanghai Center for Plant Stress Biology, CAS Center for Excellence in Molecular Plant Sciences, Chinese Academy of Sciences, Shanghai 200032, China

[2]Development Center of Plant Germplasm Resources, College of Life Sciences, Shanghai Normal University, Shanghai 200234, China

[3]University of Chinese Academy of Sciences, Beijing 100049, China

[4]Key laboratory of Plant Stress Biology, School of Life Sciences, Henan University, Kaifeng 475004, China

[5]Institute of Plant and Microbial Biology, Academia Sinica, Nangang, Taipei 11529

Correspondence and requests for materials should be addressed to: Pengcheng Wang (pcwang@cemps.ac.cn)

Abstract

Multicellular organisms such as plants contain different types of cells with specialized functions. Analyzing the characteristics of each type of cells will reveal specific cell functions and enhance understanding of how an organism organizes and works. Due to the lack of a method to enrich the particular type of cells and the limited sensitivity and capability of mass spectrometry-based technologies, comprehensive analyses of the proteome in a single plant cell are still impossible. Here we report a high-sensitive and efficient cell-type-specific proteomic pipeline, combining simplified flow cytometry-based fluorescent cell-sorting for fluorescent protoplasts and an optimized nano-scale proteomics method, which allows us to identify more than 1,000 unique proteins from 500 guard cell protoplasts. By this method, we performed an in-depth, quantitative comparison between the proteomics of guard cells and mesophyll cells, and revealed that the enrichment of signal transduction-related proteins enables guard cells to respond to various environmental stimuli quickly. This method was also applied to profile the proteome of different cells in root tips of Arabidopsis and rice seedlings. Thus, this pipeline is applicable to various types of cells in plant or non-plant systems to acquire systemic knowledge of how cells work specifically and in highly organized multiple cell organisms.

Introduction

Multicellular organisms such as plants contain different types of cells with specialized structures and functions. Different cells work coordinately to regulate complex growth/development processes and responses to the ever-changing environment. Dissecting

the specific structural and functional information in each cell type is essential to understand how a particular cell type works and how different cells integrate into a complex organism. Recently, the breakthrough of nucleic-acid-amplification-based DNA and RNA-sequencing technologies make single-cell transcriptomics a reality in plants[1-3]. Dozens of single-cell transcriptomic studies have been achieved in different species and various tissues, and researchers have chances to understand the specialized function and organization in different cells. However, proteins are major effectors in cells, whose abundance and function are further controlled by translational and post-translational regulations. Thus, determining the quantity and post-translational modifications (PTMs) at the proteome level is vital for understanding the organization and function of a particular type of cell. Due to lacking protein amplification techniques, and limited sensitivity and capability of mass spectrometry-based technologies, comprehensive analyses of the proteome in a single plant cell are still impossible[4]. The enrichment of a particular type of cells is an alternative way to study the cell-specific proteomes[5]. However, such single-cell (type) proteomics only performed in limited cell types that could easily be isolated, include pollen[6-9], guard cells[10-12], and mesophyll cells. Using the guard cell protoplasts isolated by enzyme digestion and centrifuge, several groups performed pioneer single-cell proteomic studies in Arabidopsis, *Brassica napus*, maize (*Zea mays*), and discover unique proteins, signal transduction pathways, or metabolism that determines the special function and development of guard cell [10-12]. Using a flow-activated cell-sorting (FACS) and GeLC-MS/MS (in-gel tryptic digestion followed by liquid chromatography-tandem mass spectrometry), proteomic analyses identified nearly 2,000 proteins from six different cell types in the Arabidopsis root tips [13]. A

similar study compared the proteomes in epidermal and inner cell lines in Arabidopsis root [14]. However, current methods were only appliable to limited cell types and require millions of cells, and the efficiency of cell acquirement and the sensitivity of proteomics needs to be improved.

Herein, we developed a flow cytometry-based cell-sorting method to enrich the fluorescent protoplasts. Combining with the optimized nano-scale proteomics method, we set up a cell-type-specific pipeline that applicable to the proteomic study of guard cell (GC), mesophyll cell (MC), or root epidermal cell in both Arabidopsis and rice. The quantitative comparison of guard cell protoplasts (GCP) and mesophyll cell protoplasts (MCP) revealed the enrichment of signaling components in GCPs that may enable GC to respond to various environmental stimuli quickly. This pipeline is also applicable to other types of cells in plant or non-plant systems to acquire systemic knowledge of how cells work specifically and in highly organized multiple cell organisms.

Results

We developed a flow cytometry-based cell-sorting method to enrich the fluorescent protoplasts (Fig. 1a). GC is a specialized epidermal cell that plays an important role in gaseous exchange in and out of plant leaves by regulating the opening and closing of the stomas. We used the *pGC1::GFP* transgenic plants[15], which carrying guard cell-specific expressed GFP, as a model system to test our pipeline. The *pGC1::GFP* leaves were digested into protoplasts, and after removal of tissue fragment by filtration, the protoplasts were then subjected to FACS (Fig. 1a and 1b). This simplified cell-sorting procedure works well to

isolate hundreds to millions of GCPs. We typically isolated up to 10,000 fluorescent GCPs from 0.2 g leave tissue (about 4-6 mature leaves from 3-week-old seedlings). We also optimized a nano-scale protein extraction and digestion procedure (Fig. 1a). The enriched fluorescent GCPs were lysed in SLS-SDC (SS) buffer and directly subjected into trypsin digested in a single microcentrifuge tube, to avoid protein loss in precipitation and transferring (Fig. 1a). After one-step desalting on C18 Stage-Tip, the peptides were subjected to LC-MS/MS analysis (Fig. 1a). Compared to the Gdn-Cl method optimized for medium-scale plant tissue extract[16, 17], the simplified SS method allows us to identify more peptides/proteins, especially for hundreds to thousands of protoplasts. For example, we identified 2,827 peptides, representing 1,012 proteins, from about 500 GCPs by SS method, which is nearly two-fold more than that from the Gdn-Cl method (Fig. 1d). The Gdn-Cl method acquired more peptides/proteins from 5,000 and 10,000 protoplasts, which is probably due to the precipitation and resuspension might remove metabolites and improved the subsequential enzyme digestion and MS detection.

Encouraged by this result, we further performed label-free quantitative proteomics from 1 μg total protein extract from GCPs (about 40,000 cells) and MCPs (about 20,000 cells) to acquire in-depth profiles of GCPs and MCPs. As a result, a total of 27,511 peptides, representing 4,622 proteins, are identified in GCPs, and 25,235 peptides, representing 4,237 proteins, are identified in MCPs, respectively (Fig. 2a). Among these proteins, 526 proteins are identified at least once in the three biological replicates of GCPs, but not in any sample of the MCPs, and 141 proteins are only detected in MCPs (Fig. 2a). We also perform a quantitative comparison of the GCP and MCP proteomes. As shown in Fig. 2b and 2c, 2,483

proteins show enrichment in GCPs (FC > 2, $p < 0.05$). Some proteins known to involve in

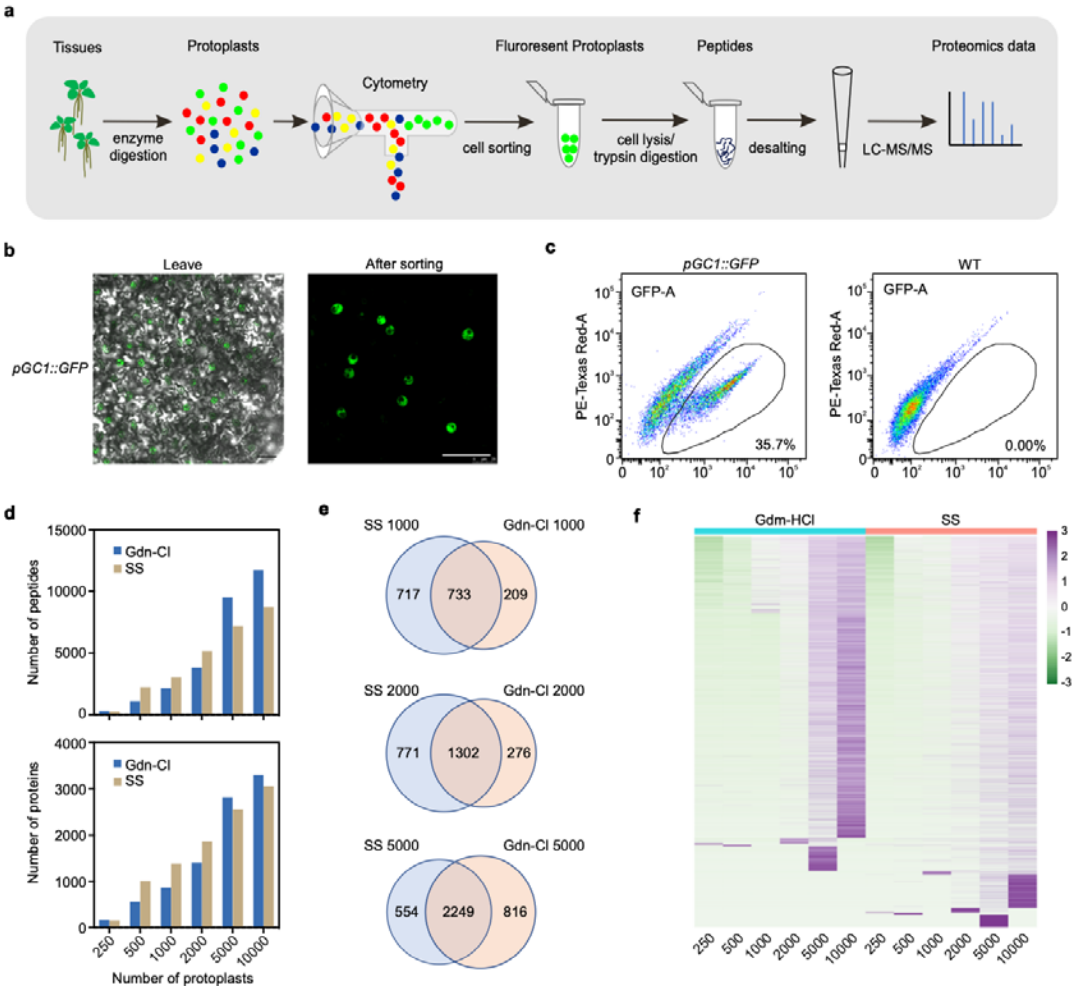

Figure 1. The cell-sorting-based nano-scale pipeline for isolate and proteomic analysis of guard cell protoplasts. a. Workflow of the cell-sorting-based nano-scale pipeline for single-cell (type) proteomics. The fluorescent protoplasts were sorted by cytometry after enzymatic digestion and filtration. Protein extraction and trypsin digestion were performed in a single microcentrifuge tube, and after desalting through the C18 Stage-Tip, the peptides were subjected to LC-MS/MS. **b.** The fluorescent image showed the GFP fluorescence of *pGC1::GFP* transgenic leaves (left, Bar=50 μm) and sorted GCPs (right, Bar=50 μm). **c.** Representative flow cytometry scatter plots are shown for *pGC1::GFP* (left) and wild type control (WT, right). **d.** The number of peptides (upper panel) and proteins (bottom panel) identified from the indicated number of GCPs. **e.**Venn diagram showing the overlap between the protein identified from the indicated number of fluorescent protoplasts used the Gdn-Cl and SS methods. **f.** Heatmap showed the number of peptides in each protein detected by SS or Gdn-Cl methods from the indicated number of GCPs. SS, SDC-SLS protocol; Gdn-Cl, Gdn-Cl protocol.

stomatal development/function are present in the list of GCP enriched proteins. FAMA and

Inducer of CBF Expression 1 (ICE1)/ SCREAM (SCRM) govern guard cell mother division and promoters GC differentiation[18]. FAMA, ICE1/SCRM, and their interacting protein, the Third Largest Subunit of Nuclear DNA-dependent Pol II (NRPB3), are present in the list of GCP enriched proteins ($p < 0.05$)[19]. After forming GC, STOMATAL CARPENTER 1 (SCAP1) is required to develop functional stomata[20]. MYB DOMAIN PROTEIN 60 (MYB60) involved in the stomatal movement is a known target of SCAP1[20]. Both SCAP1 and MYB60 only present in GCPs, not in MCPs, in our study. Besides these proteins for stomatal development, many key components of the signaling pathway in response to blue light (BL), high CO_2, and Abscisic acid (ABA), are enriched in GCPs (Fig. 2F). For example, three ABA receptors, PYRABACTIN RESISTANCE 1(PYR1)/ REGULATORY COMPONENT OF ABA RECEPTOR 11(RCAR11)[21, 22], PYR-like 2 (PYL2) and PYL4, one PP2C phosphatase ABA INSENSITIVE 2 (ABI2), and OPEN STAMATA 1(OST1), are present in the list of GCP enriched proteins. BETA CARBONIC ANHYDRASE 4 (BCA4)[23], HIGH LEAF TEMPERATURE 1 (HT1)[24], and CONVERGENCE OF BLUE LIGHT AND CO_2 1 (CBC1)/CBC2[25], mediating CO_2-induced stomatal movement, and blue light receptor CRYPTOCHROME 2 (CRY2) and PHOT2 [26], the PHOT2 substrate BLUE LIGHT SIGNALING1 (BLUS1)[27] and BLUE LIGHT-DEPENDENT H^+-ATPASE PHOSPHORYLATION (BHP)[28], also present in the list of GCP enriched proteins. A recent study suggests that glucose, but may not the malate, is the major starch-derived metabolite in guard cells for rapid stomatal opening[29]. The b-AMYLASE1 (BAM1)[29], a glucan hydrolase, and ATP-BINDING CASSETTE B14 (ABCB14)[30], the malate transporter, are enriched in GCPs. Consistently, the enriched GO terms in the GCP mainly related to transport, responses

to ABA, cold, osmotic stress. Dozens of known ABA signaling components showed a GC-enriched pattern, and only very few components, like ABA1 and PLC, have higher abundance in MCPs than that in GCPs.

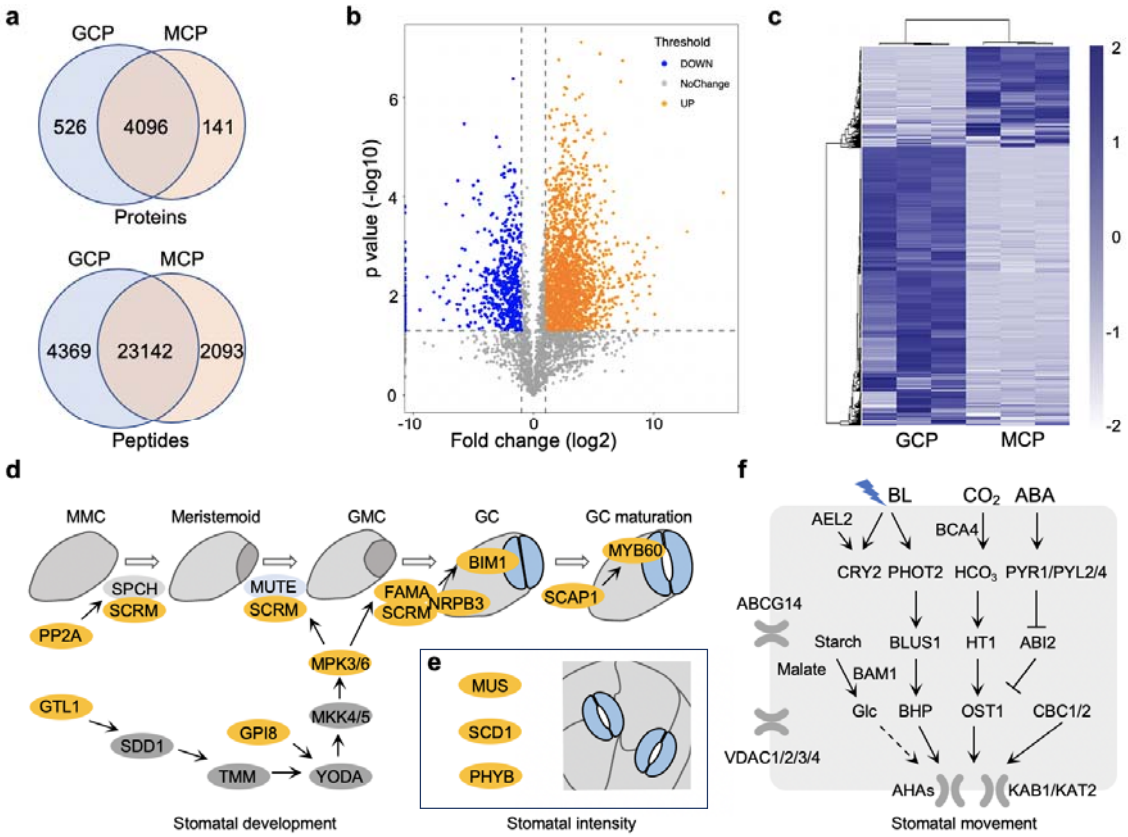

Figure 2. Quantitative comparison of GCP and MCP proteome. a. Venn diagram showing the overlap between the protein identified from GCP and MCP proteome. **b.** Volcano plot of differentially up-regulated (upper panel) or down-regulated (bottom panel) proteins in the proteome of GCPs and MCPs, respectively. **c.** Heatmap showing the quantitative comparison of proteins in GCP and MCP proteome. On the y-axis, the negative log10 values are plotted. On the x-axis, the log2 values of the fold change seen in the comparison. **d**. Proteins function in guard cell development are present in the proteome of GCPs. e. Proteins function in the regulation of guard cell intensity are present in the proteome of GCPs. Yellow circles highlight the protein detected in GCPs. **f.** Proteins function in stomatal movement respond to ABA, CO_2, Blue light (BL) are enriched in GCPs. All proteins listed in this panel show higher abundance in GCPs than that in MCPs (FC > 2, $p < 0.05$).

There are 664 proteins only present or enriched in MCPs (Fig. 2b, 2c). Notably, 546 out

of these 664 proteins are known to be the component of chloroplast (GO term 0009507). Other enriched GO of MCP enriched proteins include photosynthesis, photorespiration, pigment and chlorophyll metabolic process, electron transport chain and response to radiation and light stimulus. These data supported the dominant role of mesophyll cells in photosynthesis.

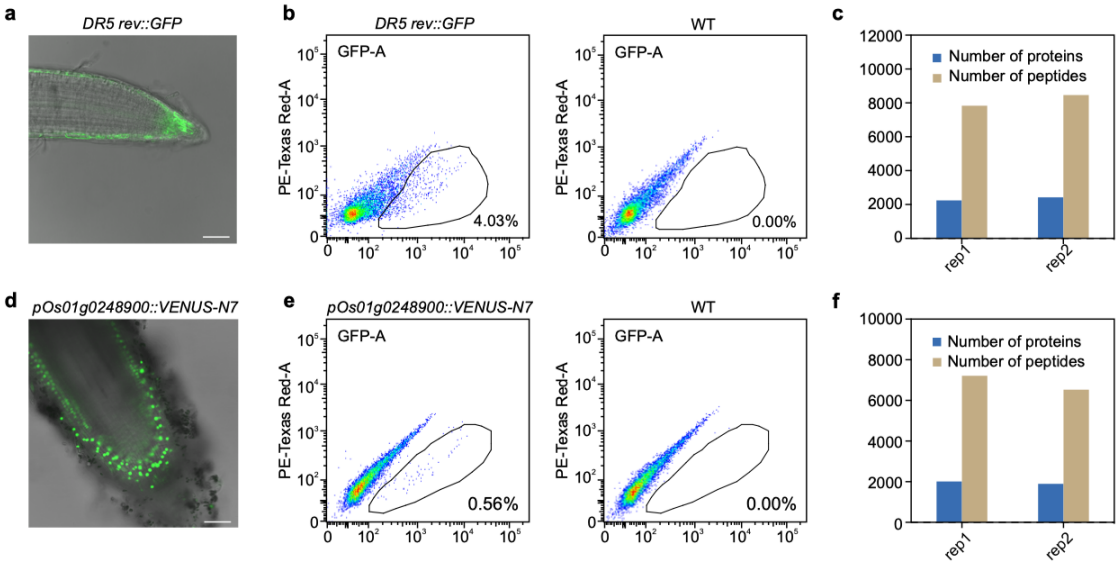

Figure 3. Proteomic analysis of DR5 rev::GFP and pOs01g0248900::VENUS-N7 fluorescent protoplasts. a. Image showed the fluorescent of *DR5 rev::GFP* transgenic seedling roots. Bar=50 μm. b. Representative flow cytometry scatter plots are shown for *DR5 rev::GFP* (left) and wild type control (right). c. The number of peptides and proteins identified from about 1,000 *DR5-rev::GFP* fluorescent protoplasts. d. The image showed the fluorescent of root in *pOs01g0248900::VENUS-N7* transgenic rice seedling. Bar=50 μm. e. Representative flow cytometry scatter plots are shown for *pOs01g0248900::VENUS-N7* (left) and wild type (WT, right). f. The number of peptides and proteins identified from about 1,000 *pOs01g0248900::VENUS-N7* transgenic rice fluorescent protoplasts.

To verify if this pipeline is appliable to other types of fluorescent cells, we enriched the protoplasts of root cells in *DR5 rev::GFP* transgenic Arabidopsis (Fig. 3a and 3b) and obtained average of 6,871 peptides, representing 1,950 proteins from about 1,000 fluorescent protoplasts (Fig. 3c). The GO analysis revealed that proteins involved in translation, response

to cold, oxidative stress, and embryo development are enriched in *DR5 rev::GFP* fluorescent protoplasts.

We also obtained 8,149 peptides, representing 2,232 proteins, from about 1,000 fluorescent protoplasts of *pOs01g0248900::VENUS-N7* that strongly expressed in atrichoblasts in rice root tips[31] (Fig. 3d-f). The enriched GO terms of fluorescent protoplasts of *pOs01g0248900::VENUS-N7* include oxoacid and cellular ketone metabolic processes, transport, and glycolysis, etc.

Discussion

In this study, we optimized the nano-scale protein extract and detection methods by using one-tube lysis and digestion procedure to avoid protein loss in sample preparation steps. This improved pipeline can detect thousands of proteins from as few as 1,000 cells and allows us to profile the proteome of various types of cells in plants, like GC, MC, and epidermal cells in roots. Compared to some pioneer works that identified about 2,000 proteins from about 1,000,000 root cell protoplasts[13] and 3×10^8 GCPs[11], our method showed a significantly improved sensitivity and efficiency. The improved pipeline allows us to acquire in-depth proteome information for thousands of fluorescent protoplasts. We showed that signaling components are enriched in GCs that allow GC to quickly respond to environmental cues.

We also applied our pipeline to root cells in both Arabidopsis and rice and obtained similar results, suggesting that this procedure may apply to different cells from different plant species and even non-plant systems. In the future, this pipeline may also be applied to the sorting and enrichment of different organelles for spatial proteomics to understand the

localization and dynamics of proteins in cells[32]. Such enriched cells or organelles also could be used for post-translational modification (PTM) proteomics to further reveal the functional regulation at the proteome level. Combine with the single-cell-transcriptomics that could identify unique marker genes for each cell group, we may generate fluorescent transgenic lines and perform specific proteomics in each type of cells in a tissue or even organism. From this information, we may be able to acquire a more systematic knowledge of how different cell types function, both as individual cells and in important interactions with other cells to form tissues and organs. This information may allow us to more fully recognize particular modes of interactions that can generate emergent properties, that are integrated together to form the intercellular signaling and organization of single cells into the functional phenotypes of multiple cell organisms.

As a FACS method, the protoplasts isolation method for each type of cells needs to be optimized. Several hours' enzymatic digestion with cellulase/macrozyme and high-concentration mannitol may affect the stability or modification of some proteins, which drawbacks the application of this pipeline in some stimulus-responsive proteomics analysis. In addition, we are failure to obtain enough amount of the quiescent center (QC) cells, which might be due to the limitation of cytometry to isolate such an extremely low abundance type of cell. The QC also could not be detected in some single-cell-transcriptome assays[31, 33, 34]. In the future, new methods like magnetic-activated cell sorting may be helpful to isolate, and proteomic analyze the such type of cells.

Methods

Growth conditions and plant materials

The *pGC1::GFP*[15] and *DR5rev::GFP*[35] seeds were sterilized in 75% ethanol and germinated on half strength Murashige and Skoog (MS) medium plates with 0.75 agar. The root tips of *DR5 rev::GFP* were harvested at indicated time. After 7 days' growth on the plates, the *pGC1::GFP* seedlings were transplanted to soil, and rosette leaves were harvested at indicated time for protoplast isolation.

The seeds of *pOs01g0248900::VENUS-N7* transgenic rice[31] were grown in half strength MS liquid medium in the growth chamber at 26 °C with 60% humidity (16 hour light/8 hour dark). After 7 days, the root tips of the seedlings were harvested for protoplast isolation.

Preparation of *Arabidopsis* GCPs and MCPs

The rosette leaves of three-week-old seedlings of *pGC1::GFP* grown in the soil are blended for 10 s five times in 100 mL ice cold water. The blended mixture is filtered through a 100 µm mesh (Falcon) to remove the broken mesophyll and epidermal cells. The peels then were transferred into 20 mL enzyme solution containing 1.35% cellulose R10 (Yakult Pharmaceutical Industry), 0.268% macrozyme R-10 (Yakult Pharmaceutical Industry), 0.1% polyvingl pyrrolidone 40,000 (PVP-40), 0.25% bovine serum albumin (BSA), and 0.5 mM L-ascorbic acid, 2.75 mM 2-[N-morpholino] ethare sulfonic acid hydrate-Tris (MES), pH 5.5, 0.275 mM $CaCl_2$, 0.275 mM $MgCl_2$, 5.5 µM KH_2PO_4, and 0.275 M sorbitol at 25 °C with shaking. After 1 h digestion, the peels were filtered with 100 µm mesh and placed into 10 ml enzyme solution II containing 1.3% cellulose "Onozuka" RS (Yakult Pharmaceutical

Industry), 0.0075% Pectolyase Y-23 (Yakult Pharmaceutical Industry), 0.25% BSA, and 0.5 mM L-ascorbic acid 5 mM MES, pH 5.5, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 μM KH_2PO_4 and 0.5 M sorbitol for 2 h. The digestion mixture was filtered with 40 μm mesh (Falcon), and the GCPs were collected by centrifuge at 200 × g for 5 min. The protoplasts were resuspended in the same solution without enzyme and sorted with the cytometry (ARIA III Cell sorter, BD bioscience) with the GFP signal and single-cell pattern to isolate the high purity (~ 100%) fluorescent protoplasts. The indicated number of cells were collected by centrifuge and used for protein extraction for LC-MS/MS.

For isolating of Arabidopsis MCPs, leaf strips were excised from the middle parts of young rosette leaves, dipped in enzyme solution containing 1.35% cellulase R10 (Yakult Pharmaceutical Industry) and 1.35% macerozyme R10 (Yakult Pharmaceutical Industry) and incubated at room temperature in the dark. The protoplast solution was diluted with an equal volume of W5 solution (2 mM MES, pH 5.7, 154 mM NaCl, 125 mM $CaCl_2$, and 5 mM KCl) and filtered through a nylon mesh. The MCPs are resuspended with 10 ml W5 buffer and sorted with the cytometry (ARIA III Cell sorter, BD bioscience), use the autofluorescence of chloroplast to isolate the high purity (~ 100%) fluorescent protoplasts. The indicated number of cells were collected by centrifuge and used for protein extraction for LC-MS/MS.

Preparation of Arabidopsis root cell fluorescent protoplasts

For isolating of *DR5 rev::GFP* fluorescent protoplasts, the root tip regions (about 0.5 cm from root tip) were cut from the roots of 7-day-old Arabidopsis seedling. The root tips were digested for 2 h in enzyme solution containing 1.5% cellulase R10, 1.5% macerozyme R10,

0.4 M mannitol, 10 mM KCl, 10 mM $CaCl_2$ and 0.1% BSA. The protoplasts were filtered with a 40 μm mesh (Falcon) and centrifuge at 500 × g for 6 min. The protoplasts were resuspended in the 8% mannitol and sorted with the cytometry (FACS ARIA III Cell sorter, BD bioscience) with the GFP signal and single-cell pattern to isolate the high purity (~ 100%) fluorescent protoplasts. The indicated number of cells were collected by centrifuge and used for protein extraction for LC-MS/MS.

Preparation of fluorescent protoplasts of rice root cells

The root tips of 6-day old rice seedlings were harvested and digested for 3 h at room temperature in enzyme solution containing 4% cellulose Onozuka RS, 1.5% macerozyme R10, 0.4 M mannitol, 0.1 M 2-(N-morpholino) ethare sulfonic acid hydrate-Tris (MES), 10 mM KCl, 10 mM $CaCl_2$ and 0.1% BSA and shaked every 30 min. The protoplasts were filtered with a 40 μm mesh (Falcon), centrifuged at 500 × g for 6 min. The protoplasts were resuspended in the 8% mannitol and sorted with the cytometry (BD bioscience, ARIA III Cell sorter) with the GFP signal and single-cell pattern to isolate the high purity (~ 100%) fluorescent protoplasts. The indicated number of cells were collected by centrifuge and used for protein extraction for LC-MS/MS.

Protein extraction and digestion

Two sample preparation workflows were compared for the efficiency of protein extraction. GCPs and MCPs were lysed in (1) SDC-SLS (SS) protocol: 12 mM SDC and 12 mM SLS or (2) Gdn-Cl protocol: 6M guanidine chloride (GdnHCl). All the lysis chemicals were

dissolved in 100 mM Tris-HCl (pH 8.5). Proteins were reduced and alkylated with 10 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) and 40 mM 2-Chloroacetamide (CAA) at 95 °C for 10 min. Proteins were then subjected to five cycles of ultrasonication pulses of 30 sec and pauses of 30 sec. For SS protocol, protein extracts were 5-fold diluted using 50 mM triethylammonium bicarbonate (TEAB), and trypsin was subsequently added to a final 1:100 (w/w) enzyme-to-protein ratio and incubated at 37 °C overnight. For Gdn-Cl protocol, proteins were precipitated using methanol-chloroform precipitation, and the precipitated protein pellet was air dried. The pellet was suspended in SDC-SLS buffer (12 mM SDC, 12 mM SLS in 100 mM Tris-HCl (pH 8.5)) and then followed the SS protocol for enzymatic digestion and detergent precipitation. For both methods, the digested peptides were desalted using a C18 StageTip.

LC-MS/MS Analysis

The peptides were analyzed using Orbitrap Fusion (Thermo Fisher Scientific) or timsTOF pro (Bruker) mass spectrometer. For the analysis of 1 μg of GCP and MCP digests, the peptides were dissolved in 4 μL of 0.2% formic acid (FA) and injected into an Easy-nLC 1200 (Thermo Fisher Scientific). Peptides were separated on a 15 cm in-house packed column (360 μm OD × 75 μm ID) containing C18 resin (2.2 μm, 100 Å, Michrom Bioresources). The mobile phase buffer consisted of 0.1% FA in ultra-pure water (Buffer A) with an eluting buffer of 0.1% FA in 80% ACN (Buffer B) run over a linear 182 min gradient of 5–35% buffer B at flow rate of 300 nL/min. The Easy-nLC 1200 was coupled online with an Orbitrap Fusion Tribrid mass spectrometer. The mass spectrometer was operated in the data-dependent

acquisition (DDA) mode in which a full-scan MS (from m/z 350–1500 with the resolution of 120,000) was followed by top speed higher-energy collision dissociation (HCD) MS/MS scans of the most abundant ions with dynamic exclusion for 60 s.

For the analysis of the digests of counted 250 to 10,000 GCP cells, the peptides were dissolved in 4 μL of 0.2% formic acid (FA) and injected into nanoflow reversed-phase chromatography. Which was performed on an EASY-nLC 1200 system (Thermo Fisher Scientific). Peptides were separated in 120 min at a flow rate of 300 nl min on a 25 cm × 75 μm column with a laser-pulled electrospray emitter packed with 1.6 μm ReproSil-Pur C18-AQ particles. Mobile phases A and B were water with 0.1 % formic acid and 80:20:0.1 % ACN: water: formic acid, respectively. The fraction of B was linearly increased from 2% to 27% in 90 min, followed by an increase to 46% in 22 min and a further increase to 100% in 3 min before re-equilibration. Liquid chromatography was coupled online to a hybrid TIMS quadrupole TOF mass spectrometer (timsTOF Pro) via a CaptiveSpray nano-electrospray ion source. The electrospray voltage appliedwas 1.60 kV. Precursors and fragments were analyzed at the TOF detector, with a MS/MS scan range from 300 to 1500m/z. The Tims TOF Pro was operated in parallel accumulation serial fragmentation (PASEF) mode. Precursors with charge states 2 to 3 were selected for fragmentation, and 4 PASEF-MS/MS scans were acquired per cycle. The dynamic exclusion was set to 24 s.

Data Analysis

The raw files were searched directly against the Arabidopsis thaliana database (TAIR10 with 35,386 entries) with no redundant entries using SEQUEST HT search algorithm on Proteome

Discoverer software (version 2.4). Peptide precursor mass tolerance was set at 10 ppm, and MS/MS tolerance was set at 0.02 Da. Three missed cleavage sites of trypsin were allowed. Search criteria included a static carbamidomethylation of cysteine (+57.0214 Da) and variable modifications of oxidation (+15.9949 Da) on methionine and phosphorylation (+79.9663 Da) on serine, threonine and tyrosine. The false discovery rates (FDR) of proteins, and peptides were set at 1% FDR.

Acknowledgements

This work was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences, Grant XDB27040106 (to P.W.), and National Natural Science Foundation of China, Grant 31771358 (to P.W.). We are grateful to Drs. Julian Schroeder of University of California, San Diego, for providing the seeds of *pGC1::GFP* transgenic plants, Jiawei Wang of CAS Center for Excellence in Molecular Plant Sciences, for providing the seeds of *pOs01g0248900::VENUS-N7*.

Author contributions

P.W. designed the research. H.W., J.R. and Q.G. and performed research. H.W., R.L., T.S., C.K., S.D., and C.-P.S. performed data analysis. H.W., R.L., C.K., C.-C.H., and C.-P.S. wrote the manuscript.

Competing Interests Statement

The authors declare no competing interests.

References

1. Shaw, R., Tian, X. & Xu, J. Single-cell transcriptome analysis in plants: advances and challenges. *Mol Plant* **14**, 115-126 (2021).
2. Rich-Griffin, C. *et al.* Single-Cell Transcriptomics: a high-resolution avenue for plant functional genomics. *Trends Plant Sci* **25**, 186-197 (2020).
3. de Souza, L.P., Borghi, M. & Fernie, A. Plant single-cell metabolomics-challenges and perspectives. *Int J Mol Sci* **21** (2020).
4. Marx, V. A dream of single-cell proteomics. *Nat Methods* **16**, 809-812 (2019).
5. Dai, S. & Chen, S. Single-cell-type proteomics: toward a holistic understanding of plant function. *Mol Cell Proteomics* **11**, 1622-1630 (2012).
6. Holmes-Davis, R., Tanaka, C.K., Vensel, W.H., Hurkman, W.J. McCormick, S. Proteome mapping of mature pollen of Arabidopsis thaliana. *Proteomics* **5**, 4864-4884 (2005).
7. Noir, S., Bräutigam, A., Colby, T., Schmidt, J. Panstruga, R. A reference map of the Arabidopsis thaliana mature pollen proteome. *Biochem Biophys Res Commun* **337**, 1257-1266 (2005).
8. Dai, S. *et al.* Proteomics identification of differentially expressed proteins associated with pollen germination and tube growth reveals characteristics of germinated *Oryza sativa* pollen. *Mol Cell Proteomics* **6**, 207-230 (2007).
9. Fíla, J. *et al.* Phosphoproteomics profiling of tobacco mature pollen and pollen activated in vitro. *Mol Cell Proteomics* **15**, 1338-1350 (2016).
10. Zhu, M., Dai, S., McClung, S., Yan, X. & Chen, S. Functional differentiation of Brassica napus guard cells and mesophyll cells revealed by comparative proteomics. *Mol Cell Proteomics* **8**, 752-766 (2009).
11. Zhao, Z., Zhang, W., Stanley, B.A. & Assmann, S.M. Functional proteomics of *Arabidopsis thaliana* guard cells uncovers new stomatal signaling pathways. *Plant Cell*, tpc.108.063263 (2008).
12. Zhang, X. *et al.* Identification of PAN2 by quantitative proteomics as a leucine-rich repeat-receptor-like kinase acting upstream of PAN1 to polarize cell division in maize. *Plant Cell* **24**, 4577-4589 (2012).
13. Petricka, J.J. *et al.* The protein expression landscape of the Arabidopsis root. *Proc Natl Acad Sci USA* **109**, 6811-6818 (2012).
14. Fukao, Y. *et al.* Quantitative proteomic analysis of the response to zinc, magnesium, and calcium deficiency in specific cell types of Arabidopsis roots. *Proteomes* **4**, 1 (2016).
15. Yang, Y., Costa, A., Leonhardt, N., Siegel, R.S. & Schroeder, J.I. Isolation of a strong Arabidopsis guard cell promoter and its potential as a research tool. *Plant Methods* **4**, 6 (2008).
16. Wang, P. *et al.* Reciprocal regulation of the tor kinase and ABA receptor balances plant growth and stress response. *Mol Cell* **69**, 100-112.e106 (2018).
17. Hsu, C.-C. *et al.* Universal plant phosphoproteomics workflow and its application to tomato signaling in response to cold stress. *Mol Cell Proteomics* **17**, 2068-2080 (2018).
18. Ohashi-Ito, K. & Bergmann, D.C. Arabidopsis FAMA controls the final proliferation/differentiation switch during stomatal development. *Plant Cell* **18**, 2493-2505 (2006).
19. Chen, L. *et al.* NRPB3, the third largest subunit of RNA polymerase II, is essential for stomatal patterning and differentiation in Arabidopsis. *Development* **143**, 1600-1611 (2016).
20. Negi, J. *et al.* A Dof transcription factor, SCAP1, is essential for the development of functional stomata in Arabidopsis. *Curr Biol* **23**, 479-484 (2013).

401 21. Ma, Y. *et al.* Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science* **324**,
402 1064-1068 (2009).
403 22. Park, S.-Y. *et al.* Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START
404 proteins. *Science* **324**, 1068-1071 (2009).
405 23. Hu, H. *et al.* Carbonic anhydrases are upstream regulators of CO_2-controlled stomatal movements in
406 guard cells. *Nat Cell Biol* **12**, 87-93 (2010).
407 24. Hashimoto, M. *et al.* Arabidopsis HT1 kinase controls stomatal movements in response to CO2. *Nat*
408 *Cell Biol* **8**, 391-397 (2006).
409 25. Hiyama, A. *et al.* Blue light and CO_2 signals converge to regulate light-induced stomatal opening. *Nat*
410 *Commun* **8**, 1284 (2017).
411 26. Kinoshita, T. *et al.* Phot1 and phot2 mediate blue light regulation of stomatal opening. *Nature* **414**,
412 656-660 (2001).
413 27. Takemiya, A. *et al.* Phosphorylation of BLUS1 kinase by phototropins is a primary step in stomatal
414 opening. *Nat Commun* **4**, 2094 (2013).
415 28. Hayashi, M., Inoue, S.I., Ueno, Y. & Kinoshita, T. A Raf-like protein kinase BHP mediates blue
416 light-dependent stomatal opening. *Sci Rep* **7**, 45586 (2017).
417 29. Flütsch, S. *et al.* Guard cell starch degradation yields glucose for rapid stomatal opening in Arabidopsis.
418 *Plant Cell* **32**, 2325-2344 (2020).
419 30. Lee, M. *et al.* The ABC transporter AtABCB14 is a malate importer and modulates stomatal response to
420 CO2. *Nat Cell Biol* **10**, 1217-1223 (2008).
421 31. Zhang, T.-Q., Chen, Y., Liu, Y., Lin, W.-H. & Wang, J.-W. Single-cell transcriptome atlas and chromatin
422 accessibility landscape reveal differentiation trajectories in the rice root. *Nat Commun* **12**, 2053
423 (2021).
424 32. Lundberg, E. & Borner, G.H.H. Spatial proteomics: a powerful discovery tool for cell biology. *Nat Rev*
425 *Mol Cell Biol* **20**, 285-302 (2019).
426 33. Satterlee, J.W., Strable, J. & Scanlon, M.J. Plant stem-cell organization and differentiation at single-cell
427 resolution. *Proc Natl Acad Sci U S A* **117**, 33689-33699 (2020).
428 34. Dorrity, M.W. *et al.* The regulatory landscape of Arabidopsis thaliana roots at single-cell resolution. *Nat*
429 *Commun* **12**, 3334 (2021).
430 35. Benková, E. *et al.* Local, Efflux-dependent auxin gradients as a common module for plant organ
431 formation. *Cell* **115**, 591-602 (2003).