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# Cell type-specific proteomics uncovers a RAF15-SnRK2.6/OST1 kinase cascade in guard cells

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**ABSTRACT**

Multicellular organisms such as plants contain various cell types with specialized functions. Analyzing the characteristics of each cell type reveals specific cell functions and enhances our understanding of organization and function at the organismal level. Guard cells (GC) are specialized epidermal cells that regulate the movement of the stomata and gaseous exchange, and provide a model genetic system for analyzing cell fate, signaling and function. Several proteomics analyses of GC are available, but these are limited in depth. Here we used enzymatic isolation and flow cytometry to enrich for GC and mesophyll cell protoplasts and perform in-depth proteomics in these two major cell types in Arabidopsis leaves. We identified ~3,000 proteins not previously found in the GC proteome and more than 600 proteins that may be specific to GC. The depth of our proteomics enabled us to uncover a guard cell-specific kinase cascade whereby Raf15 and Snf1-related kinase2.6 (SnRK2.6)/OST1(open stomata 1) mediate abscisic acid (ABA)-induced stomatal closure. RAF15 directly phosphorylated SnRK2.6/OST1 at the conserved Ser175 residue in its activation loop and was sufficient to reactivate the inactive form of SnRK2.6/OST1. ABA-triggered SnRK2.6/OST1 activation and stomatal closure was impaired in *raf15* mutants. We also showed enrichment of enzymes and flavone metabolism in GC, and consistent, dramatic accumulation of flavone metabolites. Our study answers the long-standing question of how ABA activates SnRK2.6/OST1 in

guard cells and represents a resource potentially providing further insights into the molecular basis of GC and mesophyll cell development, metabolism, structure, and function.

**Keywords:** Abscisic acid, Guard cell, Metabolomics, Phosphorylation, Proteomics, Signaling,

## INTRODUCTION

Multicellular organisms such as plants contain different cell types with specialized structures and functions. Single-cell transcriptomics, proteomics and metabolomics can be used to dissect the specific structural and functional information in each cell type, ultimately revealing how different cells integrate into a complex organism. Proteomics is key to bridging the gap between transcriptomics and phenotypes; however, comprehensively analyzing the proteome in a single cell is still challenging (Marx, 2019) and even cutting-edge mass spectrometry can only identify about 1,000 proteins in a single mammalian cell (Brunner et al., 2021; Petelski et al., 2021). In plants, enriching for a particular type of plant cell is a realizable approach for in-depth proteomics (Dai and Chen, 2012) and has been used for cell types that can easily be isolated, including pollen (Holmes-Davis et al., 2005; Noir et al., 2005; Dai et al., 2007; Fíla et al., 2016), guard cells (GCs)(Zhao et al., 2008; Zhu et al., 2009; Zhang et al., 2012), and mesophyll cells (MCs)(Zhao et al., 2008; Zhu et al., 2009).

Guard cells (GC) are specialized epidermal cells that play an important role in gaseous exchange in and out of plant leaves by regulating the opening and

closing of the stomata. Using blended intact GCs or GC protoplasts (GCPs) isolated by enzyme digestion and centrifugation, several groups performed pioneering single-cell-type proteomic studies in Arabidopsis, *Brassica napus*, and maize, and discovered unique proteins, signal transduction pathways, and metabolism that determine the function and development of GCs (Zhao et al., 2008; Zhu et al., 2009; Zhang et al., 2012; Geilfus et al., 2018; Balmant et al., 2021; David et al., 2021). However, only about 900-1,700 proteins were identified in these studies and remaining tissue fragments in crude extracts may affect the accuracy of the results. Therefore, we set out to improve the depth and accuracy of GC proteomics.

SNF1-related protein kinase 2.6 (SnRK2.6), also named OPEN STOMATA 1 (OST1), is a master regulator in stomatal movement. The *ost1* mutant shows abolished or strongly impaired stomatal closure in response to the phytohormone abscisic acid (ABA) (Mustilli et al., 2002), low-air-humidity-induced leaf-to-air vapor pressure difference (Xie et al., 2006; Merilo et al., 2018), or bacterial invasion (Melotto et al., 2006). Under unstressed conditions, OST1 is dephosphorylated and inhibited by the A Clade PP2C phosphatases (Vlad et al., 2009). Upon stress, ABA binds to ABA receptor PYR1/PYL/RCAR proteins, and the ABA/receptor complex inhibits PP2Cs (Fujii et al., 2009; Ma et al., 2009; Miyazono et al., 2009; Park et al., 2009). B2/B3 subgroup RAF kinases are essential for SnRK2 activation upon ABA or osmotic stress (Saruhashi et al., 2015; Fàbregas et al., 2020; Katsuta et al., 2020; Lin et al., 2020; Soma et al., 2020; Takahashi et al., 2020; Lin et al., 2021). Accordingly, the high-order mutant

of B2 and B3 RAFs, *OK*$^{100}$*-nonu*, shows an ABA-hyposensitive phenotype in germination and seedling growth, resembling the phenotype of *snrk2.2/2.3/2.6* triple and *pyl112458* high-order mutants (Lin et al., 2021). However, although much less sensitive to ABA than the wild type, the stomata of *OK*$^{100}$*-nonu*, which are still partially closed upon application of ABA, are not identical to those of *snrk2.2/2.3/2.6* triple and *pyl112458* high-order mutants (Lin et al., 2021). Therefore, it is unclear which of the more than 20 subgroup B RAF kinases has the predominant function in GC ABA signaling.

Here, we combined enzymatic isolation and fluorescence-activated cell sorting (FACS), which has previously been applied to cell type-specific proteomics in Arabidopsis roots (Petricka et al., 2012; Fukao et al., 2016), to enrich for highly pure protoplasts, which allowed us to profile, in depth, the proteome of GCPs and mesophyll cell protoplasts (MCPs) in Arabidopsis leaves. By quantitative comparison of more than 4,500 proteins identified in GCPs and MCPs, we showed the B1 subgroup RAF15, but not members of the B2/B3 RAF subgroups, to be enriched in GCs. Recombinant RAF15 directly phosphorylated SnRK2.6/OST1 and was sufficient to reactivate the dephosphorylated inactive form of SnRK2.6/OST1 *in vitro*. ABA-triggered SnRK2.6/OST1 activation and stomatal closure was impaired in *raf15* mutants. Our results reveal a unique GC-specific RAF15-SnRK2.6 kinase cascade, which has a crucial role in ABA-induced stomatal closure. In addition, the in-depth proteome analysis of GCPs and MCPs could be a valuable resource for understanding the molecular basis of cell type-specific structure, signaling, metabolism, and function.

## RESULTS

### In-depth proteomics analysis of GCPs

Previous studies have confirmed that GCPs retain key physiological responses observed in GCs in situ, including responsiveness to environmental signals such as light, ABA, and $CO_2$ (Zhao et al., 2008). To acquire a high purity and in-depth GC proteome, we adapted the GCP preparation method (Zhao et al., 2008) and used FACS to enrich for fluorescent GCPs from *pGC1::GFP* transgenic plants (Yang et al., 2008) (Figure 1A). We lysed the fluorescent GCPs and subjected them to trypsin digestion. We then used mass spectrometry to analyze 1 µg total protein extract from about 30,000 GCPs (Hsu et al., 2018; Wang et al., 2018). As the results, 22,687 peptides, representing 4,492 protein groups, were identified at least once in the triplicates of GCP samples (Dataset S1).

To evaluate the reliability of our proteomics study, we first compared our GCP proteomics with previous studies (Dataset S2). Of the 1,732 proteins identified by a previous GCP proteomics study (Zhao et al., 2008), we identified 1,453 proteins (83.9%) (Figure 1B, left panel). Proximity labeling (PL) enables discovery of protein neighborhoods defining functional complexes and/or organellar protein compositions (Mair et al., 2019). A recent study performed a TurboID-based PL assay and identified 398 putative interactors of the stomatal transcription factor FAMA (Mair et al., 2019). Promisingly, 246 (61.8%) of these 398 proteins were present in our GCP proteomics result (Figure 1B, left panel). We also compared our GCP proteomics with two previous studies that used blended intact GCs (Geilfus et al., 2018; David et al., 2021). In our list of GCP

proteins, we found 1,425 (78.0%) of the 1,827 proteins identified by David et al., (2021), and 750 (78.2%) of the 959 proteins identified by Geilfus et al., (2018) (Table S1 in Geilfus et al., 2018) (Figure 1B, right panel). Thus, our GCP proteomics covers most GCP proteins identified by previous proteomic studies.

We then quantitatively compared the proteins present in both our GCP proteomics and intact GC proteomics (Geilfus et al., 2018). We reanalyzed the raw data (PXD009918) and identified 1,931 proteins from sextuplicate after-dawn intact GC samples (see Dataset S3), which differs from the original results in Geilfus et al., (2018), likely because of updates to the Proteome Discover Software we used. 1,268 out of 1,931 proteins overlapped with our GCP proteomics (Dataset S3). Of the 1,268 proteins identified by both studies, 370 (29.2%) showed at least two-fold higher abundance (FC > 2, $p < 0.05$) in the intact GCs than in our GCPs, while 393 proteins (31.0%) showed at least two-fold lower abundance (FC < 0.5, $p < 0.05$) in the intact GCs than in our GCPs (Figure 1C). The relative abundance of 505 (39.9%) of these 1,268 proteins was not significantly different between the two studies (Figure 1C). Pearson's correlation analyses ($R^2 = 0.5694$, $p < 0.0001$) showed a positive association between the relative abundances of the 1,268 proteins in the two proteomic studies (Figure 1C). Considering the different growth conditions, sample preparation, and MS machines, such a high correlation indicates the reliability of our cell sorting-based GCP proteomics analyses.

We then surveyed our results for the presence of proteins known to function in GC structure and function (Figure 1D). FAMA and Inducer of CBF Expression

1 (ICE1)/ SCREAM (SCRM) govern GC mother division and promote GC differentiation (Ohashi-Ito and Bergmann, 2006). FAMA, ICE1/SCRM, and their interacting protein, the third largest subunit of nuclear DNA-dependent Pol II (NRPB3) (Chen et al., 2016), were present in our data. After forming GC, STOMATAL CARPENTER 1 (SCAP1) is required to develop functional stomata (Negi et al., 2013). MYB DOMAIN PROTEIN 60 (MYB60) involved in the stomatal movement is a known target of SCAP1 (Negi et al., 2013). Both SCAP1 and MYB60 were present in our data (Figure 1D). Proteins involved in the early development stages of GCs, like SPCH and MUTE, were not in the list of GCP proteins, indicating that our method mainly enriches for fully developed GCs in the mature leaves. Besides proteins involved in stomatal development, known regulators of GC intensity, like MUS, SCD1, and PHYB, were also present in our data (Figure 1E).

Many key components of stomatal movement regulation in response to blue light (BL), high $CO_2$, and ABA were also present in our data (Figure 1F). For example, we identified BETA CARBONIC ANHYDRASE 4 (BCA4) (Hu et al., 2010), HIGH LEAF TEMPERATURE 1 (HT1) (Hashimoto et al., 2006), and CONVERGENCE OF BLUE LIGHT AND $CO_2$ (CBC2) (Hiyama et al., 2017), light receptors CRYPTOCHROME 3 (CRY3) and PHOT2 (Kinoshita et al., 2001), the PHOT2 substrate BLUE LIGHT SIGNALING1 (BLUS1) (Takemiya et al., 2013), and BLUE LIGHT-DEPENDENT $H^+$-ATPase PHOSPHORYLATION (BHP) (Hayashi et al., 2017). A recent study suggested that glucose, not malate, is the major starch-derived metabolite in GC for rapid stomatal opening (Flütsch et al.,

2020). Consistent with this, b-AMYLASE1 (BAM1) (Flütsch et al., 2020), a glucan hydrolase was present in our data. However, we also identified ATP-BINDING CASSETTE B14 (ABCB14) (Lee et al., 2008), a malate transporter, suggesting the potential role of malate in GCs.

In summary, our GCP proteomics is consistent with previous data, indicating that protoplasting and FACS do not considerably affect the protein composition of GCs. Further, we identified around 3,000 proteins not found in previous datasets, indicating that we achieved greater depth.

**Comparison of GCP and MCP proteomes**

To identify proteins specifically enriched in GCs, we performed a label-free quantitative comparison between GCP and MCP proteomes. We subjected ~1 µg total protein extract from ~15,000 FACS-purified MCPs to MS analysis and identified 23,655 peptides representing 4,137 protein groups (Dataset S4). There were 615 proteins identified at least twice in the three biological replicates of GCP but not in any MCP sample, and 229 proteins identified at least twice in the three biological replicates of MCP but not in any GCP sample (Figure 2A–C, Dataset S5, S6). Combining with 2,260 proteins that show at least two-folds higher abundances in GCP samples than that in MCP samples (FC > 2, $p < 0.05$), a total 2,875 proteins were considered as proteins enriched in GCPs (Dataset S6). The enriched KEGG pathway and enriched gene ontology (GO) terms in the GCPs were mainly related to transport, splicing, endocytosis, and responses to ABA, cold, and osmotic stress (Figures 2D, S2; Dataset S7).

There were 915 proteins were enriched (FC > 2, $p < 0.05$) or only detected in MCPs (Figure 2B, C; Datasets S5, S6). Notably, 714 of these 915 proteins are known components of chloroplast (GO term 0009507) (Figure S2; Dataset S7). Other enriched GO terms of MCP-enriched proteins included photosynthesis, photorespiration, pigment, chlorophyll metabolic process, electron transport chain, and response to radiation and light stimulus (Figure S2; Dataset S7). These data support the dominant role of MCs in photosynthesis.

To identify proteins that may respond to protoplasting of GCP, we conducted a quantitative comparison of proteomics of the epidermal peels before and after protoplasting (Figure S3; Dataset S8), following the published procedure (Denyer et al., 2019; Shahan et al., 2022). Our results showed that the abundance of 248 (8.6%) GCP-enriched proteins significantly increased after protoplasting (FC > 2, $p < 0.05$). However, even after normalization with the fold change by protoplasting, 191 out of 248 proteins still exhibited significant GCP-enriched patterns. Therefore, protoplasting had a minimal effect on the proteins in our dataset, which we noted in Dataset S6 and S8. Overall, our data provide a reliable and valuable resource for exploring the molecular basis of guard cell- or mesophyll cell-specific functions.

**GC-enriched RAF15 phosphorylates SnRK2.6/OST1**

We then systemically surveyed the presence of known ABA signaling components in GCPs and MCPs. Dozens of known ABA signaling components showed a GC-enriched pattern, and few components had a higher abundance in MCP than in GCP. For example, the ABA receptors PYR1, PYL1, PYL2, and

PYL4 showed 154-, 6.2-, 394-, and 24.3-fold enrichment, respectively, in GCPs than that in MCPs (Figure 3A). SnRK2.6/OST1 and ABI2 showed 38.4- and 17.0-fold enrichment, respectively, in GCPs (Figure 3A). To our surprise, the B2 and B3 subgroup RAF kinases, known to phosphorylate and activate SnRK2.2, SnRK2.3, and SnRK2.6/OST1 in response to ABA (Lin et al., 2020; Takahashi et al., 2020; Lin et al., 2021), were not detected/enriched in our proteomics result (Figure 3B). This suggested that kinases other than B2 and B3 RAFs may contribute to ABA-induced SnRK2.6/OST1 activation and stomata closure.

Our GCP proteomics identified RAF15 in the B1 subgroup, BHP/RAF27 in the C1 subgroup, HT1/RAF19 in the C5 subgroup, RAF28 in the C6 subgroup, and CBC2/RAF33, and AtMRK1/RAF48 in the C7 subgroup (Figure 3B). Most of these genes showed higher expression in GCPs than in MCPs (Figure S4). Using a transient expression assay system in MCPs of *OK$^{100}$-nonu,* which lacks most members of the B2 and B3 RAFs involved in ABA signaling, and has very low expression of SnRK2.6/OST1, we evaluated the function of these B1 and C subgroup RAFs in ABA signaling (Lin et al., 2021). Co-transfection of SnRK2.6/OST1 alone did not rescue the expression of ABA-induced *RD29B-LUC* in MCPs of *OK$^{100}$-nonu* (Figure 3C). However, co-transfection of RAF15, BHP/RAF27, HT1/RAF19, or MRK1/RAF48, but not RAF28 or CBC2/RAF33, partially rescued the expression of ABA-induced *RD29B-LUC* in MCPs of *OK$^{100}$-nonu* (Figure 3C). An *in vitro* kinase assay showed that full-length HT1/RAF19 or RAF15, out of the five tested RAFs, could directly phosphorylate SnRK2.6$^{KR}$, a

kinase “dead” form of SnRK2.6/OST1 (Figure 3D). Our finding of phosphorylation of HT1/RAF19 on SnRK2.6 is consistent with previous study (Tian et al., 2015).

Using $^{18}$O-ATP as the phosphate donor, we identified the RAF15 phosphosite in SnRK2.6/OST1 by mass spectrometry. The spectrum clearly showed the $^{18}$O-phosphorylation of Ser175 in the activation loop of SnRK2.6/OST1, after incubation with RAF15 (Figure 3E). Site-direct mutagenesis showed that RAF15 mainly phosphorylates the Ser175 in SnRK2.6 and the Ser175Ala (S175A) mutation abolished the phosphorylation of SnRK2.6/OST1 by RAF15 (Figure 3F). Thus, Ser175 is the major RAF15 target site in SnRK2.6/OST1. By contrast, neither Ser171Ala nor Ser175Ala mutation affected the HT1/RAF19-mediated phosphorylation of SnRK2.6/OST1$^{KR}$ (Figure S5A), indicating that HT1/RAF19 phosphorylates SnRK2.6/OST1 at a different site. In addition, unlike the RAF24-KD in B4 subgroup that phosphorylated multiple tested SnRK2s (Figure S5B), the RAF15 only phosphorylated SnRK2.6$^{KR}$, but not the tested SnRK2.1$^{KR}$, SnRK2.4$^{KR}$, and SnRK2.10$^{KR}$ (Figure 3G).

**RAF15 mediates SnRK2.6/OST1 activation and stomatal closure**

It is well-documented that the phosphorylation of two serine residues in the activation loop of SnRK2.6/OST1, Ser171 and Ser175, is essential for SnRK2.6/OST1 activation (Vlad et al., 2010; Ng et al., 2011; Lin et al., 2020; Soma et al., 2020; Takahashi et al., 2020; Lin et al., 2021; Zhang et al., 2021). RAF15-mediated phosphorylation of Ser175 in SnRK2.6/OST1 suggests a role of RAF15 in SnRK2.6/OST1 activation in GCs. To validate this hypothesis, we

tested if RAF15 could reactivate the dephosphorylated inactive form of SnRK2.6 (de-SnRK2.6). We used an adenosine triphosphate (ATP) analog-based *in vitro* kinase assay system, which can distinguish the trans- and autophosphorylation of SnRK2.6, to monitor the activity of SnRK2.6/OST1 (Lin et al., 2021). A Met94Gly (M94G) mutation in SnRK2.6/OST1 enlarges its ATP binding pocket, which can use the ATP analog $N^6$-Benzyl-ATPγS to thiophosphorylate itself or its substrate (Lin et al., 2021). By this method, thiophosphorylation by activated SnRK2.6$^{M94G}$ can be detected with an anti-thiophosphate ester antibody that only recognizes the thiophosphorylation. As shown in Figure 4A, pre-dephosphorylated SnRK2.6$^{M94G}$ (de-SnRK2.6$^{M94G}$) had no auto-thiophosphorylation activity (lanes 2-6). Application of the recombinant kinase domain of RAF15 (RAF15-KD) quickly induced the auto-thiophosphorylation of SnRK2.6$^{M94G}$ in a time-dependent manner (lanes 7-10), suggesting that RAF15-mediated phosphorylation is sufficient to reactivate SnRK2.6$^{M94G}$ *in vitro*, in the context of the auto-thiophosphorylation activity of SnRK2.6$^{M94G}$. We then examined SnRK2.6$^{M94G}$ activity by detecting the thiophosphorylation of ABA-responsive element-binding factor 2 (ABF2), a well-studied SnRK2 substrate. Adding RAF15-KD initiated the thiophosphorylation of SnRK2.6$^{M94G}$ and ABF2 (Figure 4B, lanes 4 and 5). Thus, transphosphorylation by RAF15-KD is sufficient to reactivate the dephosphorylated SnRK2.6/OST1. Unlike RAF15-KD, HT1/RAF19-KD did not reactivate the dephosphorylated inactive de-SnRK2.6 in the *in vitro* activation system (Figure 4C, lanes 3 and 4).

We then explored the ABA-induced stomatal closure in *raf15-1* (Salk_020351) and *raf15-2* (Salk_044414), two T-DNA mutant lines of *RAF15* (Figure S5). The *raf15-1* is a knock-out allele, with no detectable *RAF15* transcription, while the *raf15-2* is a knock-down allele (Figure S6A). Both *raf15-1* and *raf15-2* showed significantly impaired stomatal closure in response to ABA, compared to wild type (unpaired two-tailed *t*-test, $p < 0.001$) (Figure S6B). We also measured the stomatal conductance of detached intact *raf15* and Col-0 leaves by time-resolved gas-exchange analyses (Ceciliato et al., 2019). Both alleles of *raf15* were hyposensitive to low concentrations (1 and 3 μM) of ABA in stomatal closure (Figures 4D, E, S6C, D). However, *raf15* mutant lines show similar ABA sensitivity in stomatal closure in higher concentration of ABA (5 μM), when compared to wild type seedlings (Figures 4D, E, S6E). Interestingly, sorbitol-induced stomatal closure in *raf15-1* and *raf15-2* was comparable to that in Col-0 wild type, and only *raf15-1*, the knock-out allele, showed slightly slowed stomatal closure (Figures 4F, S6F). We also measured the SnRK2.6/OST1-activation by in-gel kinase assay in epidermal fragments isolated by blendor method (Bauer et al., 2013). The in-gel kinase assay result showed that the ABA-induced activation of SnRK2.6/OST1 was strongly impaired in *raf15-1* and *raf15-2* mutants (Figure 4H). Consistent with the essential role of RAF15 in SnRK2.6/OST1 activation, it physically interacts with SnRK2.6/OST, in both the results of coimmunoprecipitation and split-luciferase assays (Figures 4G, S6G). These data suggested that RAF15 is essential for ABA-induced SnRK2.6/OST1 activation and stomatal closure.

We also evaluated the function of the two other members of the B1 subgroup of RAF kinases, RAF13/M3Kθ1 and RAF14/M3Kθ2, in phosphorylating and activating SnRK2s. RAF13 also phosphorylated SnRK2.6$^{KR}$ and Ser171 in the activation loop was the major RAF13 phosphosite (Figure 4I). Like RAF15, recombinant RAF13 reactivated the dephosphorylated inactive de-SnRK2.6 *in vitro* (see Figure 4C, lane 5 and 6). Another B1 RAF, RAF14, had weak kinase activity and did not phosphorylate SnRK2.6$^{KR}$ *in vitro* (Figure S5H). The abundance of *RAF13* and *RAF14* mRNA in GCs was much less than that of *RAF15* mRNA (Figure S4A), and RAF13 and RAF14 protein was not detected in our GC proteomics (Dataset S1). We also noticed that all three members of the B1 RAF subgroup failed to phosphorylate SnRK2.4$^{KR}$, one of the ABA-independent SnRK2s (Figure S6I). Thus, GC-enriched RAF15 may have a unique role in stomatal closure by specifically mediating ABA-induced SnRK2.6/OST1 activation (Figure 4J).

**Metabolomics analysis of GCP and MCP**

Comparative proteomics analysis of GCP and MCP revealed an enrichment of flavonoid biosynthesis and metabolism-related proteins in GCP (GO:0009812 and GO:0009813, respectively). This included Flavonol Synthase 1 (FLS1), Flavanone 3-hydroxylase (F3H), Transparent Testa 5 (TT5), Chalcone Isomerase Like (CHIL), and Flower Flavonoid Transporter (FFT), and ten other proteins involved in the same processes that were hyper-accumulated or only detected in GCP compared to MCP, indicating more active flavonoid biosynthetic activities in the guard cells (Figure 5A). However, most of these GCP-enriched

flavonoid metabolism-related proteins showed significantly higher transcription levels in GCP, compared to MCP (Figure 5B, S7A), or enrichment in any single-cell transcriptomics analysis of Arabidopsis leaves. Encouraged by these results, we performed untargeted metabolomics to compare the metabolomic profiles of GCP and MCP. compare the flavonoid metabolites in GCP and MCP. From eleven biological replicates each containing about 100,000 protoplasts, we detected 89 high-confidence mass features (MFs) following an established peak extraction-data filtering-relative quantification pipeline (as delineated in Materials & Methods; Dataset S9).

Principal component analysis of the filtered MF dataset showed a clear separation of the metabolomes between the two cell types, with a single GCP sample clearly deviating from the other ten biological replicates (Figure S7B). After precluding this outlier, relative abundance of each MF was compared between GCP and MCP. In result, we identified 29 MFs that were significantly hyper-accumulated in GCP compared to MCP, and no MCP-enriched MF (FC > 2, $p < 0.05$, unpaired two-tail *t*-test). By combining co-eluting GCP-enriched MFs and searching in public databases, we identified three of the GCP-specific peaks as kaempferol-derived flavonoid glycosides: kaempferol-glu-rha-rha (Figure 5C, D), kaempferol-glu-rha (Figure 5E, F), and kaempferol-rha-rha (Figure 5G, H). Other GCP-enriched MFs have been putatively annotated as flavonoid glycosides, kaempferol-glu and quercetin-glu-rha (Figure S7A–D), and two sinapic acid derivatives (Figure S7E–H) using the same spectral search strategy. Taken together, our integrated proteomics and metabolomics data highlighted

that the hyperaccumulation of flavonoids in guard cells correlated and was likely caused by higher abundance of flavonoid biosynthetic enzymes in a cell-type specific manner.

## DISCUSSION

Here, we present in-depth proteomics analysis of pure GCPs using a FACS-based protocol that enabled us to identify about 4,500 proteins from ~30,000 GCPs. By comparison, previous GC proteomics efforts that used centrifugation to enrich for GCPs identified only ~1,500 proteins from ~30 million GCPs (Zhao et al., 2008). Therefore, we revealed ~3,000 proteins not previously shown to be present in the GC proteome, as well as more than 600 proteins found in GCPs but not in MCPs. Although it is possible that some proteins may have been induced or repressed during the protoplasting process, and therefore may have been missed due to their degradation, extremely low concentration or being inappropriate for MS detection, our results still provide a valuable resource for studying the specific structure, signaling, and functions of GCP and MCP. The depth of our GCP proteomics enabled us to identify the B1 subgroup RAF15, which has not previously been identified in proteomics data. We showed that the GC-enriched RAF15 phosphorylates SnRK2.6/OST1 and mediates ABA signaling in GCs. Similar to the homologous B2 and B3 RAFs (Lin et al., 2020; Takahashi et al., 2020; Lin et al., 2021), RAF15 phosphorylates Ser175, the key phosphosite in the activation segment of SnRK2.6/OST1, and is sufficient to reactivate dephosphorylated inactive SnRK2.6/OST1. Consistently, the *raf15* single mutant shows impaired stomatal closure in response to ABA.

Two recent studies suggested that RAF6/M3Kδ5, a member of the B3 RAF subgroup, has a dominant role in the rapid stomatal closure induced by low-air-humidity-induced leaf-to-air vapor pressure difference (Hsu et al., 2021; Peng et al., 2022). It was proposed that reduced air humidity might activate SnRK2.6/OST1 through two separate ABA-dependent and ABA-independent pathways (Merilo et al., 2018). Although both *RAF6/M3Kδ5* and *RAF15* are highly expressed in GCPs (Figure S4A) and involved in stomatal response by activating OST1 (Hsu et al., 2021; Peng et al., 2022), they might function differently. RAF15 likely functions in the later stage of stress, when the ABA level has increased, while RAF6/M3Kδ5 likely functions earlier, which may be independent of ABA. Interestingly, RAF6/M3Kδ5 was not present in our GCP proteomics, or any other GCP proteomics. Therefore, enzymatic isolation in a high-concentration mannitol solution may affect the stability of RAF6/M3Kδ5. We noticed that both RAF15 and RAF6/M3Kδ5 were targeted in an artificial miRNA line that was hyposensitive to ABA in germination and seedling development (Hauser et al., 2013). Consistent with this observation, RAF15 is also expressed in germinating seeds, flower, and silique, suggesting that RAF15 may also participate in ABA signaling in other tissues/processes (Figure S4).

Previous studies have suggested that B2 and B3 RAFs participate in ABA signaling by mediating SnRK2.2/3/6 activation, while B2, B3 and B4 RAFs are activated by hyperosmolality and are essential for both ABA-independent and -dependent SnRK2 activation (Katsuta et al., 2020; Lin et al., 2020; Soma et al., 2020; Takahashi et al., 2020; Lin et al., 2021). Here we showed that in addition to

B2/3/4 RAFs, at least two members of the B1 RAFs, RAF15 and RAF13, also phosphorylate and activate SnRK2s. Thus, more than twenty RAFs, in the B1, B2, B3, B4 subgroups, and the ten SnRK2s may form complex kinase networks, and different cell types may have unique RAF-SnRK2 cascades It is worth noting that in addition to the B subgroup, other groups of RAF kinases also participate in ABA signaling. Two members of the C subgroup of RAF-like protein kinases, RAF22 and RAF36, are direct substrates of SnRK2s and negatively regulate ABA signaling (Kamiyama et al., 2021; Sun et al., 2022). RAF22 also phosphorylates ABI1 at Ser416 and forms an interactive network with OST1 and ABI1 to optimize plant growth and response to drought stress in Arabidopsis (Sun et al., 2022).

It has long been known that flavanols are enriched in GC, playing critical roles in ROS scavenging and UV/high light protection (Vierstra et al., 1982; Weissenböck et al., 1987; Geng et al., 2016; Watkins et al., 2017; Zhu and Assmann, 2017; Daryanavard et al., 2023). However, this cell type-specific accumulation has been primarily associated with GC-specific expression of FLS1 (Kuhn et al., 2011). Our proteomics analysis revealed that the GC-specific accumulation of flavonoid biosynthetic enzymes were not limited to FLS1, but included almost all of the known enzymes in this metabolic pathway (Figure 5A). Consistent with the proteomics evidence, we observed clear accumulation of various flavonoid glycosides in GCP specifically. This observation was consistent with the report that kaempferol-gal-rha being the major green fluorescing compound in the lower epidermis of *Vicia faba*, a discovery made more than 40

years ago (Vierstra et al., 1982). Our two case studies, based on GCP-specific proteomics of the RAF15-SnRK2.6/OST1 cascade and flavanol metabolism enzymes, strongly suggest that cell-type proteomics can be a valuable resource for revealing cell-type specialized signaling pathways. This resource may help to ensure that different cells, depending on their developmental stage, nutrition status, or microenvironment, respond differentially and precisely to similar environmental cues.

## MATERIALS AND METHODS

### Growth conditions and plant materials

The *pGC1::GFP* (Yang et al., 2008) seeds were sterilized in 75% ethanol and germinated on half-strength Murashige and Skoog (MS) medium plates with 0.75% agar. After 7 days' growth on the plates, the *pGC1::GFP* seedlings were transplanted to soil, and rosette leaves were harvested at the indicated time for protoplast isolation.

### Preparation of Arabidopsis GCP and MCP

The rosette leaves of three-week-old seedlings of *pGC1::GFP* grown in the soil were blended for 10 s five times in 100 mL ice-cold water. The blended mixture was filtered through a 100 μm mesh (Falcon) to remove the broken mesophyll and epidermal cells. The peels were transferred into 20 mL enzyme solution containing 1.35% cellulose R10 (Yakult Pharmaceutical Industry), 0.268% macrozyme R10 (Yakult Pharmaceutical Industry), 0.1% polyvinyl pyrrolidone 40,000 (PVP-40), 0.25% bovine serum albumin (BSA), 0.5 mM L-ascorbic acid, 2.75 mM 2-[N-morpholino] ethanesulfonic 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 27 °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, 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 at 20 °C for 2 h. The digestion mixture was filtered with 40 µm mesh (Falcon), and the GCP were collected by centrifugation at 200 × *g* for 5 min. The protoplasts were resuspended in 500 µL of the same solution without enzyme and sorted using cytometry (ARIA III Cell sorter, BD bioscience) with the GFP signal and single-cell pattern at seed of 25-50 µL/min to isolate high purity (~ 100%) fluorescent protoplasts. The indicated number of cells were collected by centrifugation and used for protein extraction for LC-MS/MS.

For isolating Arabidopsis MCP, leaf strips were excised from the middle parts of young rosette leaves, dipped in an enzyme solution containing 1.35% cellulase R10 (Yakult Pharmaceutical Industry) and 0.268% 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 MCP were resuspended with 10 ml W5 buffer and sorted using cytometry (ARIA III Cell sorter, BD bioscience), using chloroplast's autofluorescence to isolate high purity (~ 100%) fluorescent protoplasts. The

indicated number of cells were collected by centrifugation and used for protein extraction for LC-MS/MS.

To identify the proteins induced by protoplasting processes, the protoplasted and equivalent un-protoplasted epidermal peels collected at completion of the protoplasting procedure and used for protein extraction for LC-MS/MS.

**Protein extraction and digestion.**

GCP and MCP were lysed in SLS-SDC buffer (12 mM sodium lauroyl sarcosinate (SLS), 12 mM sodium deoxycholate (SDC), 100 mM Tris-Cl, pH 8.5) or 6 M guanidine chloride (Gdn-Cl). 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. 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. The digested peptides were desalted using a C18 StageTip.

**LC-MS/MS analysis of proteome.**

The peptides were analyzed using Orbitrap Fusion (Thermo Fisher Scientific) mass spectrometer. To analyze 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 a 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.

**Proteomics 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. The protein intensity was normalized by total protein intensity across all six samples. After normalization, these proteins present in at least two of three replicates in one group, but not in any sample in the other group, or proteins show at least two-folds higher intensity in one group compared to the other group (FC > 2, $p < 0.05$, two-tail unpaired *t* test) were considered as the group-enriched proteins. The GO enrichment

analysis was performed using AgriGO v2,(Tian et al., 2017) The original GO enrichment results are filtered by REViGO 1 to remove semantic redundancy. “ClusterProfiler” R package was used for KEGG pathway enrichment analysis.

**Stomatal bioassay.**

For stomatal aperture assay, rosette leaves of 4-week-old Arabidopsis seedlings were taken. Epidermal strips were peeled out and incubated in buffer containing 50 mM KCl and 10 mM MES, pH 6.15, in a plant growth chamber for 3 h before ABA treatment. Stomatal apertures were measured 2 h after the addition of 5 μM ABA. The apertures of indicated number of stomata per sample were measured by quantifying the pore width of stomata using Image J software. All the experiments were repeated at least three times.

**In-gel kinase assay.**

For in-gel kinase assays, 20 μg extract of total proteins from intact leave fragments was used for SDS/PAGE analysis with histone embedded in the gel matrix as the kinase substrate. After electrophoresis, the gel was washed three times at room temperature with washing buffer (25 mM Tris-Cl, pH 7.5, 0.5 mM DTT, 0.1 mM $Na_3VO_4$, 5 mM NaF, 0.5 mg/mL BSA, and 0.1% Triton X-100) and incubated at 4°C overnight with three changes of renaturing buffer (25 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.1 mM $Na_3VO_4$, and 5 mM NaF). The gel was then incubated at room temperature in 30 mL reaction buffer (25 mM Tris-Cl, pH 7.5, 2 mM EGTA, 12 mM $MgCl_2$, 1 mM DTT, and 0.1 mM $Na_3VO_4$) with 200 nM ATP plus 50 mCi of [$\gamma$-$^{32}$P]ATP for 90 min. The reaction was stopped by transferring the gel into 5% (w/v) trichloroacetic acid and 1% (w/v) sodium pyrophosphate.

The gel was washed in the same solution for at least 5 h with five changes of the wash solution. Radioactivity was detected with a Personal Molecular Imager (Bio-Rad).

**Time-resolved stomatal conductance measurements.**

Intact leaves of 4- to 5-week-old Arabidopsis plants were used for time-resolved stomatal conductance measurements using a portable photosynthesis system (leaf LI-6400XT 6400; Li-Cor, Lincoln, NE, USA). Stomatal conductance measurements in response to 3 μM ABA and sorbitol were carried out with detached intact leaves in which ABA or sorbitol was added to the transpiration stream via the petiole, as described (Ceciliato et al., 2019). Data presented are means ± SEM, n = 3, three leaves from individual plants for each genotype per experiment.

**Protein purification and *in vitro* kinase assay.**

For *in vitro* kinase assays, full length coding sequence of SnRK2.6 and kinase domains of RAFs were cloned into either *pGEX-4T-1, pET28a or pET-SUMO vectors* and transformed into BL21 or ArcticExpress cells. The recombinant proteins were expressed and purified as previously described (Lin et al., 2020). For the phosphorylation assay, recombinant full-length RAF15, HT1/RAF19, MRK1/RAF48, BHP/RAF27, RAF38, and the kinase domains of RAF13 (aa 492-775) and RAF14 (aa 518-781) were incubated with “kinase-dead” forms of SnRK2.6 with or without Ser to Ala mutations at Ser171 and Ser175 in reaction buffer (25 mM Tris HCl, pH 7.4, 12 mM $MgCl_2$, 2 mM DTT), with 1 μM ATP plus 1 μCi of [$\gamma$-$^{32}$P] ATP for 30 min at 30°C. Reactions were stopped by boiling in SDS

sample buffer and proteins were separated by 10% SDS-PAGE. Primers used in RAF construction are listed in Supplementary Dataset 10.

For the dephosphorylation assay, SnRK2.6$^{M94G}$ coated on Glutathione Sepharose (Cytiva) were dephosphorylated with Lambda Protein Phosphatase (λPP) for 30 min and the λPP was removed by washing three times with protein buffer (25 mM Tris HCl, pH 7.4, 150 mM NaCl). To detect the effects of RAF15 on SnRK2.6$^{M94G}$ thiophosphorylation and activity, recombinant GST-RAF15-KD (aa 548-809) was incubated with pre-dephosphorylated SnRK2.6$^{M94G}$ for 30 min in reaction buffer (25 mM Tris HCl, pH 7.4, 12 mM $MgCl_2$, 2mM $MnCl_2$, 0.5 mM DTT, 50 μM ATP, 50 μM $N^6$-Benzyl-ATPγS). Then ABF2 was added to the reaction and incubated for an additional 30 min. This phosphorylation reaction was stopped by adding EDTA to a final concentration of 25 μM. A final concentration of 2.5 mM p-nitrobenzyl mesylate (Abcam, ab138910) was added to proceed the alkylating reaction for 1 h at room temperature. Samples with SDS sample buffer were boiled and separated by SDS-PAGE, transferred to Polyvinylidene fluoride (PVDF) membrane, and immunoblotted with antibodies against thiophosphate ester (Abcam, ab92570).

**Protoplast isolation and transactivation assay**

Protoplast isolation and transactivation assays were performed as previously described (Wang et al., 2018). Briefly, protoplasts were isolated from leaves of 4-week-old plants grown under a short photoperiod (10 h light at 23°C/14 h dark at 20°C). Leaf strips were excised from the middle parts of young rosette leaves, dipped in an enzyme solution containing cellulase R10 (Yakult Pharmaceutical

Industry) and 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 flow-through was centrifuged at 100 *g* for 2 min to pellet the protoplasts. Protoplasts were resuspended in W5 solution and incubated for 30 min. 100 µL of protoplasts suspended in MMG solution (4 mM MES, pH 5.7, 0.4 M mannitol, and 15 mM $MgCl_2$) were mixed with the plasmid mix and added to 110 µL PEG solution (40% w/v PEG-4000, 0.2 M mannitol, and 100 mM $CaCl_2$). The transfection mixture was mixed completely by gently tapping the tube followed by incubation at room temperature for 5 min. The protoplasts were washed twice with 1 mL W5 solution. The *RD29B-LUC* (7 µg of plasmid per transfection) and *ZmUBQ-GUS* (1 µg per transfection) were used as an ABA-responsive reporter gene and as an internal control, respectively. For wild type and mutated *RAF*, *SnRK2.6* plasmids, 3 µg per transfection were used. After transfection, protoplasts were incubated for 5 h under light in washing and incubation solution (0.5 M mannitol, 20 mM KCl, 4 mM MES, pH 5.7) with or without 5 µM ABA. The mutations were introduced into wild type RAFs using the primers listed in Supplementary Dataset 10.

**Co-immunoprecipitation (co-IP) assay.**

For co-IP assays in tobacco, *pCambia1300-35S::SnRK2.6-flag* and *pCambia1300-35S::RAF15-eGFP* or *pCambia1300-35S::eGFP* were transferred into tobacco leaves through agrobacterium-mediated infiltration. Two days after

infiltration, tissue was ground and proteins were extracted in 1:2 (w/v) IP buffer (20 mM Tris-Cl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 2 mM DTT, 10% [v/v] Glycerol, 0.5% [v/v] Triton X-100 and protease inhibitor cocktail) for 30 min in liquid nitrogen. Cell debris was removed via centrifugation and 100 μL supernatant was collected for input controls. The remaining supernatant was incubated with Anti-GFP Magarose beads (Smart-Lifesciences, Cat. No.SM038001) for 2 h at 4 °C and washed three times in wash buffer (20 mM Tris-HCl, pH 7.5, 5 mM NaCl, 0.1% [v/v] Triton X-100) and twice with 50mM Tris-HCl, pH 7.5. Immunoprecipitated proteins and input proteins were analyzed by immunoblots using anti-GFP (1:5000; TaKaRa, JL-8) and anti-flag (1:5000; Abmart, M20008M) antibodies.

**Split luciferase (LUC) complementation assay.**

The coding sequence of *RAF15* was amplified and transferred to *pEarley-nLUC/cLUC* vectors. Split-LUC complementation assay was performed by transient expression in tobacco leaves through agrobacterium-mediated infiltration. Two days after infiltration, luciferase activity was detected with a CCD camera by applying firefly D-luciferin (NanoLight).

**Metabolomics analysis.**

Around 100,000 GCP and 100,000 MCP were suspended in 500 μL of extraction solution (90% methanol). After ultrasonic homogenization for 2 min and vortexing for 10 min, the samples were kept in the dark for 16 h, dried under vacuum, resuspended in 50 μL 40% methanol in water, and centrifuged at 15,000 × g for

30 min, 4 °C. The clear supernatants were loaded into injection vials and ready for UHPLC-MS/MS.

For UHPLC-MS/MS assay, the vanquish-flex UHPLC system was coupled to Q Exactive Plus (Thermo Fisher Scientific) for metabolite separation and detection. A Hypersil GOLD column (2.1×100 mm.1.9 um; Thermo Fisher Scientific) was used for compound separation at 30 °C, and 1 μl of sample was loaded. The mobile phase A was HPLC grade H2O with 0.1% (v/v) FA and phase B was HPLC grade ACN. The gradient elution conditions were set as follows: from 0 to 2 min, the mobile phase B increased to 10%; from 2 to 10 min, the mobile phase B increased to 50%; from 10 to 10.1 min the mobile phase B increased to 80%; from 10.1 to 13 min, the mobile phase B was kept at 80%; from 13 to 14 min the mobile phase B increased to 95%; from 14-18 min the mobile phase B decreased to 10%. The flow rate was 0.3 mL/min.The MS data acquisition was performed in the full scan MS/ddMS2 mode. The resolutions of full scan MS and ddMS2 were set at 70,000 and 17,500, respectively. The automatic gain control (AGC) target and maximum injection time in full scan MS settings were 1e6 and 100 ms, while their values were 2e5 and 50 ms in ddMS2 settings. The TopN (N, the number of top most abundant ions for fragmentation) was set to 8, and collision energy was set to 20%, 40% and 60%. A heated ESI source was used at positive and negative ion mode. The spray voltage was set as 3.5 KV for positive mode and 3.2 KV for negative mode; the capillary temperature and aux gas heater temperature were set as 320 and 350 °C,

respectively. Sheath gas and aux gas flow rate were set at 35 and 15 (in arbitrary units), respectively. The S-lens RF level was 50.

Raw MS files were converted to the universal mzxml format by the RawConverter software (v1.2.0.1), and peaks were called and quantified with the XCMS-CAMERA pipeline in the R environment as previously described (He et al., 2015; Zhou et al., 2019). Based on examination of the total ion chromatograms, peaks detected prior to 2.5 minutes or later than 11 minutes were discarded due to poor separation. Peaks annotated as likely isotopes were also precluded from subsequent comparative analysis. Mass features were compared between GCP and MCP with unpaired student's *t*-tests. Co-eluting mass features (difference in retention time < 0.02 minutes) were combined and confirmed to be fragments of the same parental ion by examining the mass spectra at the selected range of retention time. At least three high abundance *m/z* values were used as queries to search in the ReSpect for Phytochemical database (Sawada et al., 2012). Three compounds were identified based on more than three matching fragments including the parental ion, and four additional compounds were putatively annotated based on consistent parental ion and an additional matching daughter ion.

**Accession numbers**

Genome Initiative database under the following accession numbers: *OSKL3/SnRK2.2* (AT3G50500), *OSKL2/SnRK2.3* (AT5G66880), *SnRK2.4* (AT1G10940), *OST1/SnRK2.6* (AT4G33950), *RAF1/CTR1* (AT5G03730), *RAF2/M3Kδ3/EDR1* (AT1G08720), *RAF3/M3Kδ1* (AT5G11850), *RAF4/M3Kδ7*

(AT1G18160), *RAF5/M3Kδ6/SIS8* (AT1G73660), *RAF6/M3Kδ5* (AT4G24480), *RAF7* (AT3G06620), *RAF8* (AT3G06630), *RAF9* (AT3G06640), *RAF10* (AT5G49470), *RAF13/M3Kθ1* (AT2G31010), *RAF14/M3Kθ2* (AT2G42640), *RAF15* (AT3G58640), *RAF16* (AT1G04700), *RAF40/HCR1* (AT3G24715), *RAF24* (AT2G35050), *RAF18* (AT1G16270), *RAF20* (AT1G 79570), *RAF35* (AT5G57610), *RAF42* (AT3G46920), *BHP/RAF27* (AT4G18950), *HT1/RAF19* (AT1G62400), *RAF28* (AT4G31170), *CBC2/RAF33* (AT5G50000), *CBC1/RAF38* (AT3G01490), *MRK1/RAF48* (AT3G63260), *PYR1* (AT4G17870), *PYL1* (AT5G46790), *PYL2/RCAR14* (AT2G26040), *PYL4* (AT2G38310). *GC1* (AT1G22690), *FAMA* (AT3G24140), *ICE1*(AT3G26744), *NRPB3* (AT2G15430), *SCAP1* (AT5G65590), *MYB60* (AT1G08810), *SPCH* (AT5G53210), *MUTE* (AT3G06120), *MUS* (AT1G75640), *SCD1* (AT1G49040), *PHYB* (AT2G18790), *BCA4* (AT1G70410), *CRY3* (AT5G24850), *PHOT2* (AT5G58140), *BLUS1* (AT4G14480), *BAM1* (AT3G23920), *ABCB14* (AT1G28010), *MPK6* (AT2G43790), *ABI1* (AT2G26080), *ABI2* (AT5G57050), *ABF2* (AT1G45249), *RAF11* (AT1G67890), *MAPKKK7* (AT3G13530), *GTL* (AT1G33240), *GPI8* (AT1G08750), *SDD1* (AT1G04110), *TMM* (AT1G80080), *YODA* (AT1G63700), *MKK4* (AT1G51660), *MKK5* (AT3G21220), *MPK3* (AT3G45640), *AEL2* (AT3G13670), *KAB1* (AT1G04690), *KAT2* (AT4G18290), *ABCG14* (AT1G28010), *BIM1* (AT5G08130), *AEL2* (AT3G13670), *ABI2* ( AT5G57050), *SnRK2.1* (AT5G08590), *SnRK2.10* (AT1G60940), *FLS1* (AT5G08640), *F3H* (AT3G51240), *TT5* (AT3G55120), CHIL (AT5G05270), *FFT* (AT4G25640), AT4G16330, *UGT78D2* (AT5G17050), *TT15* (AT1G43620), *RPN12A* (AT1G64520),*TT7*

(AT5G07990), *UGT89C1* (AT1G06000), *CIP7* ( AT4G27430), *RHM1* (AT1G78570), *EBS1* (AT1G71220).

**General statistical analysis**

Statistical significance of relative luciferase activity or gene expression was examined by Student's *t* test (*p < 0.05, **p < 0.01, ***p < 0.001). The association between protein abundance from different proteomics studies was examined by Pearson's correlation analysis.

**Data availability**

The proteomics data were deposited to the PXD039858/PXD041345 for ProteomeXchange and JPST002022 and JPST002118 for jPOST (https://repository.jpostdb.org/preview/130037734563ddfd8fae6f9, access key: 4097; https://repository.jpostdb.org/preview/1014894330642d434879571, access key: 2721). The metabolomics data were deposited to the MetaboLights study: MTBLS3997 (www.ebi.ac.uk/metabolights/MTBLS3997).

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**CONFLICTS OF INTEREST**

The authors declare that they have no conflicts of interest associated with this work.

## AUTHOR CONTRIBUTIONS

P.W. designed the research. H.W., Y.W., Z.L., R.L., W.R., S.X., and B.Z. performed research. H.W., T.S., R.L., X.W., X.Z., S.Z., S.D., H.H., C.-P.S., and P.W. performed data analysis. H.W., R.L., S.Z., H.H., C.-P.S., and P.W. wrote the manuscript. All authors read and approved of the manuscript

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## Figure legends

### Figure 1. The cell-sorting-based nano-scale pipeline for isolation and proteomic analysis of guard cell protoplasts

**(A)** Workflow of the cell-sorting-based nanoscale 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)** Venn diagram showing the overlap between the proteins identified by our study and previous GC proteomics. **(C)** Quantitative comparison of relative abundance of common proteins identified by Geilfus et al., 2018 (PXD009918) and our study. The protein abundance was normalized by median Z-score. Each dot represents a common protein. Blue dots indicate proteins with higher abundance in Geilfus et al., 2018 (FC > 2, $P < 0.05$, two-tailed *t* test) than in this study. Red dots indicate proteins with lower abundance in Geilfus et al., 2018 (FC < 0.5, $P < 0.05$, two-tailed *t* test) than in this study. Pearson correlation test shows the correlation between the two studies. **(D)** Proteins known to function in GC development are present in the GCP proteome. MMC, meristemoid mother cell; GMC, guard

mother cell. Yellow circles highlight proteins detected in GCP proteomics, while grey circles indicate proteins not detected. **(E)** Proteins known to function in the regulation of GC intensity are present in the GCP proteome. Yellow circles highlight proteins detected in GCP proteomics. **(F)** Proteins known to function in stomatal movement in response to ABA, $CO_2$ and blue light (BL) are present in the GCP proteome.

**Figure 2. Quantitative comparison of GCP and MCP proteomes**

**(A)** Venn diagrams showing the overlap between the proteins and peptides identified from GCP and MCP proteomes. **(B)** Volcano plot of differentially enriched proteins in the GCP and MCP proteomes. On the y-axis, the negative log10 values are plotted. On the x-axis, the log2 values of the fold change are seen in the comparison. **(C)** Heatmap showing the quantitative comparison of proteins in GCP and MCP proteomes. **(D)** Cnetplot generated by ClusterProfiler indicating proteins associated with enriched KEGG pathways; dots in light brown represent KEGG pathways, with the size of dots determined by the number of proteins belonging to the pathway. Small dots of the same size represent proteins; the dot color is determined by fold changes of protein abundance of GCP/MCP.

**Figure 3. GC-enriched B1 subgroup RAF15 phosphorylates SnRK2.6/OST1**

**(A)** Relative protein abundance of ABA receptor PYR1/PYLs, PP2C phosphatases, and SnRK2s in the GC and MC proteomes. **(B)** Relative protein abundance of B and C subgroup RAFs in the GC and MC proteomes. **(C)** Activation of the reporter gene by combinations of RAFs with SnRK2.6 in the protoplasts of *OK*$^{100}$*-nonu*. The ratio of *RD29B-LUC* expression in the protoplasts with 5 μM ABA relative to that without ABA treatment was used to indicate the activation activity of different RAFs on SnRK2.6. Error bars, SEM (n = 6 individual transfections). **(D)** Recombinant full-length RAF was used to phosphorylate SnRK2.6$^{KR}$ (SnRK2.6$^{K50R}$, a kinase-dead form of SnRK2.6), in the presence of [γ-$^{32}$p]ATP. Autoradiograph (upper) and Coomassie staining (bottom) show phosphorylation and loading, respectively, of purified GST-HT1/RAF19, GST-MRK1/RAF48, GST-BHP/RAF27, GST-RAF38, HIS-RAF15, and HIS-SnRK2.6$^{KR}$. **(E)** The MS/MS spectrum showing the $^{18}$O-phosphopeptide contains the phosphoserine Ser175 in SnRK2.6. **(F)** Ser175Ala mutation abolished RAF15-mediated phosphorylation of SnRK2.6$^{KR}$. **(G)** Recombinant full-length RAF15 was used to phosphorylate SnRK2.6$^{K50R}$, SnRK2.1$^{K33R}$, SnRK2.4$^{K33R}$, SnRK2.10$^{K33R}$, the kinase-dead SnRK2.6/1/4/10, in the presence of [γ-$^{32}$p]ATP. Autoradiograph (upper) and Coomassie staining (bottom) show phosphorylation and loading, respectively, of purified RAF15 and SnRK2s.

**Figure 4. RAF15 is essential for ABA-induced SnRK2.6/OST1 activation in GC**

**(A)** RAF15-KD triggers the autophosphorylation of pre-dephosphorylated GST-SnRK2.6$^{M94G}$ (de-SnRK2.6$^{M94G}$) at the indicated time points. Anti γ-S immunoblot

(upper) and Coomassie staining (lower) show thiophosphorylation and loading, respectively, of recombinant GST-RAF15-KD and GST-SnRK2.6$^{M94G}$. **(B)** RAF15-KD activates pre-dephosphorylated GST-SnRK2.6$^{M94G}$ (de-SnRK2.6$^{M94G}$) and the reactivated SnRK2.6$^{M94G}$ phosphorylates itself and ABF2. Anti γ-S immunoblot (left) and Coomassie staining (right) show thiophosphorylation and loading, respectively, of recombinant GST-RAF15-KD, GST-SnRK2.6$^{M94G}$, and GST-ABF2. Images shown are representative of at least two independent experiments. **(C)** RAF13-KD, but not HT1, triggers the autophosphorylation of pre-dephosphorylated GST-SnRK2.6$^{M94G}$ (de-SnRK2.6$^{M94G}$). Anti γ-S immunoblot (upper) and Coomassie staining (lower) show thiophosphorylation and loading, respectively, of recombinant GST-RAF13-KD, GST-HT1/RAF19-KD and GST-SnRK2.6$^{M94G}$. **(D)** Time-resolved stomatal conductance in response to different concentration of ABA in Col-0 and *raf15* mutants. Error bars, SEM (n = 3 leaves). Data represent one of two independent sets of experiments. **(E)** The sensitivity of *raf15* mutants to different concentration of ABA. **(F)** Time-resolved stomatal conductance in response to sorbitol in Col-0 and *raf15* mutants. Error bars, SEM (n = 3 leaves). Data represent one of two independent sets of experiments. **(G)** co-immunoprecipitation assay shows the interaction between SnRK2.6-Flag and RAF15-GFP. **(H)** In-gel kinase assay showing the SnRK2 and OK$^{100}$ activities after the indicated time of treatment with 50 μM ABA in the leave epidermal fragments of Col-0 and *raf15* mutants. The anti-SnRK26 antibody were used to show the amount of SnRK2.6 in each sample. Asterisk and arrow indicate the non-induced band that could be used as a loading control. Images shown are

representative of two independent sets of experiments. **(I)** Recombinant GST-RAF13-KD was used to phosphorylate wild type and mutated SnRK2.6$^{KR}$ in the presence of [γ-$^{32}$p]ATP. Autoradiograph (upper) and Coomassie staining (bottom) show phosphorylation and loading, respectively, of purified GST-RAF13-KD and HIS-SnRK2.6$^{KR}$. **(J)** Model showing the RAF6 and RAF15 that mediate ABA and stomatal vapor pressure difference (VPD) signaling by phosphorylating SnRK2.6/OST1 in GC.

**Figure. 5. Flavonoid compounds specifically accumulate in GCPs**

**(A)** Relative abundance of proteins involved in flavonoid biosynthesis and metabolism in the proteomes of GCPs and MCPs. **(B)** Overlay of total ion chromatograms of GCP and MCP, with three GCP-specific peaks labeled by retention time. **(C), (E), (G)** The tandem MS spectra of the three GCP-specific peaks at 6.13 min (**C**), 6.90 min (**E**), and 7.43 min (**G**) and their corresponding representative extracted ion chromatogram at the parental ion channel (blue = GCP, yellow = MCP as in panel **A**). (**E), (F), (H)** Quantitative comparison of the three compounds between GCP and MCP. **(I)** A model showing the enzymes and metabolites in the flavonoid biosynthetic and metabolism processes enriched in GCPs. Blue squares and bolded letters showed the proteins and metabolites identified in our proteomics and metabolomes.

**SUPPORTING INFORMATION**

**Figure S1**. The cell-sorting of guard cell protoplasts

**Figure S2.** GO enrichment analysis of proteins enriched in GCPs and MCPs

**Figure S3.** Quantitative proteomics of epidermal peels before and after protoplasting

**Figure S4.** The relative expression of RAF genes in guard cells

**Figure S5.** HT1/RAF19 and RAF24 phosphorylate SnRK2s *in vitro*

**Figure S6.** RAF15 and RAF13 in B1 subgroup phosphorylate SnRK2.6/OST1 *in vitro*

**Figure S7.** Flavonoid compounds specifically accumulate in GCPs

**Figure S8.** Flavonoid compounds specifically accumulate in GCPs

**Dataset S1.** All peptides identified in GCP

**Dataset S2.** This proteomics study compared with previous studies

**Dataset S3.** Quantitative comparison between GCP and intact GC proteomics

**Dataset S4.** All peptides identified in MCP

**Dataset S5.** Quantitative data of proteins identified in GCP and MCP

**Dataset S6.** GCP and MCP enriched proteins

**Dataset S7.** GO and KEGG analysis of GCP and MCP.

**Dataset S8.** Quantitative proteomics of epidermal peels before and after protoplasting

**Dataset S9.** Quantification and comparative analysis statistics of filtered mass features from untargeted metabolomics data

**Dataset S10.** Sequences of primers used in this study