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Functional Plant Biology

Single-cell-type transcriptomic analysis reveals distinct gene expression profiles in wheat guard cells in response to abscisic acid

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ABSTRACT

Stomatal closure, driven by shrinking guard cells in response to the accumulation of abscisic acid (ABA) under drought stress, has a great impact on plant growth and environmental acclimation. However, the molecular regulatory mechanism underlying the turgor alteration of guard cells remains elusive, especially in cereal grasses. Here, we develop a modified enzyme digestion-based approach for the isolation of wheat (*Triticum aestivum* L.) guard cells. With this approach, we can remove mesophyll, pavement cells and subsidiary cells successively from the epidermis of the trichomeless coleoptile in wheat and preserve guard cells on the cuticle layers in an intact and physiologically active conditions. Using a robust single-cell-type RNA sequencing analysis, we discovered 9829 differentially expressed genes (DEGs) as significantly up- or down-regulated in guard cells in response to ABA treatment. Transcriptome analysis revealed a large percent of DEGs encoding multiple phytohormone signalling pathways, transporters, calcium signalling components, protein kinases and other ABA signalling-related proteins, which are primarily involved in key signalling pathways in ABA-regulated stomatal control and stress response. Our findings provide valuable resource for investigating the transcriptional regulatory mechanism underlying wheat guard cells in response to ABA.

Keywords: abscisic acid, gene expression, guard cells isolation, phytohormone signaling pathways, RNA sequencing, stomatal signalling pathways, stress response, *Triticum aestivum* L.

Introduction

Common wheat (*Triticum aestivum* L.; $2n = 6x = 42$, AABBDD) is one of the most important cereal crops for human diets in the world. However, abiotic stress, especially drought stress has negatively impacts wheat growth. As specialised structures on the surface of plant, stomata function as gateways to control water loss and CO_2 influx, and play essential roles in plant growth and acclimation to environmental stresses (Hetherington and Woodward 2003; Hedrich and Shabala 2018). Stomata are each surrounded by a pair of kidney-shaped guard cells in many dicot plants. In contrast, the mature stomata in grasses, especially agronomically important cereals, are composed of two dumb bell-shaped guard cells and two subsidiary cells on the outside, which have enabled a fast stomatal response to adapt to the changing climate (Chen *et al.* 2017; Schäfer *et al.* 2018; Nunes *et al.* 2020; Wang and Chen 2020). In response to environmental and endogenous stimuli, guard cells need to integrate multiple signals to ensure appropriate stomatal response for plant survival in an ever-changing environment. This unique plant cell type represents a dynamic cellular model system for deciphering cell signalling mechanisms and plant stress response (Yoshida *et al.* 2019). Importantly, the plant hormone abscisic acid (ABA) induces stomatal closure via an intricate intracellular signalling network in guard cells, thereby promoting plant water conservation (Chen *et al.* 2020).

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The ABA signalling pathway in guard cells has been well documented in the model dicot *Arabidopsis thaliana* L. (Munemasa *et al.* 2015). Calcium, ion channels, membrane trafficking components, protein kinases, protein phosphatases and transcription factors have been shown to play key roles in ABA signalling in guard cells (Schroeder *et al.* 2001; Hsu *et al.* 2021). Using RNA sequencing (RNA-Seq) methodology, single-cell-type transcriptomes contribute toward an understanding of the unique gene expression characteristics of different plant cell types (Libault *et al.* 2017 and references therein). For example, Wang *et al.* (2011) revealed common and unique elements of ABA-regulation of gene expression in guard cells based on data from global transcriptomes of *Arabidopsis* guard cells generated with Affymetrix ATH1 microarrays. However, despite being the most important food source in the world, our molecular knowledge concerning guard cell function of graminaceous species is still minimal (Chen *et al.* 2017; Raissig *et al.* 2017). In particular, little is known about the transcriptome and ABA signalling of wheat guard cells.

Several approaches have been developed to isolate dicot guard cells for omics analysis including protoplasting (Pandey *et al.* 2002; Leonhardt *et al.* 2004; Zhao *et al.* 2019), manual cell dissection (Bates *et al.* 2012), mechanical cell isolation (Bauer *et al.* 2013; Rasouli *et al.* 2020), laser-capture microdissection (Aubry *et al.* 2016), protoplasting and fluorescence activated cell sorting (Lee *et al.* 2019) with each system having its own advantages and disadvantages. In contrast to the kidney-shaped guard cells typical of the dicots, grass guard cells intimately connect to their neighbouring subsidiary cells. It is difficult to physically isolate intact guard cells without damaging their cell walls and associated components. Therefore, isolation of high purity and physiologically active guard cells presents challenges to study the molecular mechanism underpinning the regulation of grass guard cells.

Here, we developed a modified method for isolating and purifying guard cells from coleoptile epidermal peels to eliminate the contaminants from subsidiary cells and pavement cells, and single-cell-type RNA-Seq protocol in wheat, a major cereal crop. We generated the comprehensive transcriptome profiles of wheat guard cells in response to exogenous ABA using Illumina RNA-Seq and identified differentially expressed genes (DEGs) in response to ABA. Our study provides a new isolation approach for grass guard cells and a gene expression map of the wheat guard cells at single-cell-type resolution.

Materials and methods

Plant materials and treatments

Common wheat (*T. aestivum* L. cv. Yu 18) was grown in a growth chamber under a controlled condition, as previously described (Wang *et al.* 2014). For ABA treatment, 4–5-day-old wheat plants were sprayed with 20 μM ABA (Sigma–Aldrich, St Louis, MO, USA) solution (0.1% v/v ethanol) for 4 h. Mock treatment was conducted by spraying only 0.1% ethanol. The coleoptiles were sampled for guard cells isolation. The stomata of leaves and coleoptiles were investigated with a scanning electron microscope (TM3030Plus, Hitachi, Japan).

Stomatal aperture assays

Stomatal bioassays experiments were carried out as previously described (Shen *et al.* 2015) with some modification. The epidermal strips peeled from leaves and coleoptiles were incubated in 10 mL opening buffer (10 mM MES/KOH, 50 mM KCl; pH 6.15) for 2.5 h to induce stomatal opening. Samples were then transferred to the opening buffer containing appropriate concentrations of ABA and incubated for another 2.5 h. Stomatal apertures were measured using an inverted microscope (Eclipse Ti-U; Nikon Corporations, Tokyo, Japan).

Guard cells isolation and assessment of cell viability

Epidermal strips were peeled carefully from the coleoptiles and immediately incubated at 25°C in 10 mL RNase-free enzyme solution containing 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 μM KH_2PO_4, 1.5% cellulase R-10 (Yakult, Tokyo, Japan), 0.5% macerozyme R-10 (Yakult), 1KU RNase inhibitor (Sangon Biotech, Shanghai, China), 33 mg L^{-1} actinomycin D (Sigma-Aldrich), 100 mg L^{-1} cordycepin (Sigma-Aldrich), 5 mM MES, pH 5.5. The osmolarity of the enzyme solution was adjusted to 500 mmol kg^{-1} by the addition of D-sorbitol (Leonhardt *et al.* 2004). During 1.5 h of incubation, mesophyll, pavement cells and subsidiary cells released successively from epidermal strips except for guard cells, which remained on the surface of the thin cuticle layer. The guard cells were collected and observed under a LEICA DM4000 B fluorescent microscope (Nikon Corporations) equipped with a digital camera. The viability of guard cells was performed by fluorescence measurements with indicator dye fluorescein diacetate (FDA) (Sigma-Aldrich). The treated peels were placed into Tris-KCl buffer (10 mM Tris and 50 mM KCl, pH 7.2) containing 10 μg mL^{-1} FDA. The dye hydrolysis was observed after mixing guard cells with the incubation mixture for 10 min in the dark at 25°C. Guard cells were viewed and imaged using a LEICA DM4000 B fluorescence microscope. In each experiment, three epidermal strips were measured, each of which originated from a different coleoptile. The selected images represented the same results from approximately nine time measurements.

RNA extraction and sequencing

Coleoptile samples from 50 seedlings were pooled as one biological repeat. Three biological repeats were performed. Total RNA was extracted from guard cells using RNAqueous-Micro Total RNA Isolation kit (AMBION, ThermoFischer) according to the manufacturer's instructions. Briefly, cells were lysed with the PLANT RNA ISOLATION AID. Subsequently, samples were solubilised in a powerful chaotropic solution and RNA was purified on a glass fibre filter cartridge specifically designed for very small samples. Residual genomic DNA was efficiently removed using the DNA-free system included in the kit. The RNA was examined with the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). GC (guard cell samples) and GC + ABA (guard cell samples with ABA treatment) samples were collected for RNA-Seq. The SMART-Seq ver. 4 Ultra Low Input RNA Kit for Sequencing (Takara, Japan) was used to generate high-quality cDNA. The cDNA library was constructed according to Illumina instructions and the sequencing was conducted on an Illumina Hiseq platform using 150 bp paired-end sequencing.

Differential expression analysis and functional annotation

To obtain clean reads, raw reads and reads with only the adaptors were screened to remove low-quality reads. Clean reads were then mapped to the *T. aestivum* reference genome (http://plants.ensembl.org/Triticum_aestivum) using TopHat ver. 2.0.12 (Trapnell *et al.* 2009). DEGs were defined with the DESeq2 (1.10.1) based on $|\text{Log}_2\ \text{FoldChange}| > 1$ and *q*-value (adjusted *P*-value) <0.005 (Wang *et al.* 2010). Expression levels were calculated using FPKM (fragments per kilobase of transcript sequence per million mapped reads) method (Trapnell *et al.* 2010), and genes with FPKM values larger than 1 were considered to be expressed. The functions of DEGs were annotated by BLAST search against Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Swiss-Prot and Non-redundant (NR) protein databases. Plant transcription factor were predicted using iTAK software and Plant Transcription Factor Database (http://planttfdb.cbi.pku.edu.cn/). The cluster analysis and expression pattern assessment were performed by the R analysis based on the FPKM value.

Quantitative reverse transcription PCR

cDNAs were synthesised using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative reverse transcription PCR (qRT-PCR) was performed on an ABI QuantStudio 3 Real-Time PCR System, using UltraSYBR Mixture (CWBio, Beijing, China). The reaction procedures were as follows: denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 60 s. According to the geNorm and NormFinder algorithms, gene with the lowest *M*-value and stability value was considered to be the most stable, whereas gene with the highest *M*-value and stability value was considered to be the least stable (Sinha *et al.* 2015*a*, 2015*b*). Therefore, the wheat *ACTIN* gene was confirmed to be the most suitable reference gene in this study and acted as internal control for normalisation (Supplementary Table S1, available at the journal website). The relative expression of the detected genes was calculated using the relative $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001). The gene-specific primers used in this study are listed in Table S2.

Results

Isolation and characterisation of wheat guard cells

Preliminary research in our laboratory showed that trichomes are typical protuberances in the epidermis of wheat leaves and difficult to digest by the enzymolysis method. We found that guard cells, subsidiary cells and stomatal response to ABA of coleoptiles and leaves are highly similar, while there are no trichomes in coleoptile (Fig. S1). In order to obtain high purity guard cells, we chose the coleoptiles over leaves as raw materials. Enzyme digestion was used to remove pavement cells and subsidiary cells from the epidermal peels. The results showed guard cells remain on the surface of cuticle layer after the releases of pavement cells, subsidiary cells and mesophyll from coleoptile epidermis, and viability staining results demonstrated the high physiological activity of guard cells at a purity >98% (Fig. 1). About 200 guard cells were collected from each coleoptile and one sequencing run normally included RNA from nearly 6000 cells. Considering that enzymatic digestion induces the expression of injury stress-associated genes, the transcription inhibitors actinomycin D and cordycepin were applied to inhibit the gene expression during guard cells isolation (Leonhardt *et al.* 2004; Wang *et al.* 2011). The expression of lipoxygenase (*TaLOX*), a wounding induced gene in wheat (Mauch *et al.* 1997), was significantly suppressed by transcriptional inhibitors (Fig. 2), suggesting that the addition of transcriptional inhibitors during isolation prevented the injury induction of gene expression by the enzyme digestion.

Transcriptomic analysis of ABA-induced gene expression in guard cells

To identify the genes involved in ABA responses in wheat guard cells, GC and GC + ABA were sequenced to detect the transcriptomic level of gene expression from guard cells of coleoptiles treated with or without ABA. The total number of filtered clean reads in each library was approximately 100–113 million and the average G-C content was 55.19%. The filtered clean reads were mapped

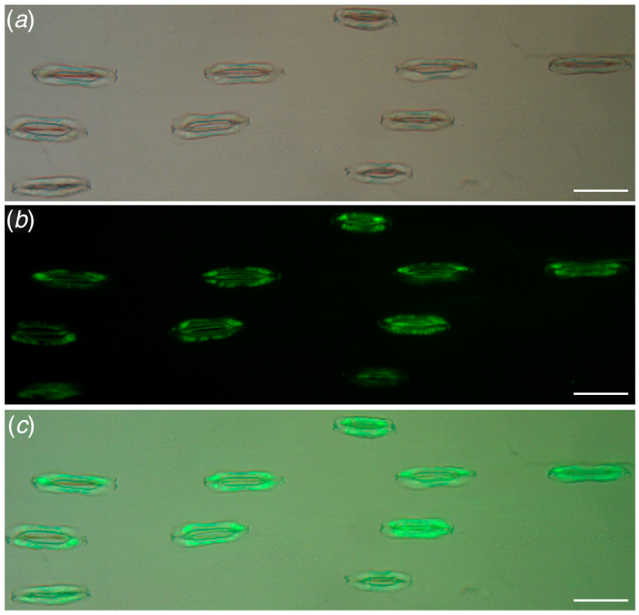

Fig. 1. Fluorescein diacetate viability staining of wheat guard cells. (*a*) Bright-field images. (*b*) Fluorescent images. (*c*) Merged images. Bar, 75 μM.

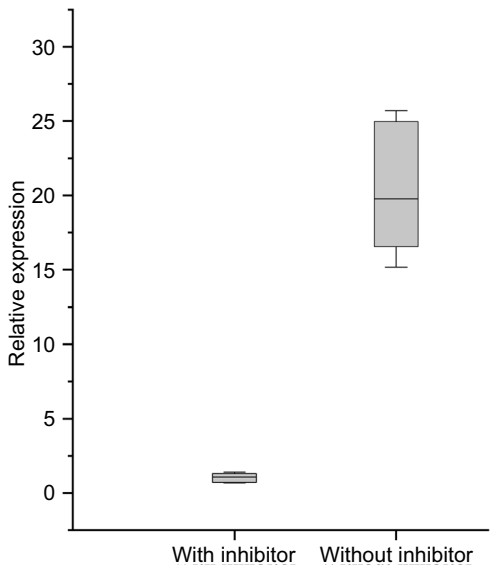

Fig. 2. Effect of transcription inhibitor on wound-responsive gene transcription during guard cells isolation. The relative expression values were calculated using the relative $2^{-\Delta\Delta C_T}$ method and expressed as fold changes, compared with those of *TaLOX* with inhibitor. The box indicates the 25th and 75th percentiles. A line across the box is depicted as the median. The bars represent 95% confidence interval and whiskers represent the maximum and minimum values.

to the *T. aestivum* reference genome (ver. 3.1). The average Q20 and Q30 of the two samples were approximately 94 and 90%, respectively (Table S3). These results indicate that all the data were qualified for downstream analysis. The expression of 9829 DEGs was significantly changed in ABA-treated guard cells compared with the untreated control. The detailed information of these DEGs is listed in File S1.

Validation of RNA-Seq data by qRT-PCR

To confirm the transcriptomic data, we selected 20 key DEGs involved in stomatal signalling and stress response for qRT-PCR analysis. The gene-specific primers were designed based on homoeologous sequence variation. Although there were quantitative differences between the two methods, the trends were consistent. The R^2 value of linear correlation analysis was 0.8776 (Fig. 3). Similar expression profiles were observed for all the selected genes as observed in RNA-Seq data, indicating the reproducibility and validity of our RNA-Seq data.

Categories of DEGs in guard cells under ABA treatment

All the DEGs were shown by heat map (Fig. 4*a*). Among them, 7989 genes were up-regulated and 1840 genes were down-regulated, thus implying that these DEGs might determine the specificity of guard cells responding to ABA. We noticed that a proportion of homeologous DEGs show contradictory expression patterns. For example, the *stress-activated protein kinases 9* (*SAPK9*) (TraesCS5A01G069500) was up-regulated, but another *SAPK9* (TraesCS5B01G075800) was down-regulated under ABA treatment. This was possibly due to differential contribution of homeologous DEGs to total gene expression levels in the three different genomes (A, B, and D) (Leach *et al.* 2014; Ramírez-González *et al.* 2018). Also, it may be a manifestation of transcriptome diversity caused by alternative splicing (Hu *et al.* 2020). GO enrichment analysis was carried out to further characterise the main biological functions of DEGs in wheat guard cells in response to ABA (Fig. 4*b*). The DEGs encoding the proteins involved in the metabolic process, biosynthetic process and single-organism process were highly represented in the biological process category. Within the cellular component category, the term of membrane was dominant. In the molecular function category, most DEGs were assigned to catalytic activity and oxidoreductase activity. To investigate the biological behaviours of the transcriptomic profiles, enrichment of DEGs in the KEGG pathway was analysed. Based on abundantly enriched DEG numbers, we listed the top 20 pathways. The KEGG annotations indicated that carbon metabolism and biosynthesis of amino acids were significantly enriched in response to ABA (Fig. 4*c*). Most notably, KEGG enrichment analyses of the identified DEGs indicated that the signalling pathways of most plant hormones including auxin, ABA, ethylene, brassinosteroid (BR), jasmonic acid

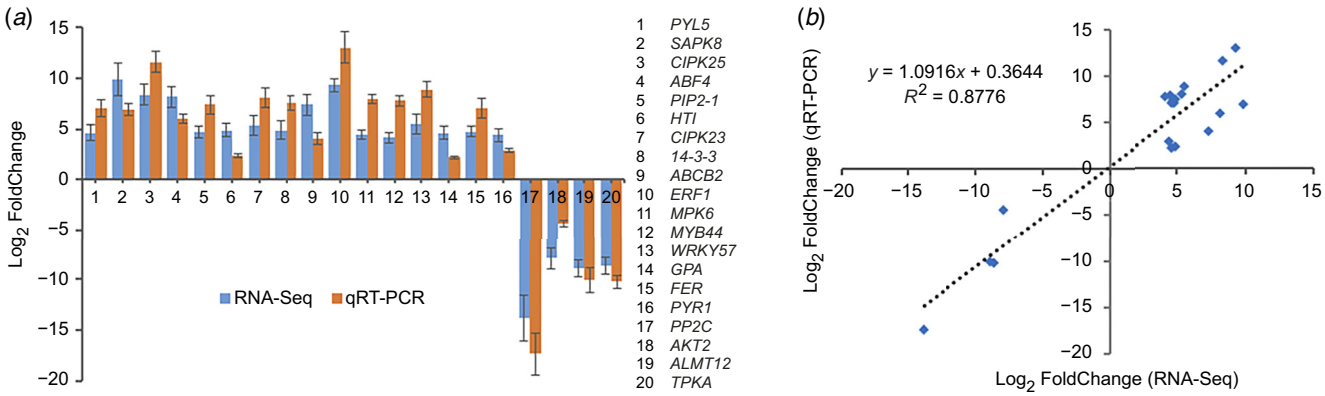

Fig. 3. Validation of transcriptomic data. (*a*) Comparison of the relative abundance of 20 transcripts as determined by qRT-PCR and RNA-Seq. Data are represented as means ± s.e. from three individual experiments, qRT-PCR was normalised by *Actin*. Details of the genes corresponding to 1–20 are shown in Table S1. (*b*) Correlation analysis of DEGs between RNA-Seq and qRT-PCR data. The scatter plot indicates the Log_2-transformed gene expression values in RNA-Seq and qRT-PCR.

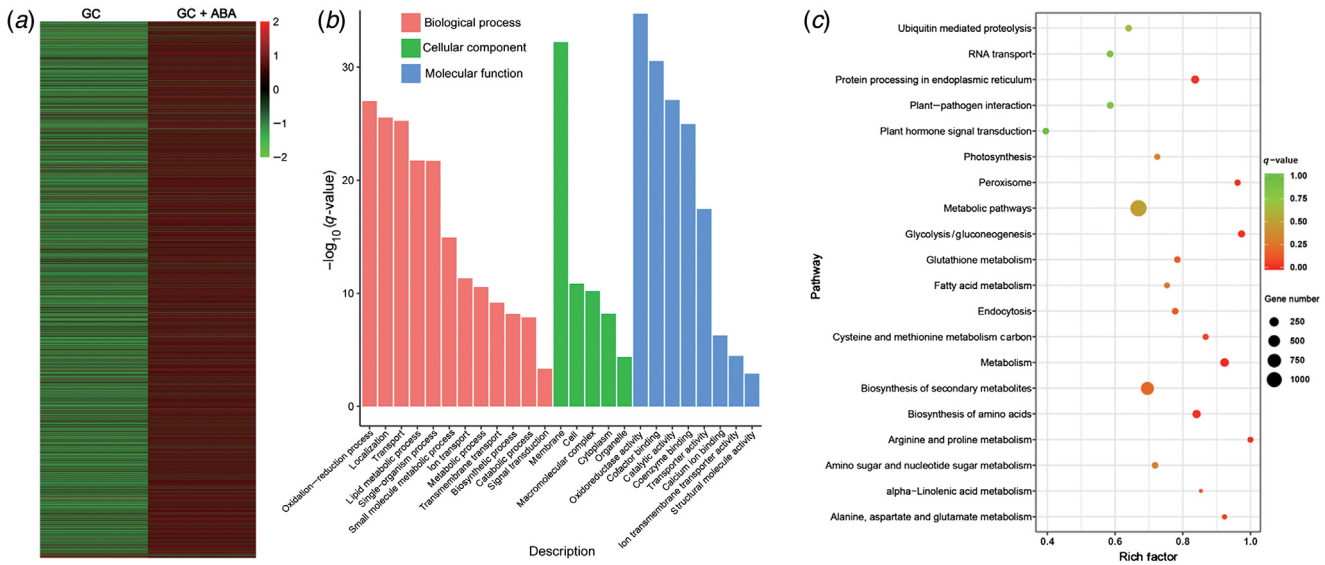

Fig. 4. Functional identification of differentially expressed genes (DEGs). (*a*) Hierarchical clustering analysis of DEGs between GC (guard cell samples) and GC + ABA (guard cell samples with ABA treatment). (*b*) GO enrichment analysis of DEGs. The 25 enriched GO terms in biological process, molecular function and cellular component branches are presented. All the adjusted statistically significant values (*q*-value) of the terms were negative 10-base log transformed. (*c*) KEGG pathway enrichment analysis of DEGs. Advanced bubble chart shows enrichment of differentially expressed genes in signalling pathways. *y*-axis label represents pathway, and *x*-axis label represents rich factor (rich factor = amount of differentially expressed genes enriched in the pathway/amount of all genes in background gene set). Size and colour of the bubble represent amount of differentially expressed genes enriched in the pathway and enrichment significance, respectively.

(JA) and salicylic acid (SA) were affected by ABA treatment (Fig. 5)

DEGs involved in multiple phytohormone signalling pathways

Under ABA treatment, not only DEGs involved in the ABA signalling pathway, but also DEGs involved in the hormone signalling pathways of JA, ethylene, BR, SA and auxin were identified in the wheat guard cell transcriptome (Fig. 6; File S2). Among the 56 DEGs involved in the core ABA signalling pathway, *zeaxanthin epoxidase 1* (*ZEP/ABA1*), *pyrabactin resistance1/PYR1-likes* (*PYR1/PYLs*), *2C-type protein phosphatases* (*PP2C*), *stress-activated protein kinases* (*SAPK*) and ABA activators, including *ABA-responsive element-binding factor* (*ABF4*), the *bZIP transcription factor*

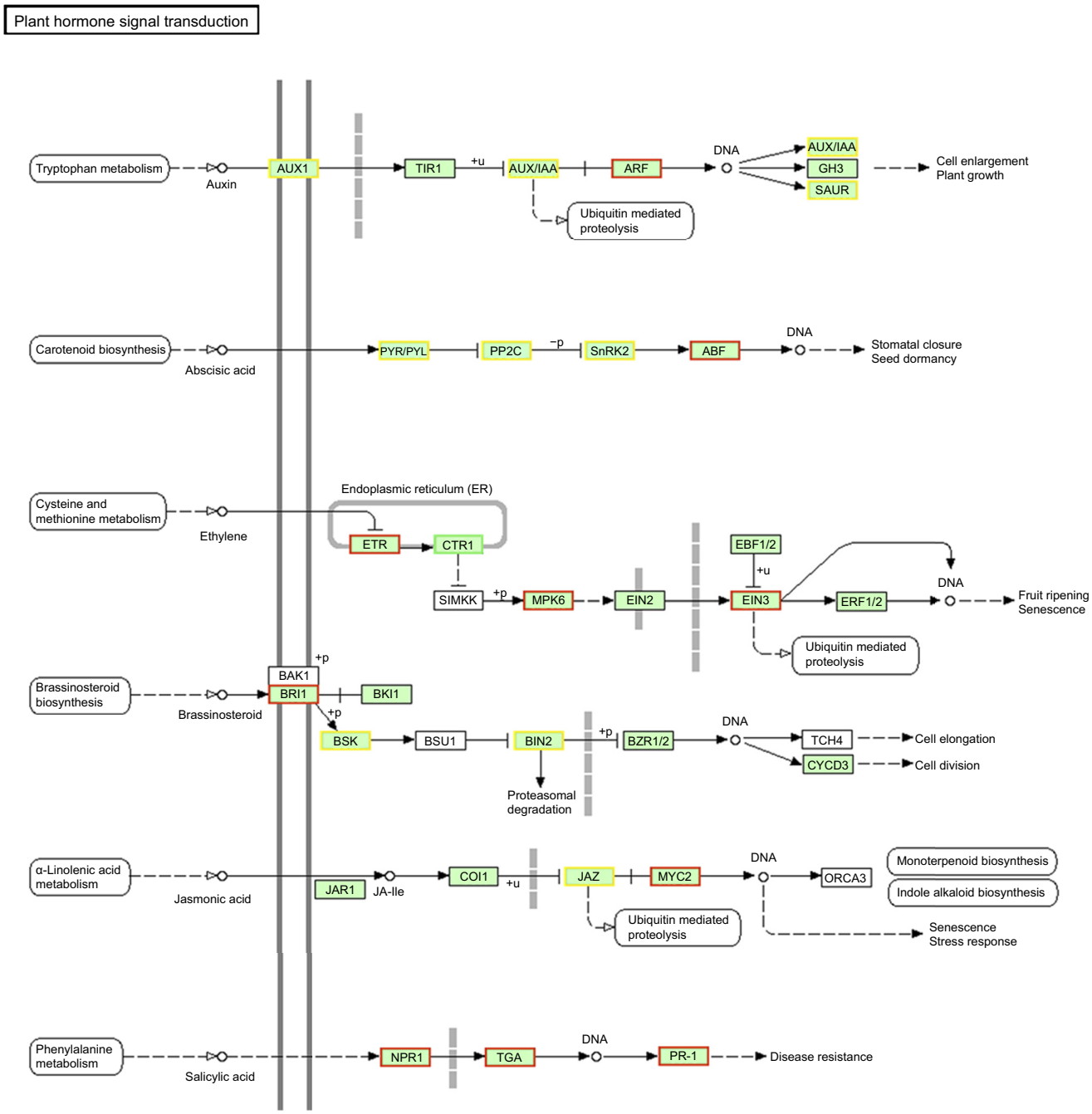

Fig. 5. KEGG pathway for plant hormone signal transduction in wheat guard cells under ABA treatment. Light green boxes represent wheat genes that have been previously identified, while white boxes represent genes that belong to the KEGG pathway, but have no currently identified in wheat until now. Light green box with black border represents non-differentially expressed genes. Light green box with red and green border represents up-regulated and down-regulated genes, respectively. Light green box with yellow border represents both up- and down-regulated genes.

(*TRAB1*) and *G-box-binding factor 4* (*GBF4*) were identified (Fig. 6*a*). Among the five ABA receptors in the wheat guard cell transcriptome, two *PYR/PYLs* (TraesCS2D01G087500 and 1A01G191700) were down-regulated. Under ABA treatment, 35 *PP2Cs* showed differences in their transcript abundance, with 29 up-regulated and six down-regulated genes. A total of 39 DEGs encoding protein involved in JA biosynthesis and signalling, such as *phospholipase A* (*PLA*), *allene oxide cyclase* (*AOC*), *allene oxide synthase* (*AOS*), *lipoxygenase* (*LOX*), *12-oxophytodienoate reductase* (*OPR*), β-oxidation *multifunctional protein* (*MFP*), *jasmonate-zim-domain protein* (*JAZ*) and bHLH transcription factor *MYC2*

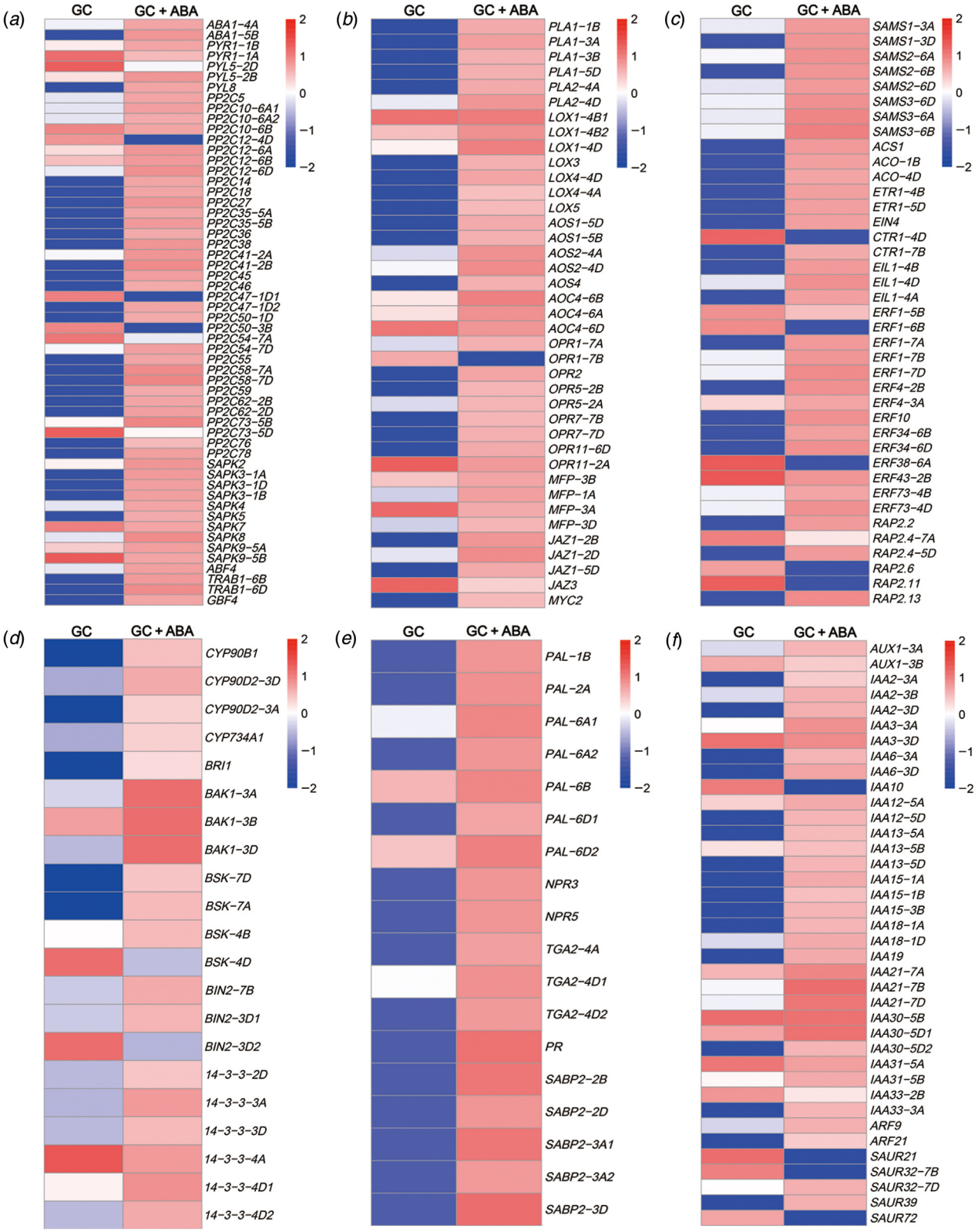

Fig. 6. Heatmap analysis of the DEGs involved in multiple plant hormone signalling pathways: (*a*) ABA, (*b*) jasmonic acid, (*c*) ethylene, (*d*) brassinosteroid, (e) salicylic acid and (*f*) auxin. The heatmap represents the normalised genes expression level measured in FPKM. The colours correspond to the value of FPKM, ranging from blue (low expression) to red (high expression). Detailed information of the DEGs is in File S2. GC, guard cell samples; GC + ABA, guard cell samples with ABA treatment.

were identified (Fig. 6*b*). Of them, three *JAZ1* were up-regulated and the *JAZ3* was down-regulated in response to ABA. We also identified DEGs encoding proteins in ethylene (39), BR (21), SA (18) and auxin (38) signalling pathways under ABA treatment, respectively (Fig. 6*c–f*). Especially, all of 18 DEGs related to SA signalling were up-regulated in guard cells in response to ABA (Fig. 6*e*).

Identification of genes involved in stomatal response to ABA

We identified 40 guard cell signalling homologous genes known to be involved in the regulation of stomatal behaviour in response to ABA (Table 1). Besides genes encoding core ABA signalling including *PYR1/PYLs*, *PP2C* and *sucrose nonfermenting 1-related protein kinase 2* (*SnRK2/SAPK*), we also discovered other key genes encoding proteins associated with kinases, transporters and ion channels involved in stomatal ABA signalling, such as *mitogen-activated protein kinases* (*MPK3*, *MPK6* and *MPK9*), *high leaf temperature protein 1*(*HT1*), *ABC transporter family member* (*ABCB2*, *ABCC4*), *potassium channels* (*AKT1*, *AKT2*, *TPKA*), *slow anion channel associated 1* (*SLAC1*) and *Aluminum-activated malate transporter 12* (*ALMT12*). It is notable that transcripts encoding transcription factors (TFs), such as *ethylene-responsive transcription factor* (*ERF1*), *MYB44* and *WRKY46*, were also found. It has been demonstrated that these TFs regulated stomatal movement and stress response in *Arabidopsis* (Jung *et al.* 2008; Cheng *et al.* 2013; Ding *et al.* 2014). Among the 40 genes involved in stomatal signalling pathway, *AKT2*, *ALMT12*, *PP2C50* and *TPKA* were down-regulated, and the others were up-regulated under ABA treatment.

Genes related to ABA signalling and stress response in guard cells

In addition to identifying core ABA signalling pathway genes, we also discovered a number of other ABA-responsive genes in wheat guard cells. A total of 346 DEGs encoding Ca^{2+} signalling (81), kinases (32), ion and water channels (53), transporters (120) and other stress-related proteins (60) were identified under ABA treatment. Specifically, there were 300 up-regulated and only 46 down-regulated DEGs (Fig. 7; File S3). Calcium signalling is important for ABA regulated abiotic stress responses. In this study, ABA-induced 81 DEGs encode Ca^{2+} and calmodulin signalling components including *calcium-dependent protein kinase* (*CDPK*), *calcineurin B-like protein-interacting protein kinase* (*CIPK*), *calcium-binding protein* (*CML*) and *cyclic nucleotide-gated ion channel* (*CNGC*) (Fig. 7*a*). The rest of the ABA-induced DEGs were related to diverse groups of transporters and ion channels relevant to stomatal regulation and stress response, such as genes encoding ATP-BINDING CASSETTE (ABC) transporters, aquaporins, CNGCs, potassium transporter and NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER (NRT1/PTR) family (Fig. 7*b*, *c*). Also, we identified 32 DEGs encoding kinase family, such as receptor-like protein kinase *FERONIA* (*FER*), *Mitogen-activated protein kinase kinase 1* (*MKK1*), MPKs and *Mitogen-activated protein kinase kinase kinase YODA* (*YDA*) (Fig. 7*d*). In addition, 60 genes expressed in the guard cells were found to be related to abiotic stress (File S3).

Transcription factors induced by ABA

TFs play essential roles in stress response by regulating the transcription of specific target genes (Nakashima *et al.* 2009). Extensive database searches of all the DEGs predicted a total of 391 DEGs encoding TFs with 319 up-regulated and 72 down-regulated in the wheat guard cell transcriptome under ABA treatment. These TFs belong to diverse TF protein families, mainly including AP2/ERF (26 transcripts), AUX/IAA (29), bHLH (19), bZIP (25), C2C2 (21), HB (24), MYB (26), NAC (27) and WRKY (26) (File S4). Among them, many TFs are involved in hormone, stomatal and stress response signalling pathways.

Discussion

Guard cells are one of the most popular cell types for studying plant signal transduction, cell function and stress responses. To reveal the unique gene expression characteristics of guard cell types, cell-specific transcriptomes have been examined in different plant species using microarray or RNA-Seq technologies (Leonhardt *et al.* 2004; Wang *et al.* 2011; Bates *et al.* 2012; Aubry *et al.* 2016; Lee *et al.* 2019). Guard cells are tightly connected to neighbouring cells, and sopotent methods are needed to obtain them. In particular, wheat stomata have a complex structure consisting of two dumb bell-shaped guard cells and two subsidiary cells of similar size (Fig. S1). In addition, trichomes possess thick cell walls that are hard to digest and extensively distributed on the surface of the wheat leaves. Therefore, the significant challenge is the isolation and separation of wheat guard cells from trichomes and subsidiary cells. Previous studies have isolated grass guard cell protoplasts for electrophysiological analysis, which is not suitable for transcriptome profiling due to limited quantity of cells (Fairley-Grenot and Assmann 1992). In the present study, we developed a modified enzyme-based approach to isolate guard cells from wheat and identified ABA-induced genes using single-cell-type RNA-Seq.

Previous studies have shown that epidermal peels can better preserve guard cells activity and integrity for studying their cellular signalling networks and omics (Chen *et al.* 2010; Pandey *et al.* 2010; Wang *et al.* 2011). In our study, we found that different cell types release from the epidermal peels successively under enzyme digestion and

Table 1. Selected 40 genes preferentially expressed in wheat guard cells involved in stomatal signalling in response to ABA.

Gene	Full-name of homologous gene	Gene_ID	Log_2 FoldChange (GC + ABAvsGC)	*q*-value (GC + ABAvsGC)
14-3-3	*14-3-3-like protein*	TraesCS4D01G046400	4.8643	8.70E−21
ABCB2	*ABC transporter B family member 2*	TraesCS6B01G291900	7.3464	1.5114E−29
ABCC4	*ABC transporter C family member 4*	TraesCS5B01G479900	3.485	0.00036425
ACA4	*Calcium-transporting ATPase 4*	TraesCS5A01G136200	6.9272	1.647E−23
AKT1	*Potassium channel*	TraesCS3B01G265500	8.8891	4.61E−07
AKT2	*Potassium channel*	TraesCS1A01G267900	−7.9207	2.7923E−136
ALMT12	*Aluminum-activated malate transporter 12*	TraesCS1B01G192000	−8.876	3.73E−37
AVP	*Pyrophosphate-energized vacuolar membrane proton pump*	TraesCS7D01G142600	9.7521	1.4413E−26
BAK1	*BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1*	TraesCS3D01G327800	12.814	1.02E−40
CAM	*Calmodulin*	TraesCS2D01G097500	3.8133	1.2174E−43
CAX2	*Vacuolar cation/proton exchanger 2*	TraesCS4B01G161500	4.7045	3.1208E−07
CBL9	*Calcineurin B-like protein 9*	TraesCS3A01G053300	4.151	0.000014377
CIPK23	*CBL-interacting protein kinase 23*	TraesCS2D01G252600	5.3669	4.0219E−10
CLC-c	*Chloride channel protein c*	TraesCS2A01G309900	6.0785	8.4638E−15
ERF1	*Ethylene-responsive transcription factor*	TraesCS7D01G158500	9.3041	2.46E−08
FER	*Receptor-like protein kinase FERONIA*	TraesCS5D01G094700	4.7219	2.7039E−07
GPA	*Guanine nucleotide-binding protein alpha-1*	TraesCS1B01G479100	4.5954	7.3676E−07
HT1	*high leaf temperature protein 1*	TraesCS6B01G183300	4.8737	7.2726E−08
MKK1	*Mitogen-activated protein kinase kinase 1*	TraesCS4D01G312300	4.2169	9.7067E−06
MPK3	*Mitogen-activated protein kinase 3*	TraesCS6B01G146300	4.4876	1.63E−06
MPK6	*Mitogen-activated protein kinase 6*	TraesCS1B01G192600	4.4619	1.95E−06
MPK9	*Mitogen-activated protein kinase 9*	TraesCS1A01G421000	5.2488	1.61E−09
MYB44	*Transcription factor MYB44*	TraesCS5B01G157300	4.138	1.55E−05
MYC2	*bHLH transcription factor*	TraesCS1A01G193200	4.3033	5.66E−06
NHX2	*Sodium/hydrogen exchanger 2*	TraesCS1B01G112700	5.3291	6.3334E−10
PIP2-1	*Aquaporin*	TraesCS2A01G198500	4.72	7.30E−30
PLDα1	*Phospholipase D alpha 1*	TraesCS3D01G130900	10.556	6.8516E−27
PLDδ	*Phospholipase D delta*	TraesCS5D01G341800	4.7833	1.6146E−07
PP2C50	*Protein phosphatase 2C 50*	TraesCS3B01G277900	−8.2157	3.78E−25
PYL5	*Abscisic acid receptor PYR1-LIKE 5*	TraesCS2B01G105300	4.5924	6.20E−09
PYL8	*Abscisic acid receptor PYR1-LIKE 8*	TraesCS1D01G126900	3.3848	0.00053479
PYR1	*Abscisic acid receptor PYRABACTINRESISTANCE 1*	TraesCS1B01G206600	4.3881	0.00025379
SAPK2	*Stress-Activated Protein Kinase 2*	TraesCS2D01G170700	5.9226	1.75E−07
SAPK3	*Stress-Activated Protein Kinase 3*	TraesCS1A01G215900	4.862	8.07E−08
SAPK8	*Stress-Activated Protein Kinase 8*	TraesCS5D01G411900	9.8762	1.35E−10
SAPK9	*Stress-Activated Protein Kinase 9*	TraesCS5A01G069500	1.9819	3.52E−05
SLAC1	*Slow anion channel associated 1*	TraesCS2B01G416100	4.4145	2.72E−06
TPKA	*Two pore potassium channel*	TraesCS5D01G401200	−8.6196	1.25E−25
VHA-A3	*V-type proton ATPase subunit A3*	TraesCS4B01G222700	6.5053	1.1406E−18
WRKY46	*WRKY transcription factor 46*	TraesCS7A01G338500	3.8322	7.92E−05

guard cells are the last cell type remained on the fragile and thin cuticle layers. This can be explained by the thick cell wall of guard cells compared with those of pavement cells and subsidiary cells. The appropriate digestion time is critical for guard cells isolation because short digestion may not degrade the unwanted cells while over degradation causes

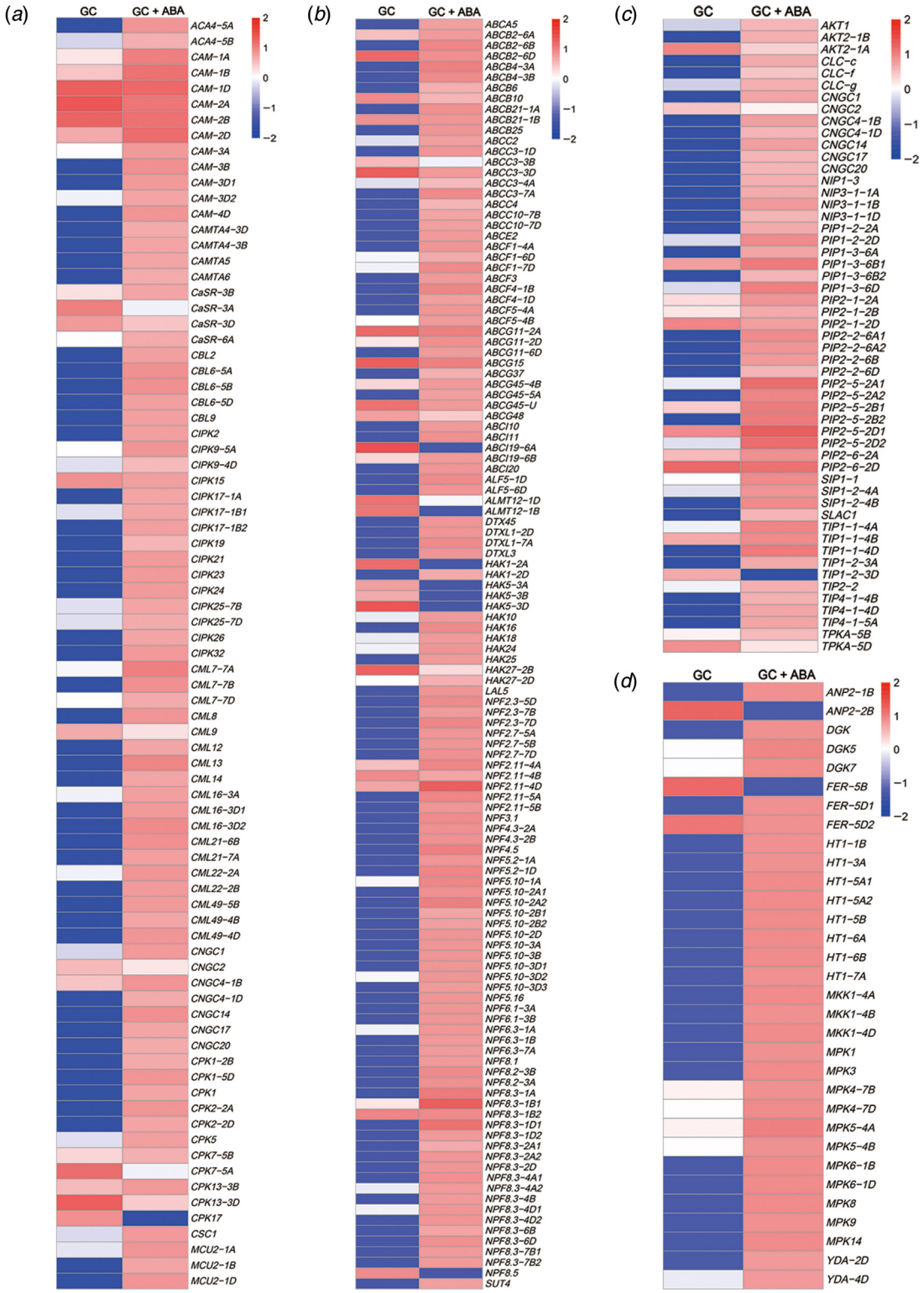

Fig. 7. Heatmap analysis of the DEGs involved in diverse signal pathways. (*a*) DEGs encoding calcium signalling components, (*b*) transporters, (*c*) water and ion channels and (*d*) kinases were identified. The heatmap represents the normalised genes expression level measured in FPKM. The colours correspond to the value of FPKM, ranging from blue (low expression) to red (high expression). Detailed information of the DEGs is in File S3. GC, guard cell samples; GC + ABA, guard cell samples with ABA treatment.

guard cell walls collapse and RNA degradation. Thus, we determined the optimum incubation times (90 min) to completely digest subsidiarity cells and pavement cells while collecting intact, healthy and sufficient guard cells for the subsequent transcriptomic analysis (Fig. 1). The establishment of this approach will accelerate the understanding of how guard cells respond to environmental stimulation and the development of stress-resistant wheat.

ABA is a primary plant hormone that triggers expeditious response of guard cells to drought and other abiotic stresses (Hsu *et al.* 2021). Therefore, we expected to find some ABA-regulated genes unique to guard cells. Here, multiple genes previously identified as essential in stomatal function and stress response were uncovered, including genes known to encode hormonal signalling components, kinases, Ca^{2+} signalling components, membrane transporters and ion channels. In wheat guard cell transcriptome, exogenous ABA can activate four main regulatory components of the core ABA signalling pathway, PYR/PYL, PP2C, SnRK2/SAPK and ABF. JA has synergistic effect with ABA in guard cell signalling pathway (Hossain *et al.* 2011) and ABA affects JA accumulation in guard cells by regulating the expression level of genes encoding key enzymes for JA biosynthesis, such as PLA, AOC, AOS, LOX, OPR1 and MFP (Wasternack 2007). The MYC2, a master regulator of the JA signalling pathway (Kazan and Manners 2013), was up-regulated in response to ABA (Fig. 6*b*). Also, it was found that ABA affects expression of genes encoding the key signalling components in SA, BR, ethylene and auxin pathway. In guard cells, the crosstalk and co-regulation of phytohormones are essential for stomatal movement and adaptation of plants to environmental changes (Acharya and Assmann 2009; Daszkowska-Golec and Szarejko 2013). In this study, single-cell-type transcriptomics of this specialised cell type provided evidence for cross-talk at the transcriptional level between ABA and other phytohormones. It would help to further understand the hormonal cross-talk and regulation of stomatal movement of crops. ABA triggers a signalling network in wheat guard cells that includes DEGs for kinases, transporters and ion channels (Fig. 7), including cation (Ca^{2+}, K^{+}) and anion channels, transporters for ABA, water, CDPKs and MAPKs, suggesting these components mediating ABA-induced stomatal movement may play the key roles in water homeostasis for wheat drought tolerance (Li *et al.* 2000; Roux and Leonhardt 2018). Many families of TFs, such as AP2/ERF, bHLH, bZIP, NAC, MYB and WRKY, play central roles in plant response to abiotic stress by regulating the expression of downstream genes (Wang *et al.* 2016; Samad *et al.* 2017). The wheat guard cell transcriptome discloses considerable DEGs for TFs (File S4), providing the further evidence that the regulation of transcription is critical for guard cell responses.

Based on the transcriptomic results of present study, ABA signalling in the wheat guard cells revealed the presence of homologous components known in *Arabidopsis*. These results seem to suggest a similar core signalling mechanism by which ABA controls stomatal movement in grasses and dicots. However, grasses have the ability to adjust stomatal aperture faster than dicots, which allows grasses to better adapt to environmental changes. Examining the reasons for this, grasses have evolved a unique stomatal complex where the guard cells and subsidiary cells act synergistically (Chen *et al.* 2017; Schäfer *et al.* 2018; Nunes *et al.* 2020; Wang and Chen 2020). Elucidating the ABA-induced specific genes expression in subsidiary cells and how guard cells and subsidiary cells communicate in wheat is worthy of exploration in the future. Furthermore, the different and even contradictory expression patterns of wheat homoeologous genes from A, B and D subgenomes may be the key to distinguishing guard cell responses to ABA in wheat from other species.

In summary, our research presented a comprehensive transcriptome analysis of wheat guard cells for stomatal regulation and stress signalling pathways under ABA treatment. This study will serve as an excellent resource for further ABA signalling studies on the regulation of stomatal movement in grasses, and also provides data that will help to develop a better understanding of abiotic stress-related mechanisms and the broad functions of transcriptional regulatory networks in agronomically important cereals.

Supplementary material

Supplementary material is available online.

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