Dawn regulates guard cell proteins in *Arabidopsis thaliana* that function in ATP production from fatty acid beta-oxidation

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Abstract

Key message Based on the nature of the proteins that are altered in abundance, we conclude that guard cells switch their energy source from fatty acid metabolism to chloroplast activity, at the onset of dawn.

Abstract During stomatal opening at dawn, evidence was recently presented for a breakdown and liquidation of stored triacylglycerols in guard cells to supply ATP for use in stomatal opening. However, proteome changes that happen in the guard cells during dawn were until now poorly understood. Bad accessibility to pure and intact guard cell samples can be considered as the primary reason behind this lack of knowledge. To overcome these technical constraints, epidermal guard cell samples with ruptured pavement cells were isolated at 1 h pre-dawn, 15 min post-dawn and 1 h post-dawn from *Arabidopsis thaliana*. Proteomic changes were analysed by ultra-performance-liquid-chromatography-mass-spectrometry. With 994 confidently identified proteins, we present the first analysis of the *A. thaliana* guard cell proteome that is not influenced by side effects of guard cell protoplasting. Data are available via ProteomeXchange with identifier PXD009918. By elucidating the identities of enzymes that change in abundance by the transition from dark to light, we corroborate the hypothesis that respiratory ATP production for stomatal opening results from fatty acid beta-oxidation. Moreover, we identified many proteins that were never reported in the context of guard cell biology. Among them are proteins that might play a role in signalling or circadian rhythm.

Keywords Guard cell · Stomatal opening · ATPase · Beta-oxidation · Triacylglycerol · Dawn

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Abbreviations

ABA	Abscisic acid
LC-MS	Liquid-chromatography-mass-spectrometry
PCA	Principal component analysis
PC	Principal component
PRIDE	PRoteomics IDentifications database
SEA	Singular enrichment analysis
SEACOMPARE	Cross comparison of SEA
DDT	Dithiothreitol
PLS	Partial least squares analysis
CSP41	Chloroplast stem-loop binding proteins
g_s	Stomatal conductance
GO	Gene ontology
GC	Guard cells
DAVID	Database for annotation, visualization and integrated discovery
GAPCP	Plastidial glyceraldehyde-3-phosphate dehydrogenases

Introduction

Stomata are tiny pores on surfaces of most terrestrial plants. Flanked by two guard cells (GCs), stomata are channels for gas exchange, which includes transpiration and CO₂ uptake. Hence, they are key determinants of plant water use efficiency and photosynthesis. At dawn, stomata open which is synchronized with the day-night circle by the circadian clock (Somers et al. 1998). The clock controls both stomatal conductance and CO₂ assimilation (Hennessey and Field 1991), which is also related to ZTL, a protein that can bind to photoreceptors (Dodd et al. 2004). Overall, the circadian rhythm comprises genes encoding proteins with different scales of transcriptome and proteome regulation (Graf et al. 2017). Approximately 6% of the *Arabidopsis* transcriptome is under circadian control (Harmer et al. 2000). Transcriptional regulators like CCA1 or LHY are circadian clock regulated with a peak in expression prior to dawn (Schaffer et al. 1998; Wang and Tobin 1998), while PRR increases expression after dawn (Matsushika et al. 2000).

At dawn, GCs open stomata in response to light through a complex network of signalling components and ion channels. Both blue and red light induce stomatal opening. Blue-light induced opening starts by perceiving the blue light signal by the blue light receptors PHOT1, PHOT2 and zeaxanthin. The signal is passed through by the signalling kinases BLUS1 and CBC1, protein phosphatases PP1 and by means of 14-3-3 proteins. This finally results in the inhibition of the activity of SLAC1-type anion channels and the activation of plasma membrane H⁺-ATPases (Baunsgaard et al. 1998; Zeiger and Zhu 1998; Kinoshita et al. 2001; Takemiya et al. 2013; Inoue and Kinoshita 2017; Geilfus 2017; Hedrich and Geiger 2017; Hiyama et al. 2017; Schäfer et al. 2018). The activity of the H⁺-ATPase creates an electrical charge-difference across the GC plasma membrane, hyperpolarizing the plasma membrane. As result, channels for K⁺ influx open and turgor pressure increases, causing GCs to swell up and stomata to open (Pilot et al. 2001; Roelfsema and Hedrich 2002; Shimazaki et al. 2007; Kollist et al. 2014; Yamauchi et al. 2016).

The way in which red light provokes stomatal responses remains to be elucidated. Roelfsema et al. (2002) conducted pioneering experiments where photosynthesis was induced by red light. The start of photosynthesis resulted in a reduction of intercellular leaf CO₂, which was suggested to cause stomatal opening. Later it was found that red light increases stomatal conductance also when intercellular leaf CO₂ remained constant (Messinger et al. 2006). This indicates that the red light response is rather

complex (Assmann and Jegla 2016). Matrosova et al. (2015) found that the protein kinase HT1 is essential for red light-induced stomatal opening, whereas red light-induced opening can be suppressed by the Rho-GTPase RopGEF2-ROP7/ROP2 pathway (Wang et al. 2017).

To deliver energy for the consuming process of stomatal opening at dawn, it was shown that GCs catabolize stored lipids (McLachlan et al. 2016), releasing substrates for oxidative phosphorylation, a pathway in which energy is released to produce ATP. Although the process of stomatal opening is likely to comprise the recruitment of many enzymes and regulatory proteins (e.g. 14-3-3 proteins; Kinoshita and Shimazaki 2002), knowledge about the identity of those enzymes is scanty. To understand the mechanism behind stomata opening at dawn, it is extremely helpful to obtain a GC protein profile and to analyse its change over the period. However, despite this importance, the GC proteome has not widely been described yet. To the best of our knowledge, only two GC proteomes have been published so far where GC protoplasts were isolated from epidermal peels based on an hours-lasting digestion with cell wall-degrading enzymes (Zhao et al. 2008; Geng et al. 2017). Moreover, proteomic changes that ensue in the GCs during opening were never examined. Technical difficulties in obtaining a sufficient amount of isolated GCs with high purity in a short period of time without protoplasting can be considered as the major reason behind this lack of data. Stomata only account for 0.5–5.0% of the leaf surface area (Hetherington and Woodward 2003) and cell volume of guard cell is about tenfold lower than that of a mesophyll cell (Taiz et al. 2015), making it very challenging to enrich and separate them from adhering mesophylls and epidermal cells. Treating epidermal peels with cell wall-degrading enzymes allows isolating GC protoplasts with a very high purity (Mach 2008). However, by requiring several hours of enzymatic digestion in artificial buffer media, it is very likely that the protoplasting has side effects on the proteome, provoking cellular responses distorting the investigation of the physiological state (Bauer et al. 2013). To overcome these constraints, a GC isolation protocol is required that enables to extract intact GCs as quick as possible without protoplasting. Bauer et al. (2013) proposed a method from Cornish and Zeevaart (1986) that can prepare epidermal GCs and enrich them in samples with ruptured pavement cells. Following the procedure by Cornish and Zeevaart (1986), GC samples were prepared for this study to extract and identify GC proteins. For this, we collected *Arabidopsis thaliana* GC samples at three time points: 1 h pre-dawn, 15 min post-dawn and 1 h post-dawn. Proteomic changes were acquired by ultra-performance-liquid-chromatography–mass-spectrometry (LC–MS) for each time point.

Materials and methods

Plant cultivation and experimental design

For *A. thaliana*, seeds were sown on 1% (w/v) agar with half strength Murashige and Skoog medium and 1% w/v sucrose. The plates were then sealed using micro-pore tape (3M, Neuss, Germany) and stratified in the dark, 4 °C for 48 h. The plates were then moved into growth cabinet (Snijders Micro Clima Series, Tilburg, the Netherlands) with a 10 h:14 h (22 °C:20 °C, 70% relative humidity, 110–120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux density) light:dark scheme. The seedlings were transplanted to soil at the 4-leaf stage. The soil was made by mixing three portions of compost (Sinclair, Lincoln, UK) and one portion of silver sand (J. Arthur Bower, Lincoln). The mixture was autoclaved at 121 °C for 40 min before used. Experiments were performed on 35–42-day-old plants. GCs were isolated from plants derived from three experimental groups: (1) plants at 1 h pre-dawn, (2) plants at 15 min post-dawn, and (3) plants at 1 h post-dawn. Dawn was induced by lightening the controlled environment chamber. For each experimental group, six biological replicates were grown in independent experiments.

Stomatal conductance

The stomatal conductance of the leaf abaxial and adaxial surfaces to water vapour was measured using a portable fluorescence gas-exchange system (LI-COR 6400 XT; LI-COR). Photon flux density was 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ as provided by an integrated fluorescence chamber head (6400-02B LED light source; LI-COR). The leaf area enclosed in the chamber was recorded for each leaf (~1 cm² of a mature leaf was measured per plant). CO₂ at a flow rate of 300 $\mu\text{mol mol}^{-1}$ CO₂ was controlled by a CO₂ injection system. Six biological replicates from each experimental group were subjected to the conductance measurement.

GC isolation

GCs were prepared according to the method described in Cornish and Zeevaart (1986), optimized for *Arabidopsis*. For this, the epidermis was peeled by hand using forceps. Peels from seven fully-grown rosette leaves were pooled to yield 20 mg of fresh weight. To remove adhering mesophyll and to rupture the epidermal plasma membrane, 20 mg of the peels were sonicated in ice-cold deionized H₂O (0.1% Tween 20) for 4 × 30 s (pulse mode; 60% of maximum wattage) using a QSONQ55 sonicator (Qsonica L.L.C, Newtown, CT, USA), equipped with 1/8" diameter probe. By

this means, the contents from the epidermal cells such as endogenous proteins leak out (Cornish and Zeevaart 1986). Light microscopy was used to ensure that peels were free of adhering intact mesophyll cells or adhering chloroplasts from broken mesophyll cells as identified by the absence of chloroplasts (Fig. 1). Vice versa, imaging proved that GCs still contained organelles such as chloroplasts or nucleolus and the plasma membrane: The cell viability stain neutral red proved that GCs and GC membranes are intact while pavement cells are disrupted (Fig. 1). Counterstaining with the membrane-impermeant Oregon Green-dextran (Thermo Fisher Scientific, Massachusetts, USA) proved that pavement cells were ruptured (see Geilfus et al. 2015). Sonicated strips were washed for 20 s in ice-cold deionized H₂O to remove Tween 20, collected with a sieve, lyophilized, weighed, ground under liquid nitrogen and stored at –20 °C.

Protein extraction and digestion

For the proteomics work, six biological replicates from each experimental group were examined. Proteins were extracted from the ground GCs following the phenol extraction/ammonium acetate precipitation method described by Carpentier et al. (2005) and Geilfus et al. (2017a, b). 150 mg of mortared frozen epidermal peels from *Arabidopsis* leaves were suspended in 750 μl of ice-cold extraction buffer (0.1 M Tris–HCl pH 8.3, 5 mM EDTA, 0.1 M KCl), 1% Dithiothreitol (DDT), 30% sucrose, 1 complete, Mini, EDTA-free protease inhibitor cocktail tablet (purchased by F. Hoffmann-La Roche Ltd, Vilvoorde, Belgium) and vortexed for 30 s. 750 μl of ice-cold Tris buffered phenol (pH 8.0) was added and vortexed for 10 min at 4 °C. After 10 min of centrifugation (12,000 rpm, 4 °C), the phenolic phase was collected and re-extracted by adding 750 μl of extraction buffer and gently vortexed. The mixture was centrifuged again for 5 min (12,000 rpm, 4 °C), the phenolic phase was collected and left overnight for precipitation with five times the volume of the collected phenolic phase of 0.1 M ammonium acetate in methanol at –20 °C. The samples were centrifuged for 60 min at 13,000 rpm at 4 °C, and after

Fig. 1 Integrity of guard cells after sonication as demonstrated by the accumulation of the cell viability stain neutral red. Ruptured epidermal cells remain colourless

removal of the supernatant the pellet was rinsed with 2 ml cold acetone/0.2% DDT and incubated for 1 h at -20°C . After centrifugation (13,000 rpm, 4°C) rinsing solution was discarded and samples were rinsed a second time with 2 ml of the same acetone/DDT mixture and centrifuged for 30 min (13,000 rpm, 4°C). The pellet was briefly air-dried and re-suspended in 75 μl buffer (8 M urea, 5 mM DTT, 30 mM TrisBase). Protein concentration was determined by a 2-D Quant Kit assay (GE Healthcare, Diegem, Belgium) using BSA as standard and measuring absorbance at 480 nm (testing each sample at least in duplicate). For tryptic digestion, DTT was added to the 20 μg protein-containing sample up to a final concentration of 20 mM, gently vortexed, and incubated for 15 min. Iodoacetamide was added to the samples up to a final concentration of 50 mM and vortexed before it was incubated for 30 min in the dark. Samples were diluted 4 times with a stock solution of 150 mM ammonium bicarbonate in order to reduce urea concentration from 8 to 2 M. Trypsin was added in a ratio of 0.2 μg trypsin/20 μg extracted protein and incubated overnight at 37°C . Samples were acidified with trifluoroacetic acid (0.1% final concentration) and de-salted using C18 solid phase extraction according to the manufacturer (Pierce™ C18 Spin Columns, Thermo Fisher Scientific, Gent, Belgium). Peptides were eluted with 40 μl 70% ACN, after which solvents were evaporated using a speed vac and dissolved in 5% ACN, 0.1% formic acid.

Peptide separation and MS analysis

The UPLC–MS/MS analysis was performed on a Q Exactive Orbitrap mass spectrometer (Thermo Scientific, USA) as described by Campos et al. (2016). The samples (5 μl containing 1 μg peptides) were separated on an Ultimate 3000 UPLC system (Dionex, Thermo Scientific) equipped with a C18 PepMap100 precolumn (5 μm , 300 $\mu\text{m} \times 5$ mm, Thermo Scientific) and an EasySpray C18 column (3 μm , 75 $\mu\text{m} \times 15$ cm, Thermo Scientific) using a gradient of 5–20% ACN in 0.1% formic acid (FA) for 10 min followed by a gradient of 10–35% ACN in 0.1% FA for 4 min and a final gradient from 35 to 95% ACN in 0.1% FA for 2.5 min. The flow-rate was set at 250 $\mu\text{l}/\text{min}$. The Q Exactive was operated in a positive ion mode with a nanospray voltage of 1.5 kV and a source temperature of 250°C . ProteoMass LTQ/FT-Hybrid ESI Pos. Mode CalMix (MSCAL5-1EA SUPELCO, Sigma-Aldrich) was used as an external calibrant and the lock mass 445.12003 as an internal calibrant. The instrument was operated in a data-dependent acquisition mode with a survey MS scan at a resolution of 70,000 (FWHM at m/z 200) for the mass range of m/z 350–1800 for precursor ions, followed by MS/MS scans of the top 10 most intense peaks with +2, +3 and +4 charged ions above a threshold ion count of 16,000 at a 35,000 resolution

using a normalized collision energy (NCE) of 28 eV with an isolation window of 3.0 m/z and dynamic exclusion of 10 s. All data were acquired with Xcalibur 2.2 software (Thermo Scientific). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD009918 and <https://doi.org/10.6019/PXD009918> (Reviewer account details: Username: reviewer01111@ebi.ac.uk; Password: GTEaCXG3).

Protein identification and functional annotation by gene ontology

All raw data were converted into mgf files using Proteome discoverer (Version 1.4, ThermoFisher Scientific). The spectra were searched using Mascot (Version 2.2.06; Matrix science, London, England) against an in-house database containing all UniProt *A. thaliana* accessions plus contaminants of keratin and trypsin (54115 sequences). The following settings were used: the enzyme was trypsin and two miscleavages were allowed, cystein-carbamidomethylation was chosen as a fixed modification and variable modifications: +1 on NQ (Deaminated), +16 on M (Oxidation) methionine-oxidation. Precursor peptide charge state was 2+ and 3+, error window on experimental peptide mass values was 10 ppm and 20 absolute milli-mass units were chosen for fragment ion mass tolerance. Peptides assigned to keratin or trypsin were not taken into account. For data validation, the false discovery rate (FDR) was calculated using Scaffold (Version: Scaffold 3.6.3; Proteome Software Inc., Portland, OR, USA). An integrated version of X! Tandem (Version: CYCLONE, 2010.12.01.1) in Scaffold was used for an additional database searching. For FDR calculation, the searching results from both Mascot and X! Tandem were combined automatically by Scaffold with the following settings: at least one peptide with confidence level of 95%.

For functional characterization of the proteins, annotations by Gene Ontology (GO) were retrieved from UniProt (<http://www.uniprot.org>) using our in-house software tool (<https://labtrop.shinyapps.io/Uniprot2Go/>). The UniProt Retrieve/ID mapping tool (<http://www.uniprot.org/uploaddlists/>) was used to convert UniProt protein ID's into TAIR ID's and AGI gene codes.

Protein quantification

Quantitative analysis was performed using Progenesis LC–MS version 4.1 (Nonlinear Dynamics) as described by Buts et al. (2014) with some modifications. For alignment, a reference run was selected, after which the files were aligned automatically, manual landmark vectors were not necessary. The sensitivity of the peak picking limits was put to four. Peptides with charges two to five were retained in the filter

step, and then the data were normalized. The accuracy of ion alignment was double-checked manually for those ions that belong to proteins that are discussed here. Feature tables of the different fractions were combined to a complete overview of the peptide quantification of the samples. Peptides with p -value < 0.05 (Progenesis) were retained for further analysis and abundances of all corresponding significant peptides were summed per protein accession number. By this, protein abundances were calculated based on peptide abundances.

Statistics of the proteomics data

The software STATISTICA (Version 8.0; Tulsa, OK, USA) was used for data analysis. Statistical evaluation started with a partial least squares analysis (PLS), see for example Carpentier et al. (2008) and Geilfus et al. (2017a, b). Based on this PLS, the most relevant variables (calculated proteins in this case), were selected for univariate ANOVA and post-hoc analysis. This selection was done according to their loadings on the component that marked them as being most influential in terms of contributing to the separation. Fisher's LSD post-hoc analysis test was used to evaluate the homogeneity of the different experimental groups ($n = 6$ biological replicates; $p < 0.05$). This resulted in sets of proteins that (1) increased or (2) decreased abundance at 15 min after dawn compared to the sampling at 1 h pre- or post-dawn or (3) increased or (4) decreased abundance at 1 h after dawn compared to the sampling at 1 h pre-dawn.

Evaluating the purity of the GC proteome

For judging on the purity of the extracted GC proteome, we conducted two analysis. First, we checked whether our list with GC proteins contains entries that are not included in a reference list from the *Arabidopsis* proteome that was retrieved from the 'PRoteomics IDentifications database' (PRIDE) (<http://www.ebi.ac.uk/pride>). Second, we analysed as to whether any functional categories of GO are overrepresented in our GC proteome relative to the PRIDE reference list. This enrichment analysis was done using the Database for Annotation, Visualization and Integrated Discovery (DAVID) tool, version 6.8 (Huang et al. 2009). In detail, enriched annotations in the GC proteome were compared to enriched annotations in the reference list, revealing significant categories and the underlying proteins that are unique in our GC proteome.

Overrepresentation tests

For identifying shared GO-terms that were enriched in the set of proteins that increased or decreased abundance at 1 h after dawn, GO was retrieved for calculating

overrepresentation test according to Mi et al. (2013) using PANTHER (<http://pantherdb.org>). This was not possible for the sets of proteins that increased or decreased abundance at 15 min after dawn because the number of significant proteins (#10) was too small for the calculation. Furthermore, a hierarchical singular enrichment analysis (SEA) was calculated to judge on term enrichment level of enriched GO terms using the agriGO SEA tool (<http://bioinfo.cau.edu.cn/agriGO/analysis.php>) with the *Arabidopsis* genemodel (TAIR9)-background according to Du et al. (2010). Lastly, the resulting lists with over-represented GO terms were subjected to cross comparison via the Cross Comparison of SEA (SEACOMPARE) tool (<http://bioinfo.cau.edu.cn/agriGO/analysis.php?method=compare>). This identified similarities and differences in the enriched annotations between the sets of proteins that increased and decreased in abundance at 1 h after dawn.

Results

Purity of GC enriched samples

Altogether, 994 different proteins were identified in the leaf GCs of at least three samples of *Arabidopsis* that were sampled from all three experimental groups (1 h before dawn, 15 min after dawn, and 1 h after dawn) (Table S1). The false discovery rate at both protein and peptide level was 0%. Three analyses indicate a high purity of the GC proteome:

- (1) Among the 994 proteins, we identified 271 unique proteins that are not included in the PRIDE reference list of the *Arabidopsis* proteome (Table S2). Thirteen of the 271 unique proteins are represented abundantly with more than 10 peptides being identified (Table 1).
- (2) Relative to the PRIDE reference *Arabidopsis* proteome, the GC proteome was overrepresented in proteins that share functional annotation to carbon metabolism, ATP binding, or glycolysis. Many proteins are located in the apoplast (Table S3).
- (3) The identified proteins were also compared to the GC transcriptome of Bauer et al. (2013); we found 124 in common to the ones listed by Bauer et al. (Table S4).

The GC proteome changes during dawn

Inducing dawn by lightening the controlled environment chamber provoked an increase in stomatal conductance from approximately $45\text{--}130\text{ mmol m}^{-2}\text{ s}^{-1}$ at 60 min after light exposure (Fig. 2a). Consistently, aperture increased over time (Fig. 3). The differences in protein concentration in dependency to the transition from pre-dawn to post-dawn remarkably demonstrated the existence of pronounced proteomic changes

Table 1 List with 13 GC specific proteins

Locus	Protein UniProt-ID	Protein name	Number of peptides used for quantification	Protein identification probability (%)	Best peptide probability (%)
AT1G33590	F4HR88	Leucine-rich repeat (LRR) family protein	17	100	100
AT1G31690	F4IAX0	Copper amine oxidase family protein	16	100	100
AT1G23130	O49304	Putative Bet v1-type pathogenesis-related protein	10	100	100
AT2G18960	P20649	H[+]-ATPase 1(HA1)	11	100	100
AT4G25900	Q940G5	Aldose 1-epimerase family protein	21	100	100
AT4G19410	Q940J8	Pectinacetyltransferase family protein	12	100	100
AT1G09750	Q94K53	Eukaryotic aspartyl protease family protein	11	100	100
AT5G63800	Q9FFN4	Beta-galactosidase 6	13	100	100
AT1G33600	Q9FW48	Leucine-rich repeat (LRR) family protein	11	100	100
AT3G16530	Q9LK72	Legume lectin family protein	12	100	100
AT5G03350	Q9LZF5	Legume lectin family protein	19	100	100
AT3G08030	Q9SFB1	Protein of unknown function	18	100	100
AT1G78060	Q9SGZ5	Probable beta-D-xylosidase 7	18	100	100

These proteins are represented abundantly with more than ten peptides being identified

Fig. 2 **a** Stomatal conductance from the intact leaf in response to transition from dark to light. Mean $\pm$ SE ($n=6$ biological replicates). **b** GC protein levels evaluated by PCA show distinct grouping of samples undergoing a transition from pre-dawn to post-dawn. Scores plot

for GC samples. GCs were collected at 1 h pre-dawn (quadrangles), 15 min after dawn (circles) and at 1 h after dawn (triangles). PC1 and PC2 explain 46% and 9% of the variation between samples, respectively ($n=6$ biological replicates)

in the GCs over dawn. As shown by the multivariate analysis, the three experimental groups (1 h before dawn, 15 min after dawn, and 1 h after dawn) are clustering separately (Fig. 2b). The first component (PC1) clearly indicated the time and light aspect, respectively. A post-hoc test revealed the identity of ten proteins that significantly decreased (Table 2) or increased (Table 3) in abundance ($p < 0.05$) uniquely at 15 min after dawn. Among the proteins that decreased after a quarter of an hour were entries of the V-type proton ATPase subunit B3 (UniProt ID: Q8W4E2) with annotations to proton transmembrane transport (GO:1902600) and vacuole (GO:0005773) (Table S5). Among the proteins that increased after 15 min were chloroplastic stem-loop binding proteins (UniProt ID:

Q9LYA9, Q9SA52; Fig. 4) with annotations to rRNA binding (GO:0019843), rRNA processing (GO:0006364), positive regulation of translation (GO:0045727) and transcription (GO:0045893) (Table S6). The same post-hoc test revealed the identity of 255 proteins that decreased (Table S7) and 84 proteins that increased (Table S10) abundance at 1 h after transition from dark to light during dawn.

Proteins that decrease at 1 h after dawn function in lipid catabolism or proton transport

Two hundred and twenty-five proteins were identified that had a lower abundance at 1 h after dawn ($p < 0.05$)

Fig. 3 Images of adaxial leaf surface show stomatal aperture at **a** 1 h before dawn, **b** 15 min after dawn, and **c** 1 h after dawn

(Table S7). Functional annotations by GO were retrieved for those proteins (Table S8). The majority of the 225 proteins shared the annotation to the biological process-category ‘metabolic process’ (GO:0008152; 62.7% of all proteins with annotations to the biological process-category; see exploded grey section in Fig. 5a). Out of this, 54 (78.3%) were annotated to ‘primary metabolic process’ (GO:0044238) (Fig. 5b) and out of this, 13 proteins (18.1%)

to ‘lipid metabolic process’ (GO:0006629) (Fig. 5c). A SEA overrepresentation test was conducted to sort the over-represented GO-terms hierarchically that were enriched in the 225 proteins according to their function. The set of 225 proteins that decreased abundance during the transition from dark to light was significantly (P value = $8.68\text{E}-04$) enriched in proteins that were functionally annotated to lipid catabolic process (GO:0016042) (Table 4).

The annotations to the ‘lipid catabolic processes’ (GO:0016042) are enriched with the highest significance score at level 9. A pivotal enzyme is the peroxisomal 3-ketoacyl-CoA thiolase that functions in the fatty acid beta-oxidation (GO:0006635) (Fig. 6a). Besides lipid catabolic processes, 4 proteins with annotations to ‘fatty acid biosynthetic processes’ (GO:0006633) were also under control of dawn. Proteins such as the 3-oxoacyl-[acyl-carrier-protein], the probable 3-ketoacyl-CoA synthase or the lipoxygenase decreased abundance during the transition from dark to light (Table S9-2).

We show next, that many of the enriched shared biological process-annotations in the set of proteins that decrease abundance at 1 h after dawn culminate in the annotation ‘ATP biosynthetic process’ (GO:0006754), which is because dawn results in the decreased abundance of five ATPases entries, of which three are functional in the mitochondrial respiration (Q9C5A9, P92549, Q96251) (Table S9-3). In good agreement, annotations to ‘proton transport’ (GO:0015992) are enriched in this data set being attributable to an effect of the dawn on abundance of proton pump entries that play a role in ‘proton transport’ (GO:0015992) (Table S9-4). Consequently, the transition from dark to light decreases abundance of GC proteins that share annotations to ‘ATP binding’ (GO:0005524), ‘hydrogen ion transmembrane transport activity’ (GO:0015078) or ‘ATPase activity, coupled’ (GO:0042623).

Most of these GC ATPase entries are located to the mitochondrial membrane (GO:0031966) (Table S9-5). Enzymes from the lipid catabolic process and β -oxidation were predicted to the peroxisome (esterase/lipase; 3-ketoacyl-CoA thiolase; Table S9-6). Enzymes that play a role in the fatty acid biosynthetic processes were located to the chloroplast envelope (3-oxoacyl-[acyl-carrier-protein] reductase; lipoxygenase; Table S9-7), the thylakoid membrane (lipoxygenase; Table S9-8) or the stroma of the chloroplast (3-oxoacyl-[acyl-carrier-protein] reductase; Table S9-9).

Proteins that increase at 1 h after dawn function in carbohydrate and amino acid metabolism

Eighty-four proteins were identified that had a higher abundance at 1 h after dawn ($p < 0.05$) (Table S10). Overrepresentation test revealed that annotations to glycolysis (GO:0006096), oxidative phosphorylation (GO:0006119) or respiratory electron transport chain (GO:0022904)

Table 2 List with ten significant proteins that transiently decrease abundance at 15 min after dawn

Locus	Protein UniProt-ID	Name	Number of peptides used for quantification	Fold change (1 h before/15 min after dawn)	Protein identification probability (%)
AT2G20990	F4IFM6	Synaptotagmin A	1	1.6	0.998
AT3G26380	F4JCI4	Melibiose family protein	1	1.4	1
AT4G12880	F4JRH9	Early nodulin-like protein 19	1	1.3	1
AT1G53210	Q8L636	Putative uncharacterized protein At1g53210	1	1.3	1
AT3G26450	Q8L942	Major latex protein, putative	2	1.8	0.998
AT3G25520	Q8LB11	60S ribosomal protein L5-1	1	1.3	1
AT2G02850	Q8LG89	Basic blue protein	3	1.5	1
AT1G12570	Q8VYV8	At1g12570/T12C24_9	1	3.0	1
AT1G20260	Q8W4E2	V-type proton ATPase subunit B3	2	1.5	1
AT3G14790	Q9LH76	Trifunctional UDP-glucose 4,6-dehydratase/UDP-4-keto-6-deoxy-D-glucose 3,5-epimerase/UDP-4-keto-L-rhamnose-reductase RHM3	1	1.5	1

Table 3 List with ten significant proteins that transiently and exclusively increase abundance at 15 min after dawn

Locus	Protein UniProt-ID	Name	Number of peptides used for quantification	Fold change (15 min after/1 h before)	Protein identification probability (%)
AT2G44210	B9DGK6	AT2G44210 protein	2	2.1	100
AT3G56240	O82089	Copper transport protein CCH	5	1.2	100
AT1G78830	Q8VZ33	Putative uncharacterized protein At1g78830	12	1.3	100
AT1G35140	Q9C6E4	Protein EXORDIUM-like 1	1	infinite	85
AT3G18080	Q9LV33	Beta-glucosidase 44	4	2.1	100
AT3G63140	Q9LYA9	Chloroplast stem-loop binding protein of 41 kDa a, chloroplastic	11	1.8	100
AT5G03350	Q9LZF5	Lectin-like protein At5g03350	19	1.3	100
AT1G09340	Q9SA52	Chloroplast stem-loop binding protein of 41 kDa b, chloroplastic	21	1.9	100
AT4G23820	Q9SUP5	Glycoside hydrolase family 28 protein/polygalacturonase (Pectinase) family protein	1	2.1	100
AT3G52500	Q9SVD1	Aspartyl protease family protein	9	1.1	100

were 32-, 28- or 14-fold enriched (Table 5). Up-regulated proteins that share annotations to all three categories are the succinate dehydrogenase [ubiquinone] flavoprotein subunit 1 (O82663), the NADH [ubiquinone] dehydrogenase flavoprotein 1 (Q9FNN5) and the cytochrome c-1 (O23138). Thus, many of the enriched shared biological process-annotations culminate in the annotation 'cellular carbohydrate metabolic process' (GO:0044262) and 'carbohydrate catabolic process' (GO:0016052) (Fig. S2A) and are located to 'chloroplast part' (GO:0044434) or 'mitochondrial part' (GO:0044429) (Fig. S2B).

Annotations to cellular amino acid metabolic process (GO:0006520) and nitrogen compound metabolic process

(GO:0006807) were 2.4- and twofold enriched, respectively (Table 5). Up-regulated proteins that share annotations to both categories are the chloroplastic methylthioalkylmalate synthase 1 (Q9FG67), the chloroplastic D-3-phosphoglycerate dehydrogenase 1 (O49485), the cytoplasmic serine-tRNA ligase (Q39230), the adenosylhomocysteinase 2 (Q9LK36) and the glutamate decarboxylase 2 (Q42472). Thus, many of the enriched shared biological process-annotations culminate in the annotation 'translation' (GO:0006412) and 'cellular amino acid metabolic process' (GO:0006520) (Fig. S2A).

Fig. 4 Detail of GC proteins that specifically increase at 15-min post dawn. GCs were sampled at 1 h pre-dawn, 15 min after dawn, and at 1 h after dawn. Full data available in Table S1. **a** Chloroplast stem-loop binding protein of 41 kDa a, chloroplastic, Uniprot-ID: Q9LYA9, gene name: *csp41a*, AGI code: At3g63140. **b** Chloroplast

stem-loop binding protein of 41-kDa b, chloroplastic, Uniprot-ID: Q9SA52, gene name: *csp41b*, AGI code: At1g09340. Letters show significant differences between the ratios of the means. Fisher's LSD test ($p=0.05$; $n=6$ biological replicates). Box plots (arbitrary units) marked with the same letter do not differ significantly

Fig. 5 During the transition from dark to light, enzymes with the biological process annotation 'metabolic process' are affected the most at 1 h after dawn. Evaluation based in the 225 proteins that decreased in abundance at 1 h after dawn. **a** PANTHER pie chart displays over-represented annotations of the biological process-category. **b** Level 1

child categories of the exploded (grey) section category annotation 'metabolic process' (GO:0008152) in (A). **c** Level 1 child categories of the exploded (orange) section category annotation 'primary metabolic process' (GO:0044238) in **b**

Table 4 Overrepresentation test based on the 225 proteins that decrease in abundance at 1 h after dawn identified biological process-class annotations that were enriched

Biological process	Fold enrichment	P value
Lipid catabolic process (GO:0016042)	9.78	8.68E−04
Response to cadmium ion (GO:0046686)	8.54	1.02E−02
Single-organism catabolic process (GO:0044712)	7.12	2.05E−07
Response to metal ion (GO:0010038)	6.82	1.59E−02
Catabolic process (GO:0009056)	5.01	1.69E−07
Organic substance catabolic process (GO:1901575)	4.80	1.50E−05
Cellular catabolic process (GO:0044248)	4.12	3.63E−02
Lipid metabolic process (GO:0006629)	3.89	3.21E−02
Response to abiotic stimulus (GO:0009628)	3.43	2.00E−02
Single-organism metabolic process (GO:0044710)	2.80	1.29E−07
Response to stress (GO:0006950)	2.63	6.26E−03
Response to stimulus (GO:0050896)	2.12	6.23E−03
Primary metabolic process (GO:0044238)	1.77	8.26E−03
Metabolic process (GO:0008152)	1.74	3.03E−04
Organic substance metabolic process (GO:0071704)	1.70	2.24E−02
Unclassified	1.20	0.00E−00

Enriched ($p < 0.05$) annotations were grouped via the PANTHER classification tool. The column 'Fold Enrichment' indicates the degree of the enrichment. For example, a number greater than 1 means that the category is overrepresented over the expected *Arabidopsis* genemodel (TAIR9)-background list

Comparison between proteins that increase and decrease abundance at 1 h after dawn

A comparison of the shared enriched GO-terms between the sets of proteins that increased and decreased revealed that annotation to 'translation' (GO:0006412) was enriched in the set of proteins that increased while non-significantly affected in the set of proteins that decreased (Table 6). Annotations to lipid or fatty acid metabolism (GO:0016042, GO:0006629, GO:0006633, GO:0006631) were enriched in the set of proteins that decreased while non-significantly affected in the set of proteins that increased. However, the GO-term 'generation of precursor metabolites and energy' (GO:0006091) was more significantly enriched in the set that increased compared to the set that decreased (Table 6). Responsible for this difference were photosynthetic proteins like the photosystem I P700 chlorophyll a apoprotein A2 (P56767), the chloroplastic ATP synthase subunit beta (P19366), the probable cytochrome c At1g22840 (O23138) the and photosystem II CP43 chlorophyll apoprotein (P56778).

Discussion

Purity of the GC proteome

For investigating the effect of the transition from dark to light during dawn on the GC proteome, we analysed the proteome. Three lines of evidence indicate that the presented proteome is markedly enriched with proteins

from GCs: First, we identified 271 unique proteins that are not included in the PRIDE total leaf reference proteome (Table S2). Second, we detected 124 proteins whose underlying genes were also found in the GC transcriptome of Bauer et al. (2013) (Table S4). Third, the GC proteome did not include any of the four epidermal biomarker proteins, namely the transcription factor WEREWOLF (At5g14750), the transcription factor CPC (At2g46410), the probable lysophospholipase BODY-GUARD 1 (At1g64670), or the defensin-like protein 4 (At2g02120) (Table S11).

Every extraction method in proteomics has a bias. Zhao et al. (2008) used protoplasting to study the enriched GC proteome. They report 1507 guard cell proteins. Meta-analysis reveals that their proteome contains some unique enrichments for the cellular component: 7.3% of their identified genes are annotated to the endoplasmic reticulum, 5.1% to the ribosome, 3.4% to the trans-Golgi network and 3.2% to the Golgi membrane (see table S12 for the complete list). We report 994 guard cell proteins (Table S1). Our proteome contains some unique enrichments for the cellular component: 31.5% of our identified genes are annotated to the extracellular region and 16.9% to the plant-type cell wall (Table S12). The method by Zhao et al. (2008) accentuates the ribosomal and Golgi network, indicating that some protein synthesis might take place during the long isolation procedure and our presented data seem to accentuate apoplastically segregated proteins. The enriched proteins from Zhao et al. (2008) are annotated to the biological process translation (6.8%),

Fig. 6 Detail of GC proteins that decrease in abundance at 1 h post dawn. Full data available in Table S1. **a** Peroxisomal 3-ketoacyl-CoA thiolase, UniProt-ID: Q56WD9, gene name: ped2, AGI code: AT2G33150. **b** 3-Oxoacyl-ACP reductase, chloroplastic, UniProt-ID: P33207, gene name: At1g24360, AGI code: AT1G24360. **c** 3-Ketoacyl-CoA synthase, UniProt-ID: O65677, gene name: kcs17, AGI code: AT4G34510. **d** Protein ECERIFERUM 1, UniProt-ID: Q39048,

gene name: cer2, AGI code: AT4G24510 **e** probable fructokinase-6, chloroplastic, UniProt-ID: Q9C524, gene name: At1g66430, AGI code: AT1G66430. Letters show significant differences between the ratios of the means. Fisher's LSD test ($p=0.05$; $n=6$ biological replicates). Box plots (arbitrary units) marked with the same letter do not differ significantly

Table 5 Overrepresentation test based on the 84 proteins that increase in abundance at 1 h after dawn identified biological process-class annotations that were enriched

Biological process	Fold enrichment	P value
Glycolysis (GO:0006096)	32.36	1.95E-03
Oxidative phosphorylation (GO:0006119)	28.45	1.88E-04
Cation transport (GO:0006812)	24.63	2.83E-04
Respiratory electron transport chain (GO:0022904)	14.38	1.91E-04
Cellular amino acid metabolic process (GO:0006520)	6.40	1.13E-03
Nitrogen compound metabolic process (GO:0006807)	2.45	1.79E-03
Cellular process (GO:000998)	2.00	2.07E-04
Unclassified	0.64	5.08E-0

Enriched ($p < 0.05$) annotations were grouped via the PANTHER classification tool (see legend in Table 4)

Table 6 Cross-comparison of enriched GO terms between the sets of proteins that increased and decreased in abundance at 1 h after dawn, as generated by the SEACOMPARE tool (only detail shown)

GO Term	Onto	Description	Decrease	Increase
GO:0016042	BP	Lipid catabolic process		
GO:0006629	BP	Lipid metabolic process		
GO:0006633	BP	Fatty acid biosynthetic process		
GO:0006631	BP	Fatty acid metabolic process		
GO:0006412	BP	Translation		
GO:0006091	BP	Generation of precursor metabolites and energy		

The coloured blocks represent the level of up-regulation of each GO term (adjusted $p \leq 0.05$) as marked with colour. The yellow-to-red or gray-scale represent the term is upregulated or non-significant, respectively. Proteins such as the photosystem I P700 chlorophyll a apoprotein A2 (P56767), the chloroplastic ATP synthase subunit beta (P19366), the probable cytochrome c At1g22840 (O23138) and the photosystem II CP43 chlorophyll apoprotein (P56778) were responsible that the GO term 0006091 was enriched in the protein set that increased at 1 h after dawn

Onto Ontology

while our proteins have as top annotations carbohydrate metabolic process (7.5%), ubiquitin dependent protein catabolism (4.6%) and lipid catabolism process (4%).

The GC proteome is highly dynamic over dawn

During the transition from dark to light during dawn, stomatal conductance (Fig. 2a) and aperture increase (Fig. 3), while the *A. thaliana* GC proteome changes (Fig. 2b). From all proteins that are in transition, we used multivariate statistics to select those that were (i) high or (ii) or low abundant at 15-min post dawn or (iii) increased or (iv) decreased in abundance at 1-h post-dawn.

When the aperture had already widened at 15 min after onset of light (Fig. 3b), the subunit B3 of the V-type proton ATPases (Q8W4E2) proved to be decreased in abundance (Table 2). Among other factors, this might contribute in part in stomatal opening because the activity of this vacuolar H^+ -ATPase is required for darkness-induced ABA-mediated stomatal closure. Stomatal closure requires cytosolic alkalization and the vacuolar H^+ -ATPase facilitates this cytosolic alkalization by pumping protons into the vacuole (Krebs et al. 2010; Liu et al. 2016).

At 1 h after dawn, 225 proteins that decreased in abundance were identified (Table S7). Dozens are enzymes that function in the lipid metabolism or ATP biosynthesis.

Proteins with annotation to lipid metabolism can be grouped on the one hand in lipid catabolic peroxisomal enzymes that catalyze for instance reactions during β -oxidation (Table S9-1; Table 4). On the other hand, abundances of lipid anabolic chloroplastidial enzymes decreased that function in fatty acid biosynthesis (Table S9-2; Table 4). The dawn-induced decrease in abundances from enzymes that function in the fatty acid biosynthesis or degradation indicates that GCs adjust the lipid metabolism, viz. turnover, at dawn. According to the recent study by McLachlan et al. (2016), GCs break down triacylglycerols to supply ATP to use in stomatal opening at dawn. Our study confirms both that GCs possess proteins that catalyse lipid degradation and that they are downregulated in response to light during dawn. Lipid degradation seems to be an important source of energy (e.g. ATP) in the dark but no longer or less important as source for ATP after dawn. We suggest that at this point at post-dawn when aperture is widened, ATP derived from both photosynthesis and photoassimilate dissimilation (glycolysis, oxidative de-phosphorylation and mitochondrial reparation) is provided in sufficient amounts for the further control of stomatal aperture during the day. In support of this assumption, photosynthetic proteins like the photosystem I P700 chlorophyll a apoprotein A2 (P56767), the chloroplastic ATP synthase subunit beta (P19366), the probable cytochrome c At1g22840 (O23138) and the photosystem II

CP43 chlorophyll apoprotein (P56778) proved to be more abundant at 1 h after light was switched on while proteins related to lipid and fatty acid metabolism were less abundant (Table 6). Second, annotations to glycolysis (GO:0006096), oxidative phosphorylation (GO:0006119) or respiratory electron transport chain (GO:0022904) were markedly enriched at 1 h after lightening (Table 5).

Dawn controls abundances of GC enzymes that function in mitochondrial ATP biosynthesis and peroxisomal lipid catabolism

Many of the proteins that decreased in abundance at 1 h after dawn were functionally annotated to mitochondrial biosynthesis of ATP (Table S9-3) and proton transport (Fig. S1A). The latter is mainly attributable to mitochondrial ATPase entries that are known to channel hydrogen ion transmembrane transport (Fig. S1B) that is required to phosphorylate ADP to ATP (Table S9-3, Table S9-4); a process that is located to the mitochondrial membrane (Fig. S1C; Table S9-5). This is in good agreement with the data from McLachlan et al. (2016) who reason for GCs that the product of β -oxidation, namely acetyl CoA, is converted to citrate and used in mitochondrial respiration during stomatal opening at dawn (see also Pracharoenwattana et al. 2005). The β -oxidation-driven respiratory gained ATP is thought to energize stomatal opening during dawn via effects of the PM-ATPase exerted on the polarization of the GC plasma membrane. In addition, we identified a peroxisomal 3-ketoacyl-CoA thiolase (UniProt-ID: Q56WD9; Fig. 6a) that was more abundant at 1-h pre-dawn than at 1-h post-dawn being functionally annotated to fatty acid beta-oxidation (Table S9-1). Esterase/lipase entries that all play a role in the lipid catabolism (Table S9-1), were higher abundant at pre-dawn compared to 1 h after dawn. The fact that enzymes that function in the mitochondrial phosphorylation of ADP or in the fatty acid beta-oxidation decrease in abundance after dawn further indicates that respiratory energy that is provided by GC fatty acid catabolism is less required in GCs when light is present. The degradation of the energy rich lipids for feeding GC respiration at pre-dawn as source for energizing aperture control seems to be downregulated when light is available.

GC enzymes from the fatty acid anabolism reduce abundance during dawn

Not only enzymes that catabolize lipids were detected to be downregulated over dawn in GCs. Here we report that the chloroplastic 3-oxoacyl-[acyl-carrier-protein] reductase (UniProt-ID: P33207; Fig. 6a) was higher abundant at 1-h

before dawn compared to 1-h after dawn. This enzyme is involved in the fatty acid biosynthesis because it catalyses the first reduction step in the fatty acid biosynthesis (Harwood 1988), which is the key condensation of an acyl-acyl-carrier-protein with a malonyl-acyl-carrier-protein to form the beta-ketoacyl-acyl-carrier-protein (Shimakata and Stumpf 1982). Besides this important key regulator enzyme from the fatty acid biosynthesis, two additional enzymes that are more abundant at pre-dawn are functionally related to fatty acid biosynthesis. They are the 3-ketoacyl-CoA synthase (O65677; Fig. 6c) and ECERIFERUM 1 (Q39048; Fig. 6d). The latter plays a role in very-long-chain fatty acid biosynthesis (Haslam et al. 2012) (Table S9-2).

Here we have reported about lipid synthesizing enzymes that reduce abundance in the GCs that undergo the transitions from dark to light at dawn. Although supposition, this may suggest that at pre-dawn, viz. very late at night, the plant might be still able to provide chemically stored energy to energize fatty acid biosynthesis. It is known that GC lipid droplets increase in volume at night when no light is available (McLachlan et al. 2016). Also not measured in this study, a source for energizing this lipid biosynthesis at night could be the breakdown of starch (Zeeman et al. 2007; Horrer et al. 2016). In agreement, we detected one protein, the probable fructokinase-6 (Q9C524), with annotation to ‘starch biosynthetic process’ (GO:0019252) (Fig. 6e) to be abundant at pre-dawn. Moreover, the DAVID overrepresentation test revealed that the GC proteome was enriched in two plastidial glyceraldehyde-3-phosphate dehydrogenases, namely GAPCP1 (Q9SAJ6) and GAPCP2 (Q5E924) (Table S3-2). During *Arabidopsis* embryo development, GAPCP is relevant for the utilization of sucrose for fatty acid synthesis in the plastid (Flores-Tornero et al. 2017). Taking into account that GC plastids are long regarded as a starch storage (Santelia and Lunn 2017), our results support the idea that plastidial glycolysis may contribute to fatty acid biosynthesis in the GCs. Further work is anticipated to elucidate this topic.

Chloroplast stem-loop binding proteins are expressed in GCs and increase abundance during dawn

The following part of the discussion deals with two proteins that were among the set of proteins that transiently increased abundance at 15-min pre-dawn. The data in this paper show that GCs contain the chloroplast stem-loop binding proteins CSP41a and CSP41b (Q9SA52, Q9LYA9) (Fig. 4). Chloroplasts transcribe their own genome and the precursor and mature RNA transcripts contain a repeated sequence that can form a stem-loop which is either required during 3' processing of pre-mRNAs or for stabilisation of mature mRNA (Stern and Grusissem 1987; Yang et al. 1996). Stern and Grusissem (1987) found that CSP41 is synthesized in the

Table 7 GO analysis categorizes 17 proteins in the GC proteome as signal transduction proteins

Locus	UniProt-name	Protein name	Gene name	GO annotations		Function	References
				GO-ID	Term		
At3g62030	F4IX26	Rotamase CYP 4	ROC4	0007165	Signal transduction	<i>ROC4 Arabidopsis</i> mutant shows repressed transcription in GCs when 100 μ M ABA are sprayed	Leonhardt et al. (2004)
At1g18080	O24456	Receptor for activated C kinase 1A	RACK1A	0004871	Signal transducer activity	Loss-of-function mutants show significant resistance to water stress	Ullah et al. (2008)
				0009967	Positive regulation of signal transduction		
				0010476	Gibberellin mediated signaling pathway		
At3g59920	O24653	Guanosine nucleotide diphosphate dissociation inhibitor 2 (AtGDI2)	GDI2	0007264	Small GTPase mediated signal transduction	?	
At5g20720	O65282	20 kDa chaperonin, chloroplastic	CPN20	0009788	Negative regulation of ABA-activated signaling pathway	<i>CPN20</i> negatively regulates the <i>ABAR-WRKY40</i> coupled ABA signaling	Zhang et al. (2013)
At5g26000	P37702	Myrosinase 1	TGG1	0009738	ABA-activated signaling pathway	<i>Tgg1</i> mutants are hyposensitive to ABA inhibition of GC inward K^+ channels and stomatal opening	Zhao et al. (2008)
At4g34870	Q42406	Peptidyl-prolyl <i>cis-trans</i> isomerase CYP18-4	CYP18-4	0007165	Signal transduction	The <i>CYP1</i> family protein AT3G66654 interacts with components of the <i>ABCG22-PL-PLC9</i> -mediated signal transduction in GCs	Jones et al. (2014) and Merilo et al. (2015)
At4g23650	Q42479	Calcium-dependent protein kinase 3	CPK3	0009738	ABA-activated signaling pathway	<i>CPK3</i> functions in ABA regulation of GC S-type anion- and Ca^{2+} -permeable channels and stomatal closure	Mori et al. (2006)
At5g63980	Q42546	SAL1 phosphatase	SAL1	0035556	Intracellular signal transduction		
				0009738	ABA-activated signaling pathway	When conjugated to PAP, SAL1-PAP retrograde pathway interact with ABA signaling to regulate stomata in <i>Arabidopsis</i>	Pornisiriwong et al. (2017)
At2g33150	Q56WD9	3-Ketoacyl-CoA thiolase 2, peroxisomal	PED1	0009789	Positive regulation of ABA-activated signaling pathway	Expression of the twin channel subunits KAT1 and KAT2 might be responsible for K^+ influx into GCs	Pilot et al. (2001)
At5g55190	Q8H156	GTP-binding nuclear protein Ran-3	RAN3	0007165	Signal transduction	?	
At3g02110	Q8L9Y0	Serine carboxypeptidase-like 25	SCPL25	0009742	Brassinosteroid mediated signaling pathway	The <i>SCPL25</i> homologue <i>SCPL48</i> was downregulated in GCs when epidermal peels were floated with 150 mM sucrose	Bates et al. (2012)

Table 7 (continued)

Locus	UniProt-name	Protein name	Gene name	GO annotations		Function	References
				GO-ID	Term		
At5g65620	Q94AM1	Organellar oligopeptidase A, chloroplastic/mitochondrial	OOP	0006465	Signal peptide processing	?	
At3g15356	Q9LJR2	Lectin-like protein LEC (AtLEC)	LEC	0009873	Ethylene-activated signaling pathway	?	
At4g30610	Q9M099	Serine carboxypeptidase 24	SCPL24	0009742	Brassinosteroid mediated signaling pathway	?	
At1g52400	Q9SE50	Beta-D-glucopyranosyl abscisate beta-glucosidase	BGLU18	0009738	ABA-activated signaling pathway	Required for GC-autonomous ABA synthesis during stomatal closure in response to low relative humidity	Bauer et al. (2013)
				0009789	Positive regulation of ABA-activated signaling pathway	Instrumental for stomatal closure in the dark, control of ABA levels and transpiration water loss during dehydration	Lee et al. (2006)
At2g36460	Q9SIQ9	Fructose-bisphosphate aldolase 6, cytosolic (AtFBA6)	FBA6	0031930	Mitochondria-nucleus signaling pathway	Knockout of <i>FBA8</i> causes alterations of GC actin cytoskeleton morphology and results in a reduced rate of stomatal closure in response to decreased humidity	Garagounis et al. (2017)
At2g20890	Q9SKT0	Protein THYLAKOID FORMATION 1, chloroplastic	THF1	0010182	Sugar mediated signaling pathway	<i>Thf1-1</i> mutation promotes stomatal closing, supporting the role of chloroplasts in GCs in stomatal regulation	Wang et al. (2016)

ABA Abscisic acid, GC Guard cell, Question mark (?) no information

chloroplast where it possibly binds to the stem-loop structure of mRNAs and forms a complex together with other stem-loop binding proteins of 55 and 26 kDa. CSP41 has a endoribonuclease activity but this RNAase activity is thought to be suppressed upon integration in that complex (Yang and Stern 1997) and, surprisingly, the authors suggest that this complex even impedes the transcripts from being degraded by other RNAases, thus, resulting in an accumulation of transcripts (Yang et al. 1996). In contrast, free chloroplastidial CSP41 is thought to cleave (RNase activity) mRNA and pre-mRNA to initiate bulk mRNA degradation (Yang and Stern 1997). This dual functions of CSP41 in controlling plastid gene expression is controlled by the availability of light via the redox state of the chloroplast (Qi et al. 2012). During exposure to light, CSP41 proteins do not form complexes (Marín-Navarro et al. 2007). However, in the dark, CSP41 proteins form complexes and Qi et al. (2012) suggest that this is to stabilize non-translated target mRNAs and precursor rRNAs at the dark when the translational process in the chloroplast is not active. The authors suggest that this would allow a rapid onset of translation when the translational machinery is triggered again by light. The induction of dawn, done in this study by lightening the controlled environment chambers, resulted in a transient increases in CSP41 abundances at 15 min after onset of light. The time course in CSP41 protein abundances implies that CSP41 may regulate GC RNA transcription in chloroplasts during dawn.

Seventeen proteins in the GC proteome are annotated as signal transduction proteins

Irrespective of responses to dawn, we found that 17 proteins from our GC proteome are annotated by GO to ‘signal transduction’, ‘signaling pathways’ or ‘signal’ (Table 7). A literature review revealed that five were never studied in the context of GC functioning. They are guanosine nucleotide diphosphate dissociation inhibitor 2 (O24653), GTP-binding nuclear protein Ran-3 (Q8H156), organellar oligopeptidase A, chloroplastic/mitochondrial (Q94AM1), lectin-like protein LEC (Q9LJR2), and serine carboxypeptidase 24 (Q9M099). The serine carboxypeptidase is functional in brassinosteroid mediated signaling pathway whereas the lectin-like protein plays a role in ethylene-activated signaling pathways (Table 7). Further work is necessary for the integration of these proteins into models of GC functioning.

Conclusion

Here we present the GC proteome of *Arabidopsis* and show that it is clearly dynamic over dawn. The work is significant because this is a presentation of the GC proteome

where GC isolation was not effected by protoplasting. Some of the 994 proteins were never before reported to be present in GCs (e.g. the chloroplast stem-loop binding protein CSP41; Fig. 4). Others have annotation to signalling pathways that are, for example, under control of brassinosteroids or ethylene (Table 7). Thus, the presented overview (Table S1) may stimulate future works that help to elucidate details of GC development or stomatal regulation.

Furthermore, our results reinforce the hypothesis that fatty acid beta-oxidation feeds the respiratory ATP production enabling the energy consuming process of GC opening at dawn. This hypothesis was presented by McLachlan et al. (2016) who found that GCs liquidate its stored lipids to supply ATP for use in stomatal opening at dawn. Beyond to the study by McLachlan et al. (2016), the presented study expands this recent finding by revealing the identity of several enzymes that function in the GC lipid metabolism and ATP synthesis over dawn. Enzymes related to peroxisomal beta-oxidation and mitochondrial ATP-synthesis are reduced in abundance when GCs are exposed to light for 1 h. At the same time, photosynthetic proteins increase in abundance. This indicates that GCs reduce lipid turnover when light becomes available. We suggest that, at this time point, energy gained by the oxidation of fatty acids is no longer needed because it is provided by photosynthetic-derived ATP or ATP derived from photoassimilate dissimilation. Future work is needed to elucidate how GC signalling pathways regulate fatty acid biosynthesis at night and their subsequent degradation during dawn.

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Author contributions JL designed the study, grew plants and collected epidermal peels; CMG collected proteomic data; CMG and SC contributed data analysis, SC calculated statistics; CMG, SC and JL wrote the paper.

References

- Assmann SM, Jegla T (2016) Guard cell sensory systems: recent insights on stomatal responses to light, abscisic acid, and CO₂. *Curr Opin Plant Biol* 33:157–167. <https://doi.org/10.1016/j.pbi.2016.07.003>
- Bates GW, Rosenthal DM, Sun J, Chattopadhyay M, Peffer E, Yang J, Ort DR, Jones AM (2012) A comparative study of the *Arabidopsis thaliana* guard-cell transcriptome and its modulation by sucrose. *PLoS ONE* 7(11):e49641. <https://doi.org/10.1371/journal.pone.0049641>

- Bauer H, Ache P, Lautner S, Fromm J, Hartung W, Al-rasheid KA, Sonnewald S, Sonnewald U, Kneitz S, Lachmann N et al (2013) The stomatal response to reduced relative humidity requires guard cell-autonomous ABA synthesis. *Curr Biol* 23:53–57. <https://doi.org/10.1016/j.cub.2012.11.022>
- Baunsgaard L, Fuglsang AT, Jahn T, Korthout HA, De Boer AH, Palmgren MG (1998) The 14-3-3 proteins associate with the plant plasma membrane H⁺-ATPase to generate a fusicoccin binding complex and a fusicoccin responsive system. *Plant J* 13:661–671. <https://doi.org/10.1046/j.1365-313X.1998.00083.x>
- Buts K, Michielssens S, Hertog ML, Hayakawa E, Cordewener J, America AH, Nicolai BM, Carpentier SC (2014) Improving the identification rate of data independent label-free quantitative proteomics experiments on non-model crops: a case study on apple fruit. *J Proteom* 105:31–45. <https://doi.org/10.1016/j.jprote.2014.02.015>
- Campos NA, Paiva LV, Panis B, Carpentier SC (2016) The proteome profile of embryogenic cell suspensions of *Coffea arabica* L. *Proteomics* 16(6):1001–1005. <https://doi.org/10.1002/pmic.201503999>
- Carpentier SC, Witters E, Laukens K, Deckers P, Swennen R, Panis B (2005) Preparation of protein extracts from recalcitrant plant tissues: an evaluation of different methods for two-dimensional gel electrophoresis analysis. *Proteomics*. <https://doi.org/10.1002/pmic.200401222>
- Carpentier SC, Panis B, Swennen R, Lammertyn J (2008) Finding the significant markers: statistical analysis of proteomic data. *Methods Mol Biol* 428:327–347. https://doi.org/10.1007/978-1-59745-117-8_17
- Cornish K, Zeevaert JAD (1986) Absciscic acid accumulation by in situ and isolated guard cells of *Pisum sativum* L. & *Vicia faba* L. in relation to water stress. *Plant Physiol* 81:1017–1021. <https://doi.org/10.1104/pp.81.4.1017>
- Dodd AN, Parkinson K, Webb AA (2004) Independent circadian regulation of assimilation and stomatal conductance in the *ztl-1* mutant of *Arabidopsis*. *New Phytol* 162(1):63–70. <https://doi.org/10.1111/j.1469-8137.2004.01005.x>
- Du Z, Zhou X, Ling Y, Zhang Z, Su Z (2010) agriGO: a GO analysis toolkit for the agricultural community. *Nucleic Acids Res*. <https://doi.org/10.1093/nar/gkq310>
- Flores-Tornero M, Anoman AD, Rosa-Téllez S, Toujani W, Weber AP, Eisenhut M et al (2017) Overexpression of the triose phosphate translocator (TPT) complements the abnormal metabolism and development of plastidial glycolytic glyceraldehyde-3-phosphate dehydrogenase mutants. *Plant J* 89(6):1146–1158. <https://doi.org/10.1111/tj.13452>
- Garagounis C, Kostaki KI, Hawkins TJ, Cummins I, Fricker MD, Hussey PJ, Hetherington AM, Sweetlove LJ (2017) Microcompartmentation of cytosolic aldolase by interaction with the actin cytoskeleton in *Arabidopsis*. *J Exp Bot* 68(5):885–898. <https://doi.org/10.1093/jxb/erx015>
- Geilfus C-M (2017) The pH of the apoplast: dynamic factor with functional impact under stress. *Mol Plant* 10(11):1371–1386. <https://doi.org/10.1016/j.molp.2017.09.018>
- Geilfus C-M, Mithöfer A, Ludwig-Müller J, Zörb C, Muehling KH (2015) Chloride-inducible transient apoplastic alkalizations induce stomata closure by controlling abscisic acid distribution between leaf apoplast and guard cells in salt-stressed *Vicia faba*. *New Phytol* 208(3):803–816. <https://doi.org/10.1111/nph.13507>
- Geilfus C-M, Carpentier SC, Zavišić A, Polle A (2017a) Changes in the fine root proteome of *Fagus sylvatica* L. trees associated with P-deficiency and amelioration of P-deficiency. *J Proteom* 169:33–40
- Geilfus C-M, Tenhaken R, Carpentier SC (2017b) Transient alkalization of the leaf apoplast stiffens the cell wall during onset of chloride-salinity in corn leaves. *J. Biol Chem*. <https://doi.org/10.1074/jbc.M117.799866>
- Geng S, Yu B, Zhu N, Dufresne C, Chen S (2017) Metabolomics and proteomics of *Brassica napus* guard cells in response to low CO₂. *Front Mol Biosci* 4:51. <https://doi.org/10.3389/fmolb.2017.00051>
- Graf A, Coman D, Uhrig RG, Walsh S, Flis A, Stitt M, Gruissem W (2017) Parallel analysis of *Arabidopsis* circadian clock mutants reveals different scales of transcriptome and proteome regulation. *Open Biol* 7(3):160333. <https://doi.org/10.1098/rsob.160333>
- Harmer SL, Hogenesch JB, Straume M, Chang HS, Han B, Zhu T et al (2000) Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. *Science* 290(5499):2110–2113. <https://doi.org/10.1126/science.290.5499.2110>
- Harwood JL (1988) Fatty acid metabolism. *Annu Rev Plant Physiol Plant Mol Biol* 39:101–138. <https://doi.org/10.1146/annurev.pp.39.060188.000533>
- Haslam TM, Mañas-Fernández A, Zhao L, Kunst L (2012) *Arabidopsis* ECERIFERUM2 is a component of the fatty acid elongation machinery required for fatty acid extension to exceptional lengths. *Plant Physiol* 160:1164–1174. <https://doi.org/10.1104/pp.112.201640>
- Hedrich R, Geiger D (2017) Biology of SLAC1-type anion channels—from nutrient uptake to stomatal closure. *New Phytol* 216(1):46–61. <https://doi.org/10.1111/nph.14685>
- Hennessey TL, Field CB (1991) Circadian rhythms in photosynthesis: oscillations in carbon assimilation and stomatal conductance under constant conditions. *Plant Physiol* 96(3):831–836. <https://doi.org/10.1104/pp.96.3.831>
- Hetherington AM, Woodward FI (2003) The role of stomata in sensing and driving environmental change. *Nature* 424:901–908. <https://doi.org/10.1038/nature01843>
- Hiyama A, Takemiya A, Munemasa S, Okuma E, Sugiyama N, Tada Y et al (2017) Blue light and CO₂ signals converge to regulate light-induced stomatal opening. *Nature Commun* 8(1):1284. <https://doi.org/10.1038/s41467-017-01237-5>
- Horrer D, Flüttsch S, Pazmino D, Matthews JS, Thalmann M, Nigro A, Leonhardt N, Lawson T, Santelia D (2016) Blue light induces a distinct starch degradation pathway in guard cells for stomatal opening. *Curr Biol* 26(3):362–370. <https://doi.org/10.1016/j.cub.2015.12.036>
- Huang DW, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4(1):44–57. <https://doi.org/10.1038/nprot.2008.211>
- Inoue SI, Kinoshita T (2017) Blue light regulation of stomatal opening and the plasma membrane H⁺-ATPase. *Plant Physiol* 174(2):531–538. <https://doi.org/10.1104/pp.17.00166>
- Jones AM, Xuan Y, Xu M, Wang RS, Ho CH, Lalonde S et al (2014) Border control—a membrane-linked interactome of *Arabidopsis*. *Science* 344(6185):711–716. <https://doi.org/10.1126/science.1251358>
- Kamata N, Okada H, Komeda Y, Takahashi T (2013) Mutations in epidermis-specific HD-ZIP IV genes affect floral organ identity in *Arabidopsis thaliana*. *Plant J* 75(3):430–440. <https://doi.org/10.1111/tj.12211>
- Kinoshita T, Shimazaki K (2002) Biochemical evidence for the requirement of 14-3-3 protein binding in activation of the guard-cell plasma membrane H⁺-ATPase by blue light. *Plant Cell Physiol* 43:1359–1365. <https://doi.org/10.1093/pcp/pcf167>
- Kinoshita T, Doi M, Suetsugu N, Kagawa T, Wada M, Shimazaki K (2001) Phot1 and phot2 mediate blue light regulation of stomatal opening. *Nature* 414:656–660. <https://doi.org/10.1038/414656a>
- Kollist H, Nuhkat M, Roelfsema MRG (2014) Closing gaps: linking elements that control stomatal movement. *New Phytol* 203(1):44–62. <https://doi.org/10.1111/nph.12832>

- Krebs M, Beyhl D, Görlich E, Al-Rasheid KAS, Marten I, Stierhof YD, Hedrich R, Schumacher K (2010) *Arabidopsis* V-ATPase activity at the tonoplast is required for efficient nutrient storage but not for sodium accumulation. *Proc Natl Acad Sci USA* 107:3251–3256
- Kurdyukov S, Faust A, Nawrath C, Bär S, Voisin D, Efremova N, Franke R, Schreiber L, Saedler H, Métraux JP, Yephremov A (2006) The epidermis-specific extracellular BODYGUARD controls cuticle development and morphogenesis in *Arabidopsis*. *Plant Cell* 18(2):321–339. <https://doi.org/10.1105/tpc.105.036079>
- Lee MM, Schiefelbein J (1999) WEREWOLF, a MYB-related protein in *Arabidopsis*, is a position-dependent regulator of epidermal cell patterning. *Cell* 99(5):473–483. [https://doi.org/10.1016/S0092-8674\(00\)81536-6](https://doi.org/10.1016/S0092-8674(00)81536-6)
- Lee KH, Piao HL, Kim HY, Choi SM, Jiang F, Hartung W, Hwang I, Kwak JM, Lee JJ, Hwang I (2006) Activation of glucosidase via stress-induced polymerization rapidly increases active pools of abscisic acid. *Cell* 126(6):1109–1120. <https://doi.org/10.1016/j.cell.2006.07.034>
- Leonhardt N, Kwak JM, Robert N, Waner D, Leonhardt G, Schroeder JJ (2004) Microarray expression analyses of *Arabidopsis* guard cells and isolation of a recessive abscisic acid hypersensitive protein phosphatase 2C mutant. *Plant Cell* 16(3):596–615. <https://doi.org/10.1105/tpc.019000>
- Liu J, Ji Y, Zhou J, Xing D (2016) Phosphatidylinositol 3-kinase promotes V-ATPase activation and vacuolar acidification and delays methyl jasmonate-induced leaf senescence. *Plant Physiol.* <https://doi.org/10.1104/pp.15.00744>
- Mach J (2008) Guard cell proteome reveals signals and surprises. *Plant Cell* 20(12):3185–3185. <https://doi.org/10.1105/tpc.108.201214>
- Marín-Navarro J, Manuell AL, Wu J, Mayfield SP (2007) Chloroplast translation regulation. *Photosynth Res* 94:359–374. <https://doi.org/10.1007/s11200-007-9183-z>
- Matrosova A, Bogireddi H, Mateo-Peñas A, Hashimoto-Sugimoto M, Iba K, Schroeder JJ, Israelsson-Nordström M (2015) The HT1 protein kinase is essential for red light-induced stomatal opening and genetically interacts with OST1 in red light and CO₂-induced stomatal movement responses. *New Phytol* 208(4):1126–1137. <https://doi.org/10.1111/nph.13566>
- Matsushika A, Makino S, Kojima M, Mizuno T (2000) Circadian waves of expression of the APRR1/TOC1 family of pseudo-response regulators in *Arabidopsis thaliana*: insight into the plant circadian clock. *Plant Cell Physiol* 41(9):1002–1012. <https://doi.org/10.1093/pcp/pcd043>
- McLachlan DH, Lan J, Geilfus C, Dodd AN, Larson T, Baker A, Hörak H, Kollist H, He Z, Graham I, Mickelbart MV, Hetherington AM (2016) The breakdown of stored triacylglycerols is required during light-induced stomatal opening. *Curr Biol* 26:1–6. <https://doi.org/10.1016/j.cub.2016.01.019>
- Merilo E, Jalakas P, Laanemets K, Mohammadi O, Hörak H, Kollist H, Brosché M (2015) Abscisic acid transport and homeostasis in the context of stomatal regulation. *Mol Plant* 8(9):1321–1333. <https://doi.org/10.1016/j.molp.2015.06.006>
- Messinger SM, Buckley TN, Mott KA (2006) Evidence for involvement of photosynthetic processes in the stomatal response to CO₂. *Plant Physiol* 140:771–778. <https://doi.org/10.1104/pp.105.073676>
- Mi H, Muruganujan A, Thomas PD (2013) PANTHER in 2013: modeling the evolution of gene function, and other gene attributes, in the context of phylogenetic trees. *Nucleic Acids Res.* <https://doi.org/10.1093/nar/gks1118>
- Mori IC, Murata Y, Yang Y, Munemasa S, Wang YF, Andreoli S et al (2006) CDPKs CPK6 and CPK3 function in ABA regulation of guard cell S-type anion- and Ca²⁺-permeable channels and stomatal closure. *PLoS Biol* 4(10):e327. <https://doi.org/10.1371/journal.pbio.0040327>
- Pilot G, Lacombe B, Gaymard F, Chérel I, Boucherez J, Thibaud JB, Sentenac H (2001) Guard cell inward K⁺ channel activity in *Arabidopsis* involves expression of the twin channel subunits KAT1 and KAT2. *J Biol Chem* 276:3215–3221. <https://doi.org/10.1074/jbc.M007303200>
- Pornsiriwong W, Estavillo GM, Chan KX, Tee EE, Ganguly D, Crisp PA et al (2017) A chloroplast retrograde signal, 3'-phosphoadenosine 5'-phosphate, acts as a secondary messenger in abscisic acid signaling in stomatal closure and germination. *ELife* 6:e23361. <https://doi.org/10.7554/eLife.23361>
- Pracharoenwattana I, Cornah JE, Smith SM (2005) *Arabidopsis* peroxisomal citrate synthase is required for fatty acid respiration and seed germination. *Plant Cell* 17(7):2037–2048. <https://doi.org/10.1105/tpc.105.031856>
- Qi Y, Armbruster U, Schmitz-Linneweber C, Delannoy E, De Longevialle AF, Rühle T, Small I, Jahns P, Leister D (2012) *Arabidopsis* CSP41 proteins form multimeric complexes that bind and stabilize distinct plastid transcripts. *J Exp Bot* 63:1251–1270. <https://doi.org/10.1093/jxb/err347>
- Roelfsema MRG, Hedrich R (2002) Studying guard cells in the intact plant: modulation of stomatal movement by apoplastic factors. *New Phytol* 153(3):425–431. <https://doi.org/10.1046/j.0028-646X.2001>
- Roelfsema M, Hanstein S, Felle H, Hedrich R (2002) CO₂ provides an intermediate link in the red light response of guard cells. *Plant J* 32:65–75. <https://doi.org/10.1046/j.1365-3113X.2002.01403.x>
- Santelia D, Lunn JE (2017) Transitory starch metabolism in guard cells: unique features for a unique function. *Plant Physiol* 174(2):539–549. <https://doi.org/10.1104/pp.17.00211>
- Schäfer N, Maierhofer T, Herrmann J, Jørgensen ME, Lind C, von Meyer K et al (2018) A tandem amino acid residue motif in guard cell SLAC1 anion channel of grasses allows for the control of stomatal aperture by nitrate. *Curr Biol* 28(9):1370–1379
- Schaffer R, Ramsay N, Samach A, Corden S, Putterill J, Carré IA, Coupland G (1998) The late elongated hypocotyl mutation of *Arabidopsis* disrupts circadian rhythms and the photoperiodic control of flowering. *Cell* 93:1219–1229. [https://doi.org/10.1016/S0092-8674\(00\)81465-8](https://doi.org/10.1016/S0092-8674(00)81465-8)
- Shimakata T, Stumpf PK (1982) Isolation and function of spinach leaf β -ketoacyl-[acyl-carrier-protein] synthases. *Proc Natl Acad Sci* 79:5808–5812. <https://doi.org/10.1073/pnas.79.19.5808>
- Shimazaki K, Doi M, Assmann SM, Kinoshita T (2007) Light regulation of stomatal movement. *Annu Rev Plant Biol* 58:219–247. <https://doi.org/10.1146/annurev.arplant.57.032905.105434>
- Somers DE, Webb AA, Pearson M, Kay SA (1998) The short-period mutant, *tocl-1*, alters circadian clock regulation of multiple outputs throughout development in *Arabidopsis thaliana*. *Development* 125(3):485–494
- Stern DB, Gruissem W (1987) Control of plastid gene expression: 3' inverted repeats act as mRNA processing and stabilizing elements, but do not terminate transcription. *Cell* 51:1145–1157. [https://doi.org/10.1016/0092-8674\(87\)90600-3](https://doi.org/10.1016/0092-8674(87)90600-3)
- Taiz L, Zeiger E, Möller I, Murphy A (2015) In: *Plant physiology and development*, 6th edn. Sinauer Associates, Sunderland
- Takemiya A, Sugiyama N, Fujimoto H, Tsutsumi T, Yamauchi S, Hiyama A, Tada Y, Christie JM, Shimazaki K (2013) Phosphorylation of BLUS1 kinase by phototropins is a primary step in stomatal opening. *Nat Commun.* <https://doi.org/10.1038/ncomms3094>
- Tominaga R, Iwata M, Okada K, Wada T (2007) Functional analysis of the epidermal-specific MYB genes CAPRICE and WEREWOLF in *Arabidopsis*. *Plant Cell* 19(7):2264–2277. <https://doi.org/10.1105/tpc.106.045732>
- Ullah H, Scappini EL, Moon AF, Williams LV, Armstrong DL, Pedersen LC (2008) Structure of a signal transduction regulator, RACK1, from *Arabidopsis thaliana*. *Protein Sci* 17(10):1771–1780. <https://doi.org/10.1110/ps.035121.108>
- Wada T, Tachibana T, Shimura Y, Okada K (1997) Epidermal cell differentiation in *Arabidopsis* determined by a Myb homolog,

- CPC. *Science* 277(5329):1113–1116. <https://doi.org/10.1126/science.277.5329.1113>
- Wang ZY, Tobin EM (1998) Constitutive expression of the CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) gene disrupts circadian rhythms and suppresses its own expression. *Cell* 93:1207–1217. [https://doi.org/10.1016/S0092-8674\(00\)81464-6](https://doi.org/10.1016/S0092-8674(00)81464-6)
- Wang Z, Wang F, Hong Y, Huang J, Shi H, Zhu JK (2016) Two chloroplast proteins suppress drought resistance by affecting ROS production in guard cells. *Plant Physiol* 172(4):2491–2503. <https://doi.org/10.1104/pp.16.00889>
- Wang W, Liu Z, Bao LJ, Zhang SS, Zhang CG, Li X et al (2017) The Rho-GTPase RopGEF2-ROP7/ROP2 pathway activated by phyB suppresses red light-induced stomatal opening. *Plant Physiol* 174(2):717–731. <https://doi.org/10.1104/pp.16.01727>
- Yamauchi S, Takemiya A, Sakamoto T, Kurata T, Tsutsumi T, Kinoshita T, Shimazaki K (2016) The plasma membrane H⁺-ATPase AHA1 plays a major role in stomatal opening in response to blue light. *Plant Physiol* 171:2731–2743. <https://doi.org/10.1104/pp.16.01581>
- Yang J, Stern DB (1997) The spinach chloroplast endoribonuclease CSP41 cleaves the 3'-untranslated region of petD mRNA primarily within its terminal stem-loop structure. *J Biol Chem* 272:12874–12880. <https://doi.org/10.1074/jbc.272.19.12874>
- Yang J, Schuster G, Stern DB (1996) CSP41, a sequence-specific chloroplast mRNA binding protein, is an endoribonuclease. *Plant Cell* 8:1409–1420. <https://doi.org/10.1105/tpc.8.8.1409>
- Zeeman SC, Delatte T, Messerli G, Umhang M, Stettler M, Mettler-Altmann T, Streb S, Reinhold H, Kötting O (2007) Starch breakdown: recent discoveries suggest distinct pathways and novel mechanisms. *Funct Plant Biol* 34(6):465–473
- Zeiger E, Zhu J (1998) Role of zeaxanthin in blue light photoreception and the modulation of light-CO₂ interactions in guard cells. *J Exp Bot* 49(1):433–442. <https://doi.org/10.1093/jxb/49>
- Zhang XF, Jiang T, Wu Z, Du SY, Yu YT, Jiang SC, Lu K, Feng XJ, Wang XF, Zhang DP (2013) Cochaperonin CPN20 negatively regulates abscisic acid signaling in *Arabidopsis*. *Plant Mol Biol* 83(3):205–218. <https://doi.org/10.1007/s11103-013-0082-8>
- Zhao Z, Zhang W, Stanley BA, Assmann SM (2008) Functional proteomics of *Arabidopsis thaliana* guard cells uncovers new stomatal signaling pathways. *Plant Cell* 20:3210–3226. <https://doi.org/10.1105/tpc.108.063263>