Multi-omic analysis of the Arabidopsis clock activator mutant *rve 4 6 8* reveals connections to carbohydrate metabolism and proteasome regulation

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ABSTRACT

Plants are able to sense changes in their light environments, such as the onset of day and night, as well as anticipate these changes in order to adapt and survive. Central to this ability is the plant circadian clock, a molecular circuit that precisely orchestrates plant cell processes over the course of a day. REVEILLE proteins (RVEs) are recently discovered members of the plant circadian circuitry that activate the evening complex and PRR genes to maintain regular circadian oscillation. The RVE 8 protein and its two homologs, RVE 4 and 6, have been shown to limit the length of the circadian period, with *rve 4 6 8* triple-knockout plants possessing an elongated period along with increased leaf surface area, biomass, cell size and delayed flowering relative to wild-type Col-0 plants. Here, using a multi-omics approach consisting of phenomics, transcriptomics, proteomics, and metabolomics we draw novel connections between RVE8-like proteins and a number of core plant cell processes. In particular, we reveal that loss of RVE8-like proteins results in altered carbohydrate, organic acid and lipid metabolism, including a starch excess phenotype at dawn. We further demonstrate that *rve 4 6 8* plants have lower levels of 20S proteasome subunits and possess significantly reduced proteasome activity, potentially explaining the increase in cell-size observed in RVE8-like mutants. Overall, this robust, multi-omic dataset, provides substantial new insights into the far reaching impact RVE8-like proteins have on the diel plant cell environment.

INTRODUCTION

The circadian clock is a central regulator of plant growth and development that modulates plant responses to both internal and external cues (Greenham and McClung, 2015; Nohales and Kay, 2016; McClung, 2019). It is comprised of a number of transcription factors that function as a series of interlocking molecular circuits that precisely time the 24-hour photoperiod (Creux and Harmer, 2019). Correspondingly, knockout mutants of circadian clock transcription factors have been extensively studied at the phenotypic, genetic and transcript-level in the model plant *Arabidopsis thaliana* (Creux and Harmer, 2019; McClung, 2019; Nakamichi, 2020), with upwards of 30% of genes shown to be under circadian control (Covington et al., 2008). This extensive body of work has revealed a number of roles for the circadian clock in plants, including in the timing of flowering (Shim et al., 2017), disease resistance (Lu et al., 2017) and mitigation of abiotic stress (Simon et al., 2020; Kidokoro et al., 2021) amongst others. However, despite the extensive use of genetic and transcriptomic technologies to understand the molecular impacts of core circadian clock components in *Arabidopsis*, much less is known about how, and/or if, these transcriptional changes manifest at the protein-level (Choudhary et al., 2015; Choudhary et al., 2016; Krahmer et al., 2022; Uhrig et al., 2019; Mehta et al., 2021; Uhrig et al., 2021).

Protein abundance and post-translational protein modification (PTM) changes are critical control mechanisms for biological systems. To date, quantitative proteomic analysis of circadian clock mutants *lhy cca1*, *prr7 prr9*, *gi* and *toc1* at end-of-day (ED; ZT12) and end-of-night (EN; ZT0) time-points (Graf et al., 2017) and time-course experimentation involving $CCA1_{ox}$ (Krahmer et al., 2022) and wild-type (WT) Col-0 (Uhrig et al., 2021) plants over free-running and 12:12 light:dark (LD) conditions have provided the first insights into where diel changes in the proteome manifest. Similarly, time-course and ED versus EN analyses of the phosphoproteome from $CCA1_{ox}$ (Krahmer et al., 2022) and WT Col-0 (Uhrig et al., 2019) plants point to a critical, but largely unexplored, role for protein phosphorylation and other PTMs in diel plant cell regulation; particularly at ED and EN. Beyond proteomics, quantitative metabolomic analyses of core circadian clock mutants can also help define the role the circadian clock plays in regulating growth and

development through the modulation of primary metabolism (Fukushima et al., 2009; Flis

et al., 2019).

REVEILLE (RVE) genes are recently discovered additions to the circadian clock that

have been found to function as core circadian clock activators, modulating the regulation

of the evening complex (EARLY FLOWERING 3 (ELF3), 4 (ELF4) and LUX

ARRHYTHMO (LUX)) in addition to TIMING OF CAB EXPRESSION 1 (TOC1) and

PSEUDO RESPONSE REGULATOR 5 (PRR5) (Farinas and Mas, 2011; Rawat et al.,

2011; Hsu et al., 2013; Gray et al., 2017). Findings that *rve 8* (Farinas and Mas, 2011;

Rawat et al., 2011) and *rve 4 6 8* (Gray et al., 2017) plants possess an elongated circadian

period demonstrate how RVE proteins are important to maintaining the pace of the

circadian clock, while phenotypic characterization of the *rve 4 6 8* plants revealed greater

plant growth and cell size, coupled with a delay in flowering (Gray et al., 2017).

Despite these findings, how RVE8-like proteins regulate the diel plant cell environment

at the protein- and metabolic-level remains unresolved. To address this gap in knowledge,

we have undertaken a multi-omic analysis of *rve 4 6 8* plants relative to WT Col-0 at

critical diel / circadian time-points; EN / ZT0 and ED / ZT12. Here, we demonstrate that

loss of RVE 4, 6 and 8 results in extensive diel proteome, phosphoproteome and

metabolome changes spanning a wide array of critical plant cell processes. In particular,

our results suggest that RVE8-like proteins impact critical elements of primary metabolism

such as transient diel leaf-starch / carbohydrate levels. Perhaps most intriguingly, we find

that *rve 4 6 8* plants maintain extensive protein- and PTM-level perturbations in

proteasome subunits, coupled with reduced proteasome activity and altered amino acids

levels, which together, likely form the basis of the increased growth phenotype of *rve 4 6*

8. As RVE8-like proteins are associated with multiple agronomically important traits such

as biomass and flowering time, elucidating their role in modulating the diel proteome,

PTMome and metabolome is critical for their potential implementation in crop

improvement strategies.

RESULTS

Examination of *rve 4 6 8* mutant phenotypes reveals starch excess at dawn.

In order to ensure our molecular analysis would provide robust results, we first phenotypically characterized *rve 4 6 8* plants relative to WT Col-0 (Col-0) under our growth conditions (12:12 LD photoperiod facilitated by LED lights). Here, we were able to reproduce multiple growth and development phenotypes previously observed (Gray et al., 2017), indicating that our growth conditions are comparable (Supplemental Figure 1). We monitored plant area and perimeter using real-time imaging over 5 days and found significant differences between *rve 4 6 8* and WT Col-0 arise by day 17 post-imbibition, with *rve 4 6 8* plants possessing increased leaf area and perimeter (Supplemental Figure 1A & B). We then performed fresh and dry weight measurements of *rve 4 6 8* versus WT Col-0 plants at day 19 post-imbibition revealing that *rve 4 6 8* has greater biomass (Supplemental Figure 1C & D). Lastly, under our 12:12 LD conditions, we found *rve 4 6 8* plants have delayed flowering relative to WT Col-0 (Supplemental Figure 1E).

Given these growth phenotypes and the previously observed response of *rve 4 6 8* to sucrose (Gray et al., 2017), we next stained *rve 4 6 8* and WT Col-0 rosettes for the accumulation of transient leaf starch over a 12:12 LD photoperiod examining ZT0, 6, 12 and 18 time-points. This revealed that *rve 4 6 8* plants possess a starch excess phenotype at ZT0 (Figure 1A). We then validated this analysis by performing an enzymatic quantitation of starch levels in WT Col-0 and *rve 4 6 8* plants (Figure 1B). Samples were collected hourly from ZT21 to ZT3 in order to enzymatically assess if the starch excess observed was due to the long-period phase shift of the *rve 4 6 8* mutant clock. Here, our results indicate that the observed starch excess is in fact independent of the long-period phase shift of the *rve 4 6 8* mutant (Figure 1B). We then further examined three key starch metabolism related enzymes for changes in their gene expression using NanoString RNA quantification: *PHOSPHOGLYCERATE MUTASE 1* (*PGM1*), *GRANUAL BOUND STARCH SYNTHASE 1* (*GBSS1*) and *STARCH EXCESS 4* (*SEX4*). *GBSS1* and *SEX4* transcript levels were significantly lower in *rve 4 6 8* compared to WT Col-0 at ZT0 and ZT12, respectively, while no change was observed in PGM expression (Figure 1C and D). Together with our phenotypic data, these results suggest a potential disconnect between the timing of starch degradation and biosynthesis that results in greater transient starch accumulation at dawn.

Gene expression analysis provides molecular insights into *rve 4 6 8* growth phenotypes.

Using NanoString RNA quantification, we next examined the expression of a panel of core circadian clock genes and genes involved in processes impacted by the circadian clock (Figure 2; Supplemental Figure 2 Supplemental Data 1). Studies examining RVE 8-like proteins (RVE 4, 6 & 8) have observed changes in multiple core circadian clock and clock-associated genes (Creux and Harmer, 2019), including *PSEUDO RESPONSE REGULATOR 5* (*PRR5*), *TIMING OF CAB1 EXPRESSION 1* (*TOC1*), *EARLY FLOWERING 4* (*ELF4*), *PHYTOCHROME INTERACTING FACTOR 4* (*PIF4*) and *5* (*PIF5*). We aimed to consolidate this understanding by examining changes in these genes, amongst others, in *rve 4 6 8* plants at ZT0 and ZT12 time-points under 12:12 LD conditions. Here, we find a decrease in *TOC1* and *ELF4* transcript levels combined with an increase in *PRR5*, *PIF4* and *PIF5* expression in *rve 4 6 8* at ZT12 (Figure 2A). We also observed a decrease in *CIRCADIAN CLOCK REGULATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) expression at ZT0 along with increases in *PSEUDO RESPONSE REGULATOR 7* (*PRR7*) and *GIGANTEA* (*GI*) at ZT12 that are also consistent with an elongated period (Hsu et al., 2013; Flis et al., 2016). While our PRR5 result accords with previous findings, our Nanostring quantification shows reduced TOC1 transcript levels while previous qPCR results have shown increased TOC1 levels at ZT12 (Hsu et al, 2013). Similarly, while we observe reduced transcript levels of LHY and CCA1 in the mutant, the previous study does not observe any difference. These differences between the two studies is likely due to the different measurement techniques used (qPCR vs Nanostring) and the relatively larger variation in expression recorded in Hsu et al. 2013. Collectively, our results align with previous research examining RVE8-like proteins, however, more time-points are needed to precisely define the impact of RVE8-like proteins on the phasing and period lengths of core clock transcripts.

Photoreceptors are crucial in circadian clock entrainment by light, but are in turn also controlled by the clock (Bognar et al., 1999; Sanchez et al., 2020). Correspondingly, we next examined *rve 4 6 8* plants for changes in the expression of key photoreceptor genes, quantifying changes in the expression of *PHYTOCHROME* (*PHY*) *A* and *B*, *PHOTOTROPIN* (*PHOT*) *1* and *2, CRYPTOCHROME 1* (*CRY1*) and the circadian clock

associated photoreceptor *ZEITLUPE* (*ZTL*) in *rve 4 6 8* versus WT Col-0 at ZT0 and ZT12.

Here, we found a significant decrease in *PHYA* and a significant increase in *PHOT1*

expression in *rve 4 6 8* plants at ZT12, with no change in *PHYB*, *PHOT2*, *CRY1* and *ZTL*

(Figure 2B).

Since *rve 4 6 8* plants demonstrate a number of growth phenotypes, we additionally

examined the expression of genes upstream of, or involved in, phytohormone related-

processes. Here, we find significant changes in *GERANYLGERANYL*

PYROPHOSPHATE SYNTHASE 1 (*GGPPS1*) at both ZT0 and ZT12 as well

ARABIDOPSIS RESPONSE REGULATOR 7 (*ARR7*) at ZT12 (Supplemental Figure 2).

While these genes represent only a sampling of the phytohormone metabolism / signaling

landscape, both their enzyme products have a common connection to Dimethylallyl

Pyrophosphate (DMAPP) / Isoprenyl Pyrophosphate (IPP), a critical metabolic

intermediate. GGPPS1 catalyzes the entry point reaction for downstream production of

multiple phytohormones, including: abscisic acid, gibberellin and stringolactone

biosynthesis (Ruiz-Sola et al., 2016). Cytokinin is also derived from the DMAPP / IPP

metabolic branch point (Astot et al., 2000) and ARR7 represents a cytokinin response

factor that is activated in the presence of cytokinins (Huang et al., 2017).

Quantitative proteome and phosphoproteome analyses reveal extensive protein-

level impacts of RVE8-like protein loss.

Quantifying proteome-level changes in the plant cell environment in response to

perturbation (e.g. gene deletion) is a robust means of measuring the impact of a protein

on a system. However, the high dynamic range nature of plant tissues has typically

hampered our ability to sufficiently sample the dynamically fluctuating proteome, biasing

analyses towards high abundant proteins (Mehta et al., 2022; Mehta et al., 2021). Given

this, we deployed a combination of BoxCarDIA (proteome abundance) and conventional

DDA (phosphoproteomics) mass spectrometry acquisition methods to perform label-free

quantitation (LFQ) of protein abundance and phosphoproteomic changes in *rve 4 6 8*

plants at ZT0 and ZT12. The ZT0 and ZT12 time-points were selected for analysis as they

have been shown to exhibit dynamic protein-level changes (e.g. abundance and PTMs)

across multiple plant organs, tissues and growth conditions in a variety of wild-type and

circadian clock mutant plants (Graf et al., 2017; Krahmer et al., 2022; Uhrig et al., 2019;

Uhrig et al., 2021). Through this analysis we quantified 3,934 proteins and 1,137

phosphoproteins for changes in abundance and phosphorylation status, of which 536 and

346 demonstrated significant fluctuations, respectively (Table I; Supplemental Data 2 and

3). Further, we quantified 46% of all proteins exhibiting a significant change in their

phosphorylation status at the proteome-level (159 / 347 phosphoproteins), with 8.6% of

phosphoproteins demonstrating a corresponding change in protein abundance (30 / 347

phosphoproteins).

Next, using significantly changing proteins and phosphoproteins, we performed a

gene ontology (GO) enrichment analysis for biological processes, subcellular localization

and molecular function to reveal how the loss of RVE8-like proteins impacts the diel plant

cell environment at the protein-level (Figure 3; Supplemental Data 4). Here, we found

changes in 'proteolysis' (GO:0006508), which was complemented by the enrichment of

'proteasome core complex' (GO:0005839) and 'proteasome complex' (GO:0000502)

subcellular localizations, in addition to 'response to acid chemical' (GO:0001101), which

represents a high-level category for phytohormones and 'polysaccharide metabolism'

(GO:0005976), amongst others (Figure 3A). Similarly, phosphoproteomic data revealed

enrichment in 'response to acid chemical' (GO:0001101) and 'response to abscisic acid'

(GO:0009737) in addition to 'cell cycle' (GO:0007049), 'cell division' (GO:0051301),

'macromolecule metabolism' (GO:0043170) and 'response to lipids (GO:0033993),

amongst others (Figure 3B). While proteome-level *rve 4 6 8*-dependent changes included

both increases and decreases in protein levels, we found a global increase in protein

phosphorylation in the *rve 4 6 8* mutant compared to WT Col-0 (Figure 3B).

Lastly, we compared our significantly changing phosphopeptides at ZT0 and ZT12

with phospho-sites previously found to oscillate under free-running conditions in WT Col-

0 (Krahmer et al., 2022). Here we found that 77 of our phosphopeptides (*q-value* ≤ 0.05)

significantly changed in *rve 4 6 8* compared to WT Col-0 (JTK *p-value* ≤ 0.05;

Supplemental Data 5). These oscillating phosphopeptides are from diverse, but critically

important proteins such as nuclear localized RETINOBLASTOMA-RELATED 1 (RBR;

AT3G12280) – growth and development, cytosolic PHOSPHOENOLPYRUVATE

CARBOXYLASE 2 (PEPC2; AT2G42600) and chloroplast-targeted RUBISCO

ACTIVASE (AT2G39730) - metabolism and plasma membrane associated CELLULOSE

SYNTHASE-LIKE D3 (CSLD3; AT3G03050) – cell wall biosynthesis (Supplemental Data

5). This indicates that RVE8-like proteins influence the timing of diel phosphorylation

events on proteins involved in core growth and development processes.

Time-of-day association network analysis finds multiple gene networks affected in

***rve 4 6 8* at the protein-level.**

To contextualize the changing proteome and phosphoproteome, we next generated

STRING-DB association networks (minimum edge score ≥ 0.7) for all significantly

changing proteins and phosphoproteins in *rve 4 6 8* plants at ZT0 and ZT12 relative to

WT Col-0 to better understand the protein-level changes that underpin observed *rve 4 6*

8 phenotypes. Proteome quantification revealed decreases in the abundance of multiple

starch degradation enzymes including STARCH EXCESS 1 (SEX1; AT1G10760) and

SEX4 as well as cold-stress proteins COLD-REGULATED 15A (COR15A; AT2G42540),

15B (COR15B; AT2G42530) and 6 (COR6; AT5G15970). Conversely, we observed

increases in primary metabolic and sulfur assimilation enzymes such as

PHOSPHOFRUCTOKINASE 5 (PFK5; AT2G22480), PYRUVATE ORHOPHOSPHATE

DIKINASE (PPDK; AT4G15530) 5'-ADENYLYLPHOSPHOSULFATE REDUCTASE 2

(APR2; AT1G62180) and ADENYLYL-SULFATE KINASE (APK; AT2G14750), along with

a multitude of spliceosomal, ribosomal and ubiquitin ligating proteins. Together, these

proteomic data support our observed starch excess phenotype in *rve 4 6 8* at ZT0, while

suggesting RVE8-like proteins also impact agronomically important processes such as

sulfur assimilation and cold stress response (Figure 4). We also observed distinct

changes in proteasome complex proteins, whereby four 19S proteasome proteins

(AT1G04810, AT1G75990; AT4G19006, AT4G24820) are up-regulated and twelve 20S

proteasome proteins are down-regulated in *rve 4 6 8* plants (Figure 4; Supplemental Data

6). Further examination of the transcripts corresponding to the proteasome subunits

changing at the protein-level using DiurnalDB (http://diurnal.mocklerlab.org/) found that 9

of the 12 20S subunits and 2 of the 4 19S subunits possess daily oscillations in their

transcripts (≥ 0.8 cutoff), with most peaking in abundance at ZT12 (Supplemental Data

6).

Association network analysis also revealed that the loss of RVE8-like proteins results
in major phosphoproteome perturbations, with large increases in the phosphorylation
status of a number of proteins at both ZT0 and ZT12 in *rve 4 6 8* (Figure 5).
Correspondingly, we observed abundance changes in protein kinases (PKs) CALCIUM
DEPENDENT PROTEIN KINASE 3 (CDPK6/CPK3; AT4G23650), 29 (CPK29;
AT1G76040), CYTOPLASMIC TRNA EXPORT PROTEIN (CTEXP; AT2G40730), WALL
ASSOCIATED KINASE 1 (WAK1; AT1G21250), amongst others (Figure 4), in addition to
phosphorylation changes on 23 protein kinase superfamily proteins including CPK1
(Figure 5). Overall, we observed phosphorylation changes on proteins involved in similar
processes and protein complexes as those found in our quantitative proteome data
including multiple spliceosomal- and ribosome- complex proteins, ubiquitin-related
proteins and primary metabolic enzymes (Figure 5). Exclusive to our phosphoproteome
data however, are perturbations in the phosphorylation status of a number of nuclear-
and secretion-related proteins along with extensive changes in proteins related to light
harvesting, photosynthesis and light signaling, such as CAB1 (AT1G29930), PHOT2
(AT5G58140) and HY5 (AT5G11260).

Next, we performed a phospho-motif enrichment analysis using our significantly
changing phosphopeptides to identify potential PKs regulated by RVE8-like proteins. We
found 5 enriched phosphorylation motifs relating to MITOGEN ACTIVATED PROTEIN
KINASES (MAPKs), CASEIN KINASE II (CKII) and CALCIUM DEPENDENT PROTEIN
KINASES (CDPK/CPKs) (Supplemental Data 7). Using the phosphoproteins containing
these motifs, we then created a STRING-DB association network (minimum edge score
≥ 0.7) depicting the phosphorylated substrates of these PKs found in our study to resolve
potential functional relationships as well as intersections between different PKs
(Supplemental Figure 3). Again, a stringent minimum edge score was used to resolve
robust network connections, however here, all phosphoproteins recognized as having one
of these 5 motifs is shown. This analysis resolved a number of networks, including: 26S
proteasomal complex proteins - MAPK, ribosome - CKII / CDPK/CPK, spliceosome -
CDPK/CPK, RNA processing - MAPK and golgi-ER secretion - CDPK/CPK relationships
(Supplemental Figure 3), collectively providing new cell signaling – biological process
connections for further characterization.

Wide ranging primary metabolite changes highlight altered metabolism in _rve 4 6 8_ plants.

As multiple proteins involved in metabolism demonstrated changes at the proteome and phosphoproteome level, we next examined diel changes in _rve 4 6 8_ metabolism compared to WT Col-0 at ZT0, 6, 12 and 18 using gas chromatography mass spectrometry (Figure 6; Supplemental Data 8). Here, we found dynamic changes (_rve 4 6 8_ vs. WT Col-0) in amino acids (AA), organic acids (OA), fatty acids (FA) and sugars, amongst others, over the course of the day, with the largest perturbations occurring around ZT0 - ZT6. These diel changes are highlighted by large increases in phenylalanine (AA), glutamic acid (AA), shikimic acid (OA), sinapinic acid (OA), α-linolenic acid (FA), sucrose (sugar), myo-inositol (other), 5-oxoproline (other) and phytol (other) at ZT0, along with an increase in mannose (sugar) staring at ZT6. Further, we observe that _rve 4 6 8_ plants maintain a consistent increase in glyceric acid (OA) and fumaric acid (OA) coupled with a consistent decrease in serine (AA) and threonine (AA) across all four ZT time-points harvested.

rve 4 6 8 **plants possess constitutively reduced proteasome activity**

The enrichment of proteolysis and proteasome related GO categories in our proteomics dataset, coupled with an extensive cluster of proteasome-related proteins in our STRING-DB association network, prompted us to examine whether the _rve 4 6 8_ mutant showed differences in proteasome function compared to WT Col-0. We therefore carried out a proteasome activity assay using a 7-Amino-4-methylcoumarin (AMC) flurophore tagged LLVY peptide substrate (Üstün et al., 2017) and used the well characterized MG132 proteasome inhibitor as a negative control to validate the assay. Proteasome activity was measured in crude extracts of 8 independent wild-type and _rve 4 6 8_ rosettes harvested at both ZT23 and ZT11. Our results show a significant reduction in proteasome activity in the _rve 4 6 8_ mutant compared to wild-type (adjusted _p-value_ ≤ 0.05; Bonferroni & Dunn multiple testing correction) (Figure 7A). Further, plants with impaired proteasome activity are also found to display a hypersensitivity to chemical proteasome inhibition (Gladman et al., 2016; Han et al., 2019). Correspondingly, when

331 grown on plates containing 50μM of the MG132 proteasome inhibitor, *rve 4 6 8* seedlings
332 possessed impaired growth, similar to that previously observed in mutants of both the
333 core 20S proteasome and the NAC transcription factors that activate the proteasome
334 stress regulon (Figure 7B, Supplemental Figure 4) (Gladman et al., 2016; Han et al.,
335 2019).
336
DISCUSSION
Multi-omics analysis reveals altered carbohydrate metabolism in *rve 4 6 8* plants
Through our combined use of phenomics, transcriptomics, proteomics and
metabolomics, we found that *rve 4 6 8* plants possess a starch excess phenotype at ZT0
that relates to altered both gene expression and protein abundance changes in starch
biosynthesis and degradation enzymes. Initial examination of the transcriptional changes
of select genes saw significant decreases in *GBSS1* at ZT0 and *SEX4* at ZT12 in *rve 4 6*
8 relative to WT Col-0, respectively. Subsequent proteomic analysis then found extensive
decreases in nearly all facets of the starch degradation machinery at either ZT0 and/or
ZT12 including decreases in the abundance of SEX1 (AT1G10760), SEX4, GWD3
(AT5G26570), ISA3 (AT4G09020), PHS2 (AT3G46970), LDA (AT5G04360) and DPE2
(AT2G40840) (Streb and Zeeman, 2012). Correspondingly, our metabolite data
demonstrated decreased glucose and sucrose levels at ZT6, ZT12 and ZT18, which is
consistent with altered carbon metabolism related to excess starch levels at ZT0. Plants
deficient in core clock proteins PRR7 and PRR9 (*prr7 prr9*), which also maintain a long
circadian period, have also been shown to possess a subtle starch excess phenotype
(Chew et al., 2017; Flis et al., 2019); however, unlike *prr7 prr9* plants, *rve 4 6 8* plants
seem to asymmetrically possess excess starch at ZT0 only. The mutant of
disproportionating enzyme (DPE1; AT5G64860), a key starch degradation protein,
however, possesses a similar phenotype whereby starch levels are in excess at dawn but
accumulate closer to wild-type plants by dusk (Critchley et al, 2001). We also see DPE1
asymmetrically down-regulated in protein abundance at ZT0, suggesting a connection to
RVE8-like proteins. Hence, future work connecting RVE8-like proteins to DPE1 and other
starch degradation enzymes is certainly of interest.

Interestingly, we see very few PTM-level changes occurring on starch-related
enzymes in *rve 4 6 8* plants despite many of these enzymes being previously found to be
phosphorylated (PhosPhat; http://phosphat.uni-hohenheim.de/; (Heazlewood et al.,
2008)). Unlike the extensive protein abundance changes observed, only two starch-
related enzymes were found to have a significant change in their phosphorylation status.
These were STARCH SYNTHASE 2 (SS2; AT3G01180) and BETA-AMYLASE 1 (BAM1;
AT3G23920), of which both exhibited an increase in their phosphorylation status at ZT0
and ZT12 in *rve 4 6 8* relative to WT Col-0. The role(s) of protein phosphorylation in
regulating starch-related enzyme complexes has been well defined in sink tissues (Tetlow
et al., 2004), but remains poorly understood in source tissues (e.g. leaves), where starch
is produced and degraded on a daily basis (Smith and Zeeman, 2020). Recent
biochemical characterization of SS2 phosphorylation using phospho-ablative SS2
mutants (S63A and S65A) found no enzymatic or protein complex consequences to the
loss of SS2 phosphorylation despite being phosphorylated by CKII; (Patterson et al.,
2018)). Our data finds SS2 to be phosphorylated at Ser65; part of a canonical CKII
phosphorylation motif (*p*S[D/E][D/E][D/E]) (Purzner et al., 2018), exclusively at ZT0 in the
rve 4 6 8 mutant. We also observe BAM1 to be phosphorylated, but at a *p*SP MAPK-like
motif (Purzner et al., 2018). There are no currently defined roles for BAM1
phosphorylation despite its key role in regulating plant growth, promoting stomatal
opening and facilitating starch degradation under osmotic stress in the light (Thalmann et
al., 2016).

Of the carbohydrate-related changes we found in *rve 4 6 8*, the consistent and
substantial increase in mannose, a multifunctional glucose analog, was particularly
interesting. Previous investigations of the function of mannose have found that it can: 1)
stimulate stomatal closure (Hei et al., 2018), 2) be incorporated into glycolysis through
fructose 6-phosphate via hexokinase and phosphomannose isomerase (Sharma et al.,
2014), 3) protect against oxidative damage, 4) impact flowering time through ascorbate
production (Kotchoni et al., 2009) and 5) contribute to cell wall biosynthesis and protein
glycosylation (Qi et al., 2017) - all molecular changes that relate to the phenotypes
displayed by *rve 4 6 8*. Interestingly, mannose has a high affinity for hexokinase (Herold
and Lewis, 1977), leading to the induction of glucose-like signaling responses such as

stomatal closure (Hei et al., 2018) and bud elongation in *Arabidopsis*, pea and rose

(Barbier et al., 2021). It is possible that a similar mechanism contributes to the elongated

rosette leaf morphology observed in *rve 4 6 8,* both in this study, and others (Gray et al.,

2017).

397   RVE8-like protein regulation of TOC1 may control the production of fumarate and

398   other organic acids

Arabidopsis plants over-expressing RVE8 have previously been shown to stimulate

the expression of *TOC1* (Hsu et al., 2013) and *toc1-2* plants have been found to possess

increased levels of fumarate at ZT7 and Z19 (Cervela-Cardona et al., 2021). In this

context, our finding that *rve 4 6 8* plants possess both reduced *TOC1* gene expression at

ZT12 and increased fumarate levels throughout the diel cycle, suggests RVE8-like

proteins impact fumarate metabolism via TOC1. Further, this finding aligns with our

observation that multiple other mitochondrial organic acids are, to varying degrees,

impacted by RVE8-like proteins, including malate, citrate and succinate. Change in these

organic acids align with the previous findings that *prr5 prr7 prr9* mutants have increased

fumarate, malate, citrate and succinate stemming from the down-regulation of TCA-cycle

genes (Fukushima et al., 2009). At the protein-level however, we find no significant

change in FUMARASE 1 (FUM1; AT2G47510), FUMARASE 2 (FUM2; AT5G50950),

MITOCHONDRIAL MALATE DEHYDROGENASE 1 (mMDH1; AT1G53240), mMDH2

(AT3G15020), CITRATE SYNTHASE 2 (CSY2; AT3G58750), CSY3 (AT2G42790),

SUCCINATE DEHYDROGENASE 1-1 (SDH1-1; AT5G66760), SDH5 (AT1G47420) and

SDH6 (AT1G08480) protein abundance and/or PTM levels in *rve 4 6 8* versus WT Col-0

at either ZT0 or ZT12. This indicates that RVE8-like protein control of fumarate production

is not direct, but likely through the previously identified TOC1-FUM2 mechanism (Cervela-

Cardona et al., 2021). It also suggests that if diel protein-level regulation of mitochondrial

TCA enzymes is occurring, it manifests through alternative means, such as a production-

degradation mechanism that maintains steady state levels of TCA enzymes or through

PTMs other than phosphorylation. While our current dataset is unable to directly answer

these possibilities, previous work examining mitochondrial protein turnover has implicated

FTSH, LON and CLPXP proteases (Li et al., 2017; Huang et al., 2020), of which we

observe FTSH11 (AT5G53170) and CLPX (AT5G53350), two putatively mitochondrial
targeted proteases, to possess an increase in their phosphorylation status (Heazlewood
et al., 2004; Senkler et al., 2017). Ultimately how TCA enzymes are coordinately
regulated at the transcript, protein and/or PTM-levels, in addition to possible feed-forward
or feed-back regulation by metabolites, requires targeted analyses that are beyond the
scope of this study.

RVE8-like proteins are constitutive regulators of the proteasome

Both GO enrichment and STRING-DB association network analysis of differentially
expressed proteins in *rve 4 6 8* at ZT0 and ZT12 revealed substantial protein-level
changes in the abundance of a number of proteasome complex subunits, coupled with a
limited corresponding change in phosphorylation status. In plants, connections between
the circadian clock and E3 ubiquitin ligases targeting proteins for proteasomal
degradation through K63 polyubiquitylation have emerged, indicating that diel protein
turnover is a circadian controlled phenomenon (Feke et al., 2019; Feke et al., 2020; Zhu
et al., 2020a). Further, using N^{15} labeling methods, efforts made to quantify protein
turnover rates have demonstrated that diel protein turnover involves a multitude of
proteins that are processed at variable rates over a wide-ranging time frame (Li et al.,
2017; Huang et al., 2020). To date however, no connections between the circadian clock
and the direct regulation of the proteasome complex have been uncovered.

The mature 26S proteasome is comprised of a 20S core protease (CP) which
maintains non-specific ubiquitin-independent proteolytic activity and 19S regulatory
particle (RP) subunits that determine specificity to ubiquitylated proteins (Sadanandom et
al., 2012). We observe a reduction in 12 out of the 14 CP subunits, including 5 alpha and
7 beta ring subunits, in the *rve 4 6 8* mutants. In contrast, we see an increase in the
abundance of 4 RP subunits, as well as CSN4, a member of the COP9 signalosome, a
conserved regulator of ubiquitin-dependent proteolysis and a key player in
photomorphogenesis (Qin et al., 2020). Studies investigating proteasome subunits in
Arabidopsis have shown that reduced CP proteolytic function is associated with cell
expansion (Kurepa et al., 2009a) and that one member of the 19S RP subunit, RPN12a,
is involved in cytokinin-mediated signaling for cell division (Smalle et al., 2002). This

aligns with our GO enrichment of cell cycle and cell division biological process amongst
proteins exhibiting a significant change in abundance as well as our observed increase in
cytokinin response factor ARR7. Further, these findings are consistent with a hypothesis
that proper balancing of the 20S and 19S subunits of the proteasome is key for
maintaining growth and cell division (Kurepa et al., 2009b), and that alterations in the
levels of these subunits in *rve 4 6 8*, at least partially, explain its growth and large cell size
phenotypes.

In mice, connections between the circadian clock and proteasomal degradation of
CRYPTOCHROME 1 (CRY1) have also been defined, with enhanced CRY1 degradation
associated with elevated glucose levels and a loss of core clock regulation (Toledo et al.,
2018). In plants, CRY proteins entrain the clock (Devlin and Kay, 2000), with *cry1* mutants
having longer circadian periods under blue light (Somers et al., 1998). Interestingly, in *rve*
4 6 8 plants, which similarly possess a long circadian period that increases with blue light
fluence (Gray et al., 2017), we observe increased CRY1 protein levels at both ZT0
(Log_2FC = 1.05) and ZT12 (Log_2FC = 0.81), coupled with a starch excess phenotype and
wide-ranging carbohydrate metabolism perturbations, indicating a potentially similar
circadian clock intersection between CRY1 and primary carbohydrate metabolism that
appears to involve RVE8-like proteins. Additionally, the diel amino acid perturbations
measured in *rve 4 6 8* plants may also correspond to RVE8-like proteins impacting the
diel plant cell environment through the regulation of proteasome subunits and protein
turnover.

Ultimately, a connection between the circadian clock and diel protein turnover is
logical given the consistent lack of coordinately fluctuating diel transcripts and proteins in
Arabidopsis across multiple experiments (Baerenfaller et al., 2012; Graf et al., 2017;
Mehta et al., 2021; Uhrig et al., 2021). Overall, our results implicate RVE8-like proteins
as being involved in diel protein turnover, adding important context to the well-known
disconnect between circadian transcript and protein abundancies. Future assessment of
core circadian clock mutants for changes in protein turnover rates may help resolve the
degree to which diel protein turnover contributes to daily protein-level plant cell regulation.
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Proteome changes in *rve 4 6 8* plants suggest RVE8-like proteins may modulate adaptive plant stress responses

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The enrichment of GO categories pertaining to Abscisic acid in the *rve 4 6 8* proteome,
provides protein-level evidence that RVE8-like proteins may modulate adaptation to
drought-like stresses (e.g. osmotic, salinity, and cold). A potential role for RVE8-like
proteins in drought-like stress response(s) is supported by the measured increase in
mannose-levels, which is known to influence stomatal conductance. Further, α-linolenic
acid, and its derivatives, are known to increase upon drought and salinity stress to
mitigate associated cell membrane issues by remodeling cell membranes to increase
their fluidity (Zhang et al., 2005; Upchurch, 2008; Torres-Franklin et al., 2009; He and
Ding, 2020). Here, this seems to center on FATTY ACID DESATURASE 7 (FAD7;
AT3G11170), which utilizes linoleic acid to produce α-linolenic acid (Gibson et al., 1994),
and is significantly more abundant in *rve 4 6 8* plants at ZT0, corresponding to peak levels
of α-linolenic acid.

In addition to augmenting membrane fluidity, α-linolenic acid can also fuel the
production of jasmonic acid (JA) (He and Ding, 2020). Interestingly, we find that critical
jasmonic acid biosynthetic enzymes AOC1, AOC2 and 12-OXOPHYTODIENOATE
REDUCTASE 3 (OPR3; AT2G06050) are significantly more abundant in WT Col-0
relative to *rve 4 6 8*, suggesting that RVE8-like protein deficient plants may possess
susceptibility to certain biotic stresses. A diel connection between RVE8-like proteins and
JA biosynthetic enzymes is further supported by the altered diel phosphorylation of the
peroxisomal ABC transporter COMATOSE (CTS; AT4G39850), which imports JA
precursor *cis*-12-oxophytodienoic acid that is then utilized by OPR3 (Wasternack and
Hause, 2019), which peaks in protein abundance at ZT12.

Further support for a role of RVE8-like proteins in drought-like stress response(s) is
the increased abundance of key sulfur assimilating enzymes APR2 (AT1G62180) and
APK (AT2G14750) in *rve 4 6 8* plants. These proteins are critical for the production of
ABA and glutathione (GSH), in addition to a number of other critical plant metabolites
(Aarabi et al., 2020). Correspondingly, we see an increase in a number of glutathione
transferases (GSTs); GSTF2, GSTF6 (AT1G02930) and GSTF7 (AT1G02920), which are
a subclass of the plant-specific Phi-family of GSTs implicated in mitigating oxidative

(GSTF2) and salt (GSTF6/7) stress, respectively (Lee et al., 2014; Seok et al., 2020). In previous proteomic and phosphoproteomic analyses of persistent osmotic and salt-stress, increased abundance of sulfur metabolic enzymes (Rodriguez et al., 2021), indicates a potential connection between sulfur metabolism / assimilation and drought-stress tolerance. In maize, the application of sulfur-supplemented fertilizers to drought stressed plants was shown to mitigate the effects of drought (Usmani et al., 2020).

RVE8-like proteins were most recently implicated in the direct transcriptional activation of DEHYDRATION RESPONSE ELEMENT B (DREB) expression under cold-stress (Kidokoro et al., 2021). DREB transcription factors serve as master controllers of cold-induced gene expression, regulating COLD REGULATED (COR) genes to enhance freezing tolerance (Jaglo-Ottosen et al., 1998). Correspondingly, RVE8-like protein deficient plants (*rve 4 8*) possessed diminished cold tolerance (Kidokoro et al., 2021). Interestingly, our proteomics data resolved a cluster of cold-stress related proteins that are reduced in abundance at ZT12 / ED under normal growth temperatures, suggesting that some cold response proteins are diel regulated, likely possessing additional diel roles beyond cold-stress response (Maszkowska et al., 2018; Zhu et al., 2020b). These include: chloroplast targeted COR15A (AT2G42540) and COR15B (AT2G42530) (Artus et al., 1996) as well as cytoplasmic-targeted KIN2/COR6.6 (AT5G15970), ERD10 (AT1G20450), AT1G54410 and AT4G30650. In conjunction with our analyses, these findings directly connect RVE8-like proteins to observable proteome-level changes in genes associated with drought-related stresses.

Protein kinases impacted by RVE8-like proteins

In addition to catalyzing the phosphorylation of target proteins, protein kinases (PKs) themselves are often phosphorylated, resulting in changes in their activity, function or localization, forming a complex signaling web that can have far-reaching ramifications for plant cell function. Our combined quantitative proteomic and phosphoproteomic analysis uncovered a number of PKs that change in either diel abundance or phosphorylation status in response to the loss of RVE8-like proteins. Of particular interest is the increased abundance of CASEIN KINASE II (CKIIα; AT2G23070), CALCIUM DEPENDENT PROTEIN KINASE 6 (CDPK6/CPK3; AT4G23650) and WALL ASSOCIATED KINASE 1

(WAK1; AT1G21250) and the phosphorylation of CPK1 (AT5G04870), as these PKs have

been implicated in multiple plant cell processes related to *rve 4 6 8* phenotypes as well

as osmotic- and salt-stress tolerance.

CKII is a well-known clock associated kinase that directly affects the stability of core

clock component CCA1 in *Arabidopsis* (Sugano et al., 1998; Lu et al., 2011). The role of

CKII in modulating the circadian clock is conserved across eukaryotes, with both

mammalian (Tamaru et al., 2009) and Drosophila (Akten et al., 2003) circadian clocks

being directly impacted by CKII. Most recently, multiple CASEIN KINASE 1 family kinases

were discovered to phosphorylate PRR5 and TOC1 leading to their degradation (Uehara

et al., 2019). Our finding that CKII levels are elevated in *rve 4 6 8* at ZT12 hints at the

possibility of RVE8-like proteins similarly directing CKII-mediated phospho-regulation of

circadian function. Beyond the clock, CKII has been implicated in phosphorylating

numerous light signaling proteins, such as HY5 (Hardtke et al., 2002), which we see here

possessing an increase in its phosphorylation status in *rve 4 6 8* plants at Ser36 at both

ZT0 and ZT12. Recently, HY5 phosphorylation at Ser36 was found to be phosphorylated

by SUPPRESSOR OF PHYTOCHROME A-105 (SPA) kinases (Wang et al., 2021),

suggesting that perhaps other CKII-motif containing proteins resolved here may also

represent SPA targets. Lastly, CKII is also characterized as phosphorylating ABA / abiotic

stress induced pathway proteins (Mulekar and Huq, 2014), which aligns with our

enrichment of ABA-related GO terms in our phosphoproteomic data.

CDPK6/CPK3 is a positive regulator of ABA-induced regulation of stomatal closure

(Mori et al., 2006) and is induced under high salt conditions (Mehlmer et al., 2010),

suggesting that its increased abundance in *rve 4 6 8* plants may convey signaling events

related to osmotic- / salt-stress response. Although CDPK6/CPK3 has not been shown to

directly impact the circadian clock, its connection to stomatal regulation, coupled with

known diel fluctuations in calcium levels (Marti Ruiz et al., 2020), suggests a connection

to the regulation of diel plant cell processes. Similar to CDPK6/CPK3 salt-stress induction,

over-expression of native CPK1 resulted in increased salinity tolerance in cell culture

(Veremeichik et al., 2021), reinforcing a potential link between diel CPK-mediated

phosphorylation events and the ability to mitigate abiotic stress. Alternatively, WAK1 has

been implicated in maintaining cell wall integrity (Leschevin et al., 2021), which has diel

plant cell growth implications that may contribute to the large mesophyll cell phenotype
of *rve 4 6 8* plants (Gray et al., 2017).
These results, when combined with our phospho-motif analysis that shows an
enrichment in CKII and CDPK/CPK target motifs, suggest that CKIIα and CDPK6/CPK3
may be connected to a substantial portion of the observed RVE8-like dependent
phosphoproteome. STRING-DB association network analysis of proteins with these
enriched phospho-motifs demonstrates connections to multiple nuclear protein networks
including one involving RETINOBLASTOMA-RELATED 1 (RBR; AT3G12280), a critical
growth and development protein, in addition to protein translation and secretion.
Collectively, these data provide novel insights into RVE8-like protein dependent phospho-
networks and the corresponding kinases that may be involved.
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CONCLUSION
Our robust, multi-omic analysis of *rve 4 6 8* plants further establishes RVE8-like
proteins as targets of agronomic interest by defining the proteomic and metabolic
underpinnings of their observable phenotypes. Through these analyses, we found a
number of connections to clock related cell processes that refine our understanding of *rve*
4 6 8 growth phenotypes. This includes altered metabolism that either directly (e.g.
carbohydrates and organic acids) or indirectly (e.g. precursors for growth phytohormones)
drives growth outcomes. In particular, carbohydrate metabolism, for which we found a
significant decrease in multiple starch degradation enzymes leading to starch excess at
dawn. As well, our findings reveal that RVE8-like proteins control the 26S proteasome
through altered 20S and 19S subunit abundance and phosphorylation status, which our
metabolite data suggests may be tied to changes in resource pools important for growth
and development (e.g. amino acids). This novel connection between the proteasome and
the circadian clock through RVE8-like proteins was further supported by a reduction of
proteasome activity in *rve 4 6 8* plants, something that in proteasome mutants, leads to a
large cell size phenotype similar to that observed in *rve 4 6 8* plants. This suggests that
RVE8-like proteins regulate the proteasome as a means of controlling cell division and
growth. Lastly, our multi-omic analyses hint at potential new connections between RVE8-
like proteins and abiotic stress response (e.g. osmotic, salt and/or cold – already

established), which we see manifesting throughout our proteome, phosphoproteome and

metabolome data. Overall, our combined use of quantitative transcriptomics, proteomics,

PTMomics and metabolomics have elucidated a number of new connections between

RVE8-like proteins and critical plant cell processes, laying a substantial foundation from

which to build a comprehensive understanding of the roles RVE8-like proteins play in

directing diel plant cell regulation.

MATERIALS AND METHODS

Plant Growth and Phenotyping

Plant Growth – Wild-type *Arabidopsis thaliana* Col-0 and *rve 4 6 8* seeds were

obtained from Dr. Stacy Harmer (University of California - Davis). Seeds were sterilized

in 70% (v/v) ethanol for 2 min followed by a 30% (v/v) bleach (Chlorox 7.5%) treatment

for 7 min and three washes with distilled water. The seeds were then grown on 0.5x MS

media and 7 g/L of agar at pH 5.8 (with KOH). All seeds were cold treated for 3 days at

4°C in the dark and exposed to light for a week before being transferred to soil (Sungro,

Sunshine Mix® #1). After 21 days of light exposure, entire plants were collected for

analysis. Growth chambers were equipped with a programmable Perihelion LED fixture

(G2V Optics Inc.) and lined with Reflectix® to ensure a good light diffusion. Plants were

grown under a 12h light and 12h dark photoperiod with a temperature of 21°C during the

day and 19°C at night. Six LED types (Cree LED XPE, XPE2 XPG families) were used in

the fixtures, with characteristic wavelengths of 444 nm, 630 nm, 663 nm, 737 nm, 3000

K white, and 6500 K white.

Phenomics measurements – Each chamber was equipped with a Raspberry Pi 3 B+

and an ArduCam Noir Camera (OV5647 1080p) with a motorized IR-CUT filter and two

infrared LEDs. Pictures were taken every 15 min over 7 d (between 14 d and 21 d post

seed imbibition) and were used to extract plant area and perimeter measurement using

PlantCV as previously described (Gehan et al., 2017).

Proteasome inhibitor sensitivity seedling growth experiment – Col-0 and *rve 4 6 8*

seedlings were germinated on 0.5x MS control plates and 0.5x MS plates supplemented

with 50 μM MG132 (Millipore Sigma; 474787) and grown under 12:12 photoperiod

conditions as described above for 6 days prior to imaging.

Starch Iodine Staining and Enzymatic Assay

Starch Iodine Staining and quantification – Starch was stained and visualized in *Arabidopsis* rosettes by Lugol's iodine stain. Entire rosettes were decolorized by incubation in 80% ethanol at 80°C for 30 minutes. Rosettes were rinse 3 times in distilled water and stained 20 min in a 5% (v/v) Lugol's iodine solution (Fisher Scientific). After 3 rinses in distilled water, rosettes were washed for an 1h in distilled water to remove the excess of iodine. Rosette leaves were flattened between plastic sheet and excess water removed. Each rosette was analyzed with ImageJ (Abramoff et al 2004; https://imagej.nih.gov/ij/index.html) to record mean values in starch staining pixel intensity as described by Betti et al., (2016).

Starch Extraction and Quantification – Starch was extracted and assayed as previously described (Smith and Zeeman, 2006). Frozen rosette leaves were ground to a fine powder and homogenized in perchloric acid 8% (Ricca Chemical). The insoluble fraction was pelleted and washed 3 times in ethanol 80% and resuspended in water. Prior quantification, the samples were boiled 15 min at 95°C to gelatinize the starch and hydrolyzed with α-amylase and amyloglucosidase (Roche) to release the glucose blocks. Glucose content was then measured by spectrophotometry after a 2 enzymes reaction with hexokinase and glucose-6-phosphate dehydrogenase (Roche).

RNA isolation and NanoString

Total RNA was isolated and purified using a modified single step TRIzol protocol (Chomczynski and Sacchi, 1987). Plants were flash frozen and homogenized with two glass beads using a Geno/Grinder® for 30s at 1500 x g. Immediately after, 1 mL of TRIzol was added to the sample, and the whole was vortexed and incubated for 10 min at room temperature. The samples were then centrifuged at 13000 x g for 10min to remove extracellular material and glass beads. The supernatant was transferred to a new tube. Subsequently, 200 μL of chloroform was added to the mixture and shaken vigorously by hand for 15 s and the samples were kept for 3 min at room temperature. The samples were centrifuged at 13000 x g for 15 min at 4°C and the supernatant thus obtained was transferred to a new tube by gentle pipetting, avoiding the interphase slurry. RNA was

precipitated by adding 500 μL of 100% isopropanol and the tubes were incubated 10 min at RT and centrifuged at 13000 x g for 10 min at 4°C. After decanting isopropanol, the pellet was washed with 1 mL of 75% ethanol in nuclease-free water (IDT™) and briefly centrifuged at 7500 x g for 5 min at 4°C. Total RNA was suspended in nuclease-free water and stored at 80°C for further use. RNA quantification was performed using a NanoDrop ND 1000 spectrophotometer. The RNA was treated with DNase I, Amp grade (Invitrogen) following the instructions provided by the manufacturer. The purified RNA thus obtained was reverse to cDNA using the RevertAid RT Kit (Thermo Scientific), according to manufacturer's instructions. A minimum of 150ng of purified RNA in 7.5ul was sent to NanoString (NanoString Technologies, Seattle, USA, https://www.nanostring.com) for analysis. The NanoString probes were designed and synthesized by NanoString (Supplementary Data 1). UBC21 (AT5G25760) was used as reference gene.

Liquid Chromatography Mass Spectrometry (LC-MS)

Protein Extraction - Quick-frozen rosette tissue was ground to a fine powder under liquid N_2 using a mortar and pestle. Ground samples were aliquoted into 400 mg fractions. Aliquoted samples were then extracted at a 1:2 (w/v) ratio with a solution of 50 mM HEPES-KOH pH 8.0, 50 mM NaCl, and 4% (w/v) SDS. Samples were then vortexed and placed in a 95°C table-top shaking incubator (Eppendorf) at 1100 RPM for 15 mins, followed by an additional 15 mins shaking at room temperature. All samples were then spun at 20,000 x g for 5 min to clarify extractions, with the supernatant retained in fresh 1.5 mL Eppendorf tubes. Sample protein concentrations were measured by bicinchoninic acid (BCA) assay (23225; Thermo Scientific). Samples were then reduced with 10 mM dithiothreitol (DTT) at 95°C for 5 mins, cooled, then alkylated with 30 mM iodoacetamide (IA) for 30 min in the dark without shaking at room temperature. Subsequently, 10 mM DTT was added to each sample, followed by a quick vortex, and incubation for 10 min at room temperature without shaking. Total proteome peptide pools were then generated using a KingFisher Duo (Thermo Scientific) automated sample preparation device as outlined by Leutert et al. (2019) without deviation. Sample digestion was performed using sequencing grade trypsin (V5113; Promega), with generated peptide pools quantified by Nanodrop, normalized to 400 μg for phosphopeptide enrichment and acidified with formic

acid (FA) to a final concentration of 5% (v/v) prior to being desalted using 1cc tC18 Sep

Pak cartridges (WAT036820; Waters) as previously described (Uhrig et al., 2019).

Phosphopeptide Enrichment - SepPak eluted peptides were topped up to 65% (v/v)

acetonitrile (ACN) and 5% (v/v) trifluoroacetic acid (TFA) prior to enrichment using a

KingFisher Duo (Thermo Scientific) automated sample preparation device as outlined by

Leutert et al. (2019). We used 500 μg of MagReSyn Ti-IMAC HP matrix (Resyn

BioSciences) to enrich phosphorylated peptides. Unbound peptides (total proteome) and

phosphopeptides were dried then dissolved in 3% (v/v) ACN / 0.1% (v/v) TFA, desalted

using ZipTip C18 pipette tips (ZTC18S960; Millipore) as previously described (Uhrig et

al., 2019). All peptides were then dried and re-suspended in 3% (v/v) ACN / 0.1% (v/v)

FA immediately prior to MS analysis.

BoxCarDIA Mass Spectrometry - Changes in the protein abundancies was assessed

using trypsin digested samples analysed using a Fusion Lumos Tribrid Orbitrap mass

spectrometer (Thermo Scientific) in a data independent acquisition (DIA) mode using the

BoxCarDIA method (Mehta et al., 2022). Dissolved peptides (1 μg) were injected using

an Easy-nLC 1200 system (LC140; Thermo Scientific) and separated on a 50 cm Easy-

Spray PepMap C18 Column (ES803A; Thermo Scientific). A spray voltage of 2.2 kV,

funnel RF level of 40 and heated capillary at 300°C was deployed, with all data acquired

in profile mode using positive polarity, with peptide match turned off and isotope exclusion

selected. All gradients were run at 300 nL/min with analytical column temperature set to

50°C. Peptides were eluted using a segmented solvent B gradient of 0.1% (v/v) FA in

80% (v/v) ACN from 4% - 41% B (0 - 107 min). BoxCar DIA acquisition was performed as

previously described (Mehta et al., 2022). MS^1 analysis was performed by using two

multiplexed targeted SIM scans of 10 BoxCar windows each, with detection performed at

a resolution of 120,000 at 200 m/z and normalized AGC targets of 100% per BoxCar

isolation window. Windows were custom designed as previously described (Mehta et al.,

2022). An AGC target value for MS^2 fragment spectra was set to 2000%. Twenty-eight

38.5 m/z windows were used with an overlap of 1 m/z. Resolution was set to 30,000 using

a dynamic maximum injection time and a minimum number of desired points across each

peak set to 6.

BoxCarDIA Data Analysis - All acquired BoxCar DIA data was analyzed in a library-
free DIA approach using Spectronaut v14 (Biognosys AG) using default settings. Key
search parameters employed include: a protein, peptide and PSM FDR of 1%, trypsin
digestion with 1 missed cleavage, fixed modification including carbamidomethylation of
cysteine residues and variable modifications including methionine oxidation. Data was
Log$_2$ transformed, globally normalized by median subtraction with significantly changing
differentially abundant proteins determined and corrected for multiple comparisons
(Bonferroni-corrected *p-value* ≤ 0.05; *q-value ≤ 0.05*).

DDA Mass Spectrometry - Changes in the protein phosphorylation status was
assessed using trypsin digested samples analyzed using a Fusion Lumos Tribrid Orbitrap
mass spectrometer in a data dependent acquisition (DDA) mode using the Universal
method (Thermo Scientific). Dissolved phosphopeptides were analyzed as described
above, using a linear gradient of solvent B (0.1% (v/v) FA in 80% (v/v) ACN): 5% to 22%
B, 0 – 110 min; 22% - 35% B, 110 – 120 min; 35 - 95% B, 120 – 125 min at a flow rate of
0.3 µl/min at 50°C. Full scan MS1 spectra (350 - 2000 m/z) were acquired with a resolution
of 120,000 at 200m/z with a normalized AGC Target of 125% and a maximum injection
time of 50 ms. DDA MS2 were acquired in the linear ion trap using quadrupole isolation
in a window of 2.5 m/z. Selected ions were HCD fragmented with 35% fragmentation
energy, with the ion trap run in rapid scan mode with an AGC target of 200% and a
maximum injection time of 100 ms. Precursor ions with a charge state of +2 - +7 and a
signal intensity of at least 5.0e3 were selected for fragmentation. All precursor signals
selected for MS/MS were dynamically excluded for 30s.

DDA Data Analysis – All acquired DDA phosphoproteomic data was analyzed using
MaxQuant 1.6.14.0 (http://www.maxquant.org/; (Cox and Mann, 2008)) with the following
parameters. A protein, peptide and PSM FDR of 1%, trypsin digestion with 1 missed
cleavage, fixed modification including carbamidomethylation of cysteine residues and
variable modifications including methionine oxidation and phosphorylated serine,
threonine and tyrosine. Data was then imported into Perseus version 1.6.14.0 (Tyanova
et al., 2016) for further analysis. This involved the removal of reverse hits and
contaminants, log$_2$-transformation, assembly into treatment groups and filtering based on
the presence of measured data in at least 2/3 replicates of at least one group. Data were

then median normalized, followed by imputation of missing values based on the normal

distribution function set to default parameters. All PTM analyses utilized a PTM site

localization score threshold ≥ 0.75. Significantly changing differentially abundant

phosphopeptides were determined by ANOVA and corrected for multiple comparisons

(Benjamini-Hochberg corrected *p-value* ≤ 0.05; *q-value*).

All proteomic and phosphoproteomic raw data and search parameters have been

uploaded to ProteomeXchange (http://www.proteomexchange.org/) via the Proteomics

IDEntification Database (PRIDE; https://www.ebi.ac.uk/pride/). Project Accession:

PXD029234.

772   Gas Chromatography Mass Spectrometry (GC-MS)

Metabolite Extraction and preparation - Metabolite extraction and preparation was

performed with modifications as previously described (Hill and Roessner, 2013; Liu et al.,

2016). Tissue was harvested and directly flash frozen in liquid nitrogen. Samples of 100

mg (+/- 1 mg) of pulverized tissue were prepared and homogenized in 700 μl of iced-cold

methanol (80% v/v). Internal standard 25 μl ribitol at 0.4 mg.ml^{-1} in water. Samples were

incubated 2 h at 4°C with shaking and then 15 min at 70°C at 850 rpm in a Thermomixer.

Tubes were centrifuged 30 min at 12000 rpm and the supernatants were transferred in

new tubes. Polar and non-polar phases were separated by the addition of 700 μl of water

and 350 μl of chloroform, then vortexed thoroughly and centrifuged for 15 min at 5000

rpm. The upper methanol/ water phase (150 μl) was transferred to a new tube and dried

in a vacuum centrifuge at RT. Samples were derivatized with 100 μl of methoxylamine

hydrochloride (20 mg.ml^{-1} in pyridine) for 90 min at 30°C at 850 rpm in thermomixer and

followed by incubation with 100 μL of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) at

80°C during 30 min with shaking at 850 rpm in thermomixer.

GC-MS analysis - Finally, samples were injected in splitless mode and analyzed using

a 7890A gas chromatograph coupled to a 5975C quadrupole mass detector (Agilent

Technologies, Palo Alto, CA, USA). In the same manner 1 μl of retention time standard

mixture Supelco C7–C40 saturated alkanes standard (1,000 μg.ml^{-1} of each component

in hexane) diluted 100 fold (10 μg.ml^{-1} final concentration) was injected and analyzed.

Alkanes were dissolved in pyridine at 0.22 mg.ml^{-1} final concentration. Chromatic

separation was done with a DB-5MS capillary column (30 m × 0.25 mm × 0.25 μm; Agilent

J&W Scientific, Folsom, CA, USA). Inlet temperature was set at 280°C. Initial GC Oven

temperature was set to 80°C and held for 2 min after injection then GC oven temperature

was raised to 300°C at 7°C min^{-1}, and finally held at 300°C for 10 min. Injection and ion

source temperatures were adjusted to 300°C and 200°C, respectively with a solvent delay

of 5 min. The carrier gas (Helium) flow rate was set to 1 ml.min^{-1}. The detector was

operated in EI mode at 70 eV and in full scan mode (m/z 33–600).

Metabolite identification and quantification - Compounds were identified by mass

spectral and retention time index matching to the mass spectra of the National Institute

of Standards and Technology library (NIST20, https://www.nist.gov/) and the Golm

Metabolome Database (GMD, http://gmd.mpimp-golm.mpg.de/). Metabolites

quantification was performed using MassHunter Software from Agilent. Peaks were

integrated and after blank subtraction were normalized by dividing them by the peak area

of the internal standard ribitol and by the sample weight.

Bioinformatics Analysis and Data Visualization

Visualizations were made using *R* version 3.6.1 and R package *ggplots* in combination

with Affinity Designer (https://affinity.serif.com/en-us/designer/; ver. 1.9.1.179). All data

was plotted using GraphPad - Prism (ver. 8; https://www.graphpad.com/scientific-

software/prism/). Networks were created in Cytoscape (ver 3.7.1; https://cytoscape.org/),

using the STRING-DB (https://string-db.org/) and enhancedGrahphics (ver. 1.5.4;

https://apps.cytoscape.org/apps/ enhancedgraphics) cytoscape-apps. All association

networks were built without data sub-selection and using an edge threshold ≥ 0.7). Gene

Ontology Enrichments were performed using TheOntologizer (http://ontologizer.de/)

using all identified proteins as the background for enrichment analyses (*q-value* ≤ 0.05),

while phosphorylation motif enrichment analysis was performed using Motif-X (Chou and

Schwartz, 2011) on the MoMo MEME suite (https://meme-suite.org/meme/) as previously

described (Mehta et al., 2021). Subcellular localization information for proteins was

obtained from SUBA4 (https://suba.live/).

Proteasome activity assay

Proteasome activity was measured using the fluorogenic substrate N-Succinyl-Leu-
Leu-Val-Tyr-7-Amido-4-Methylcoumarin (Millipore Sigma; S6510) as described Üstün et
al. (2017). Briefly, 100 µg of rosette tissue of 3 week old plants was extracted with 250
µL of extraction buffer (2 mM ATP, 50 mM HEPES-KOH (adjusted to pH 7.2 with 1 N
KOH); 2 mM DTT; 0.25 M sucrose). After clarifying by centrifugation at 18,000 g for 15
minutes, total protein content in the extracts was quantified with a Bradford assay (Bio-
Rad; 5000006). The assay was performed using 50 µg of total protein extract and 300
µM of substrate in assay buffer (100mM HEPES-KOH pH 7.8, 5mM $MgCl_2$, 10 mM KCl,
and 2 mM ATP) at 30°C. Fluorescence was measured at 1.5 minute intervals with an
excitation at 360nm and emission wavelength at 460nm for a period of 2 hours after an
initial 2 hour incubation. Activity was reported as Relative Fluorescence Units/min based
on the slope of the linear portion of the emission curve (MeanV in the Gen5 software).
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852 **LITERATURE CITED**

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TABLE CAPTIONS

Table I: Quantified and significantly changing proteome and phosphoproteome.

Protein groups and peptides are shown (n=4; *q-value* ≤ 0.05; fold-change (FC) ≥ 1.5).

FIGURE CAPTIONS

Figure 1: Phenotypic analysis of *rve 4 6 8* plant growth and development. (A) Iodine
starch staining of rosettes at 28 d post-imbibition. (**B**) Enzymatic quantification of starch
levels in rosettes. All phenotyping was conducted under a 12 h : 12 h light : dark
photoperiod using 100 µmol m-2 s-1 light. Stars denote Bonferroni adjusted *p-value*
significance: (***) *p-value* ≤ 0.005 and (*) *p-value* ≤ 0.05. **(C)** Starch related genes PGM1,
GBSS1 and SEX4. All time-points and genotypes were analyzed in biological triplicate
(n=3). Stars denote Student's t-test *p-value* significance: (***) *p-value* ≤ 0.005, (**) *p-value*
≤ 0.01 and (*) *p-value* ≤ 0.05. Primer pairs used in the nanostring analysis are described
in Supplemental Data 1.
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Figure 2: Diel mRNA gene expression analysis of circadian clock and
photoreceptor related genes in *rve 4 6 8* and Col-0 plants. Select genes from different
circadian / diel implicated plant cell processes were examined for their expression level
at end-of-night (ZT0) and end-of-day (ZT12) by NanoString expression analysis (see
Materials and Methods). **(A)** Expression of circadian clock / RVE8-like related genes
CCA1, LHY, TOC1, PRR5, ELF4, PIF4 and *PIF5*. **(B)** Expression of photoreceptors *PhyA,*
PhyB, CRY1, PHOT1, PHOT2 and *ZTL*. All time-points and genotypes were analyzed in
biological triplicate (n=3). Stars denote Student's t-test *p-value* significance: (***) *p-value*
≤ 0.005, (**) *p-value* ≤ 0.01 and (*) *p-value* ≤ 0.05. Primer pairs used in the nanostring
analysis are described in Supplemental Data 1.
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Figure 3: Analysis of the quantified proteome and phosphoproteome changes at
ZT0 or ZT12 in *rve 4 6 8* versus Col-0. The significantly changing **(A)** proteome and **(B)**
phosphoproteome (n=4; fold-change ≥ 1.5, *q-value* ≤ 0.05) were plotted by venn diagram
and scatterplot to visualize the overlap and Log_2 fold-change (Log_2FC) between *rve 4 6*
8 and WT Col-0 plants at ZT0 and ZT12. Gene ontology (GO) enrichment of biological
processes, subcellular localization and molecular function was then performed to
systemically contextualize proteome and phosphoproteme changes (see Materials and
Methods).
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Figure 4: Association network analysis of significant genotypic proteome
fluctuations at ZT0 and ZT12. The association network depicts significant Log_2FC in
protein abundance between *rve 4 6 8* and WT Col-0 at ZT0 and ZT12 (*q-value* ≤ 0.05;
Supplemental Data 2). Networks were generated using Cytoscape and a combination of
the STRING-DB and enhancedGraphics App (see Materials and Methods) using all
datatypes and an edge score ≥ 0.7. Nodes with no edges ≥ 0.7 were removed. Brighter
red (*rve 4 6 8*) or blue (WT Col-0) coloration indicates the relative increase in measured
Log_2FC protein abundance in that corresponding genotype. Highlighted node clusters
were manually annotated using a combination of ThaleMine
(https://bar.utoronto.ca/thalemine/begin.do), TAIR (https://www.arabidopsis.org/) and
Cytoscape Functional Annotation.

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Figure 5: Association network analysis of significant genotypic phosphoproteome
fluctuations at ZT0 and ZT12. The association network depicts significant median
Log_2FC phosphoproteome changes between *rve 4 6 8* and WT Col-0 at ZT0 and ZT12
(*q-value* ≤ 0.05; Supplemental Data 3). Networks were generated using Cytoscape and a
combination of the STRING-DB and enhancedGraphics App (see materials and methods)
using all datatypes and an edge score ≥ 0.7. Nodes with no edges ≥ 0.7 or only edges ≤
0.6 were removed. Brighter red (*rve 4 6 8*) or blue (WT Col-0) coloration indicates the
relative increase in measured Log_2FC protein abundance in that corresponding genotype.
Highlighted node clusters were manually annotated using a combination of ThaleMine
(https://bar.utoronto.ca/thalemine/begin.do), TAIR (https://www.arabidopsis.org/) and
Cytoscape Functional Annotation.

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1202 **Figure 6: Relative Log_2FC fold-change change in diel metabolite levels.** Rosette
1203 tissue (28 d post-imbibition) was sampled at ZT0, ZT6, ZT12 and ZT18 as outlined in the
1204 Materials and Methods. Brighter red (*rve 4 6 8*) or blue (WT Col-0) coloration indicates
1205 the relative increase in measured Log_2FC metabolite abundance in that corresponding
1206 genotype.

1207

Figure 7: Proteasome function is reduced in *rve 4 6 8*. (A) Proteasome activity in relative fluorescence units per minute (RFU/min) measured in crude extracts of three week old *rve 4 6 8* and WT Col-0 plants. (**** Bonferroni adjusted *p-value* <0.0001; * Bonferroni adjusted *p-value* < 0.05) (**B**) Seedling growth experiments with proteasome inhibitor treatment (50 µM MG132). Seedlings were germinated, stratified, and then grown for 6 d under 12:12 LD conditions. A total of six replicate plates per condition were assayed (Additional plates depicted in Supplemental Figure 4).

SUPPLEMENTAL FIGURES

Supplemental Figure 1. Confirmation of known *rve 4 6 8* phenotypes. Phenomics experimentation was designed to parallel that resolved by previously by Gray *et al.,* 2017 to ensure growth conditions produced comparable phenotypes prior to molecular experimentation. **(A & B)** Rosette area (mm^2) and perimeter (mm) over 5 d of growth (15 d – 19 d post-imbibition). Data was extracted using PlantCV (n=20). **(C & D)** Rosette fresh and dry weight (mg) at 28 d post-imbibition (n=10). **(E)** Days to bolting (n=20). Stars denote Student's t-test *p-value* significance: (***) *p-value* ≤ 0.005 and (*) *p-value* ≤ 0.05.

Supplemental Figure 2: Diel mRNA gene expression analysis of circadian clock and phytohormone related genes in *rve 4 6 8* and Col-0 plants. Select genes from different circadian / diel implicated plant cell processes were examined for their expression level at ZT0 and ZT12 by quantitative NanoString expression analysis (see Materials and Methods). **(A)** Expression of circadian clock / RVE8-like related genes *RVE4*, *RVE8*, *PRR7*, *GI* and *CO*. **(B)** Expression of phytohormone genes *GGPPS1*, *ARR7* and *IAA4*. All time-points and genotypes analyzed in biological triplicate (n=3). Stars denote Student's t-test *p-value* significance: (***) *p-value* ≤ 0.005, (**) *p-value* ≤ 0.01 and (*) *p-value* ≤ 0.05. Primer pairs used in the nanostring analysis are described in Supplemental Data 1.

Supplemental Figure 3. Association network analysis of significantly changing phosphoproteins maintaining an enriched protein kinase motifs. The phopshopeptides corresponding to one or more of the 5 enriched phosphorylation motifs

were used to generate an association network that relates protein kinase motifs to the
underlying protein networks which exhibit genotypic changes in phosphorylation
(Supplemental Data 5). Networks were generated using Cytoscape and a combination of
the STRING-DB and enhancedGraphics App (see Materials and Methods) using all
datatypes and an edge score ≥ 0.7. Protein localization was determined using SUBA4
(https://suba.live/). Highlighted node clusters were manually annotated using a
combination of ThaleMine (https://bar.utoronto.ca/thalemine/begin.do) and TAIR
(https://www.arabidopsis.org/).
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1248 **Supplemental Figure 4:** Additional replicates for the proteasome inhibitor sensitivity
1249 growth assay described in Figure 7B.
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SUPPLEMENTAL DATA
Supplemental Data 1: Primer sets used in Nanostring analysis.
Supplemental Data 2: All quantified and significantly changing proteome data.
Supplemental Data 3: All quantified and significantly changing phosphoproteome
data.
Supplemental Data 4: Gene Ontology enrichment analysis.
Supplemental Data 5: Comparative analysis of significantly changing
phosphorylation sites to significantly cycling
phosphorylation sites (JTK-cycle *p-value* ≤ 0.05).
Supplemental Data 6: Diel changes in proteasome.
Supplemental Data 7: MotifX phosphor-motif enrichment analysis using MoMo
MEME suite.
Supplemental Data 8: Gas Chromatography Mass Spectrometry (GC-MS) data
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Table I

	Quantified		*Significantly Changing	
	Protein Groups	Peptides	Protein Groups	Peptides
Proteome	3934	n/a	536	n/a
Phosphoproteome	1137	1872	346	425

* (n=4; *q-value* ≤ 0.05; FC ≥ 1.5)

Figure 1

Figure 2

A. Circadian Clock / RVE8-Like Related

B. Photoreceptors

Figure 3

A.

ZT0   ZT12

134   63   156

Y-axis: ZT12 (*rve 4/6/8* / Col-0) (Log$_2$FC)

X-axis: ZT0 (*rve4/6/8* / Col-0) (Log$_2$FC)

Gene Ontology Enrichment

Biological Processes:
- Macromolecule catabolism
- Cellular catabolic processes
- Response to acid chemical
- Protein catabolic processes
- Proteolysis
- Polysaccharide metabolism

Subcellular Location:
- Proteasome core complex
- Peptidase complex
- Proteasome complex
- Cytoplasm

Molecular Function:
- Threonine-type peptidase activity
- Pattern binding
- Carbohydrate binding
- Phosphotransferase activity

B.

ZT0   ZT12

29   271   38

Y-axis: ZT12 (*rve 4/6/8* / Col-0) (Log$_2$FC)

X-axis: ZT0 (*rve 4/6/8* / Col-0) (Log$_2$FC)

Gene Ontology Enrichment

Biological Processes:
- Macromolecule metabolism
- Response to alcohol
- Organelle organization
- Response to abscisic acid
- Cell cycle
- Cell division
- Response to acid chemical
- Response to lipid
- Chromosome segregation

Subcellular Location:
- Nucleus
- Cytoplasm
- Plasma membrane

Molecular Function:
- Hydrolase activity
- Transferase activity

Figure 4

Figure 5

Figure 6

	Metabolite	zt0	zt6	zt12	zt18
AMINO ACIDS	Proline	0.11876	-0.3251	0.14782	-0.0452
	Glycine	0.33635	-0.1793	0.07189	0.00667
	Alanine	0.42136	-0.0392	-0.3874	0.06209
	Valine	0.17249	-0.3952	-0.2908	-0.0968
	Serine	-0.0516	-0.668	-0.3435	-0.2243
	Threonine	-0.2489	-0.7273	-0.4982	-0.6009
	Oxoproline	0.76778	0.28482	0.37774	0.5045
	Glutamic acid	0.90973	0.34547	0.43199	0.56407
	Phenylalanine	1.37177	-0.0823	-0.4578	-0.2088
	Asparagine	0.04382	0.53934	-0.5256	0.41541
ORGANIC ACID	Shikimic acid	0.73752	0.20973	0.35809	0.45792
	Citric acid	0.22559	-0.0101	-0.0188	0.02254
	Malic acid	0.27184	-0.1122	0.14143	0.3441
	Sinapinic acid	0.65492	0.29656	0.37613	0.52211
	Succinic acid	0.29573	0.15162	0.44533	0.52778
	Glyceric acid	0.29827	1.3348	1.68119	0.82126
	Fumaric acid	1.50538	0.69384	1.05776	1.42337
FA	Stearic acid	0.07598	-0.0329	0.18202	0.42968
	α-Linolenic acid	1.79841	0.60266	0.11563	0.57337
	Palmitic acid	0.18004	0.01183	0.17715	0.28635
SUGARS	Ribose	0.426	0.08479	0.39341	0.26825
	Glucopyranoside	0.9053	0.29352	0.47691	0.52401
	Sucrose	0.92758	-0.0673	-0.2546	-0.1345
	Fructose	-0.1311	0.58635	0.29415	-0.171
	Glucose	-0.2645	0.12747	0.11925	-0.7815
	Mannose	0.01893	2.86785	1.69011	1.82642
	Mannopyranose	0.426	2.92767	1.6949	1.71009
	Galactose	0.33122	-0.4348	-0.2996	-0.2505
OTHER	Myo-inositol	0.75642	-0.0377	0.49368	0.45784
	Glycerol	-0.1105	-0.1713	0.16128	-0.1205
	Phytol	0.64536	-1.0376	-0.6581	-0.9052

Figure 7