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Supplementary Information for

Superoxide is promoted by sucrose and affects amplitude of circadian rhythms in the evening

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Datasets 1 to 6

Supplementary Information Text

Materials and Methods

Plant materials and growth conditions. Col-0 was used as wild-type *Arabidopsis thaliana. CCR2p:LUC, CCA1p:LUC, PRR7p:LUC* and *TOC1p:LUC* transgenic lines have been described previously (1). Mutants *rboha, rbohb, rbohc/root hair defective2-1* and *rbohd rbohf* and *WRKY11p:GUS* and *WRKY30p:GUS* transgenic lines were obtained from Arabidopsis Biological Resource Centre (ABRC). Mutant *tps1-12* (2) was backcrossed twice to Col-0.

Seeds were surface sterilised with 30% (v/v) bleach, 0.02% (v/v) Triton X-100, washed three times with sterile deionised water and sown on ½ strength Murashige & Skoog (½ MS), pH 5.7 or modified Hoagland media, pH 5.7 (3) solidified with 0.8% (w/v) agar Type M (Sigma). After 2 d in the dark at 4ºC, seedlings were grown at 20ºC in 12 h light/12 dark cycles (LD) under 100-140 µmol m^{-2} s^{-1} light. Concentrations of DPI, MV and 3-AT were based on a previous study (4)

For dark growth assays, seeds were germinated on ½ MS in LD for 48 h. Within 1 h of dawn before photomorphogenesis, germinated seeds were transferred to ½ MS with 1% (w/v) agar containing treatments, wrapped in foil and grown vertically for 3 d. Plates were photographed and root and hypocotyl lengths were quantified with ImageJ (NIH).

RNA-seq. Col-0 seeds were sown on nylon membrane on modified Hoagland's solution and grown at 45º angle. Two week old seedlings were wrapped in aluminium foil before dawn and grown in the dark for 72 h. Under dim green light, dark-adapted seedlings were transferred to Hoagland's media containing 10 mM mannitol or 10 mM sucrose and maintained in the dark or 10 mM mannitol with or without 20 µM DCMU and returned to the light. Shoots of 40 seedlings were collected at 0, 0.5, 2 and 8 h after treatments, snap-frozen in liquid nitrogen and stored at -80ºC until processing. The RNA-seq samples were taken from two independent experiments; the first produced three biological replicates for all conditions, and the second, three further replicates for the dark-grown 0, 2 and 8 h conditions. RNA was extracted with RNeasy Plant Mini Kit including on-column DNase treatment (Qiagen). RNA quantity and purity were confirmed using a Nanodrop spectrophotometer (ThermoScientific), and samples were run on an Agilent 2100 Bioanalyzer, with RNA 6000 Nano kit, to confirm RNA integrity (all samples displayed RINs of > 7). mRNA sequencing libraries were prepared from 1 µg total RNA using the NEBNext RNA Ultra Directional Library preparation kit for Illumina (New England BioLabs Inc.), in conjunction with the NEBNext Poly(A) mRNA Magnetic Isolation Module and NEBNext multiplex oligos for Illumina (dual 8 bp indexing primers set 1), according to the manufacturer's instructions. Libraries were pooled at equimolar ratios, and the pool was sent for 2 x 150 base paired end sequencing on a HiSeq 3000 at the University of Leeds Next Generation Sequencing Facility. Each sample was sequenced twice on two separate lanes, except replicate 3 of the light 2 h condition, which failed and was resequenced on one lane only, and replicate 1 of the 0 h condition in experiment 2, which also failed and was not resequenced. Raw reads have been uploaded to the European Nucleotide Archive, ENA accession PRJEB40453 (https://www.ebi.ac.uk/ena/browser/view/PRJEB40453).

RNA-seq samples were quantified with Salmon v0.8.2 (5) using options -l ISR, --seqBias, --gcBias, --useVBOpt and --numBootstraps 30 and providing both lanes of sequencing for each sample as input. The reference was Araport11 files Araport11_genes.201606.cdna.fasta.gz and Araport11_GFF3_genes_transposons.201606.gtf.gz, downloaded from https://www.arabidopsis.org/download/index-auto.jsp?dir=%2Fdownload_files%2FGenes%2FAraport11_genome_release on 26 April 2017. A map of transcript names to gene names to use with Salmon option -g was created with the following Unix one liner:

```
cut -f9 Araport11_GFF3_genes_transposons.201606.gtf | sort | uniq |
perl -ne 'print "$1\t$2\n" if /transcript_id "(.+)"; gene_id "(.+)";/'
> Araport11_GFF3_gene_transposons.201606.salmon.geneMap.tsv
```

Salmon output was converted to sleuth-compatible format with wasabi (https://github.com/COMBINE-lab/wasabi, commit f31c73e). These files are available in a Dryad repository (https://datadryad.org; https://doi.org/10.5061/dryad.v41ns1rv9).

Differential expression was analysed with Sleuth v0.29.0 (6) with multiple testing correction by stageR v0.1.0, commit 59af4d7 (7), against the Araport11 gene annotation (8) imported from Ensembl Genomes release 36 (9) with biomaRt (10). Models were run with a log2 transformation function on the counts (log2(x+0.5)). A Sleuth model was built for each pairwise comparison (Dark vs Sucrose 0.5 h, Dark vs Sucrose 2 h, Dark vs Sucrose 8 h, Light vs DCMU 0.5 h, Light vs DCMU 2 h, Light vs DCMU 8 h) with differentially expressed genes detected with a Wald test for each comparison. A full model was run on all samples including control 0 h samples with differentially expressed genes detected with a likelihood ratio test. Screening p-values for stageR were taken from the full model's likelihood ratio test and confirmation p-values from the pairwise models' Wald tests. stageR results targeted a 10% overall false discovery rate using the Holm method for family-wise error rate correction. R code to run Sleuth and stageR analyses is provided in the Dryad repository (doi:10.5061/dryad.v41ns1rv9; run_sleuth.R, run_stageR.R). Comparisons between gene lists were made using a Venn diagram tool http://bioinformatics.psb.ugent.be/webtools/Venn/. Gene ontology (GO) enrichment of these lists used PANTHER Classification System (11) accessed through The Arabidopsis Information Resource (TAIR).

qRT-PCR. cDNA was prepared from 0.5 µg RNA in 10 µl reactions using Tetro cDNA synthesis kit (Bioline). 0.5 ng/µl of cDNA was used in each PCR reaction with 0.2 µM primers in the SensiFAST SYBR no-ROX kit (Bioline) on a CFX96 Touch Real-time PCR detection system (Bio-Rad). PCR reaction efficiencies were determined for each primer pair using LinRegPCR (12) and transcript levels were determined for target and reference genes using (mean PCR efficiency)$^{-Ct}$. Primer sequences are listed in Dataset 6.

Transcriptome Clustering. Genes were clustered based on Sleuth scaled_reads_per_base abundance values for each sample, using scikit-learn's BayesianGaussianMixture (13) https://scikit-learn.org/stable/modules/generated/sklearn.mixture.BayesianGaussianMixture.html) with maximum 1000 iterations. Numbers of clusters from 2 to 20 were tested, with the 14 cluster output chosen for further analysis. Gene Ontology Enrichment analysis for each cluster was performed with R's clusterProfiler (14) https://bioconductor.org/packages/release/bioc/vignettes/clusterProfiler/inst/doc/clusterProfiler.html). R code for clustering is provided in the Dryad repository (doi:10.5061/dryad.v41ns1rv9; cluster_analysis.R).

Histochemical stains. Seeds were sown on ½ MS and grown in LD and 11 d old seedlings were wrapped in aluminium foil at dusk. After 72 h, at subjective dusk under dim green light, seedlings were transferred into 0.5 ml liquid ½ MS containing 0.1% (v/v) DMSO or chemical treatments in 48-well plates. At the following subjective dawn in dim green light, 0.5 ml of 60 mM mannitol or sucrose was added (30 mM final sugar concentration). For H_2O_2 stains, 1 mg/ml (w/v) 3'3-diaminobenzidine tetrahydrochloride hydrate was dissolved in 50 mM Tris acetate (pH 5.0). For O_2^- stains, 2 mg/ml (w/v) nitroblue tetrazolium was dissolved in 10 mM potassium phosphate buffer (pH 7.8), 10 mM NaN_3. Seedlings were vacuum infiltrated for 1 min in freshly prepared staining solutions and incubated in the dark for 24 h. Samples were cleared by boiling for 5 min in 1:1:4 lactic acid:glycerol:ethanol then transferred to 1:4 glycerol:ethanol. GUS-stains of transgenic lines was performed overnight as previously (15). Stained seedlings were mounted under coverslips on microscope slides and imaged immediately with a SMZ800 stereomicroscope (Nikon) or a V370 Photo flatbed scanner (Epson). DAB and NBT stain intensity were quantified in whole shoots by dividing integrated density by area of individual seedlings and subtracting background signal in ImageJ (NIH).

L-012 luminescence assay. Clusters of 7 d old seedlings grown on ½ MS or 6 mm leaf discs from 4 week old plants grown in LD were transferred to 96-well luminescence plates (Greiner) containing 250 µl liquid ½ MS before dusk (ZT12), wrapped in aluminium foil and placed in the dark for 72 h. At subjective dawn under dim green light, media was replaced with 100 µl 100 µM L-012, 20 µg/ml horseradish peroxidase containing 0.01% DMSO, 10 µM DPI, 2 µM MV, 0.2 mM 3-AT, 20 µM VAS2870, 500 µM apocynin or 500 µM allopurinol. After 1 h of chemical pre-treatment 100 µl of 60 mM sucrose or mannitol was added to each well (final sugar concentration 30 mM). Luminescence was measured in the dark at 90 s intervals in a Lumistar Omega plater reader (BMG) using a 4 mm orbital well scan.

Luciferase luminescence assays. For sugar-response assays, *CCR2p:LUC* seeds were sown on ½ MS and grown in LD. Pairs of 10 d old seedlings were transferred into 96-well luminescence plates (Greiner) containing 200 µl ½ MS with agar at dusk, wrapped in foil and grown in the dark. 1 mM D-luciferin, K-salt (Promega) was applied twice under dim green light. After 84 h in the dark (subjective dawn), 20 µl of 0.5% (v/v) DMSO, 50 µM DPI, 10 µM MV or 1 mM 3-AT was applied to seedlings under dim green light, 1 h before addition of 30 µl of 30 mM mannitol or sucrose. For the dose response curves, seedlings were transferred under dim green light to ½ MS media containing DMSO, DPI, VAS2870, apocynin or allopurinol 12 h before application of sugar at subjective dawn. Luminescence was measured in the dark at 1 h intervals in a Lumistar Omega plate reader (BMG) using a 4 mm orbital well scan.

To measure circadian rhythms, clusters of 5 seeds were sown on ½ MS and grown in LD. Clusters of 7 d old seedlings were transferred at dawn to ½ MS containing 30 mM mannitol or sucrose with 0.1% (v/v) DMSO, 10 µM DPI, 2 µM MV or 0.2 mM 3-AT. 1 mM D-luciferin, K-salt (Promega) was applied to seedlings twice prior to imaging. Luciferase was imaged in 10 min integrations following 120 s of dark at 1 hr intervals with an HRPCS5 intensified CCD camera (Photek) fitted with LB3 red (640 nm) and blue (470 nm) LED arrays providing light at 60 µmol m^{-2} s^{-1} for 1 LD followed by continuous low light at 10 µmol m^{-2} s^{-1}. Luminescence counts were extracted from ROIs using Image32 software (Photek) and circadian rhythms were analysed by Fast Fourier Transform Non-linear Least Squares using Biodare2 (16).

Sugar quantification. Seedlings were grown as for the RNA-Seq experiment or pairs of seeds were sown on ½ MS and grown in LD. Seven d old seedlings were wrapped in foil at dusk and grown in the dark. After 72 h, seedlings were transferred under dim green light into 96 well plates containing ½ MS with 0.1% DMSO or 10 µM DPI. At subjective dawn, seedlings were treated with 30 µL 30 mM mannitol or sucrose. 30 seedlings were harvested per biological replicate, frozen in liquid N and stored at -80ºC until processing. Soluble sugars were extracted in 80% (v/v) ethanol measured using a Sucrose/Glucose/Fructose calorimetric assay kit (Megazyme) scaled down for 96-well plates.

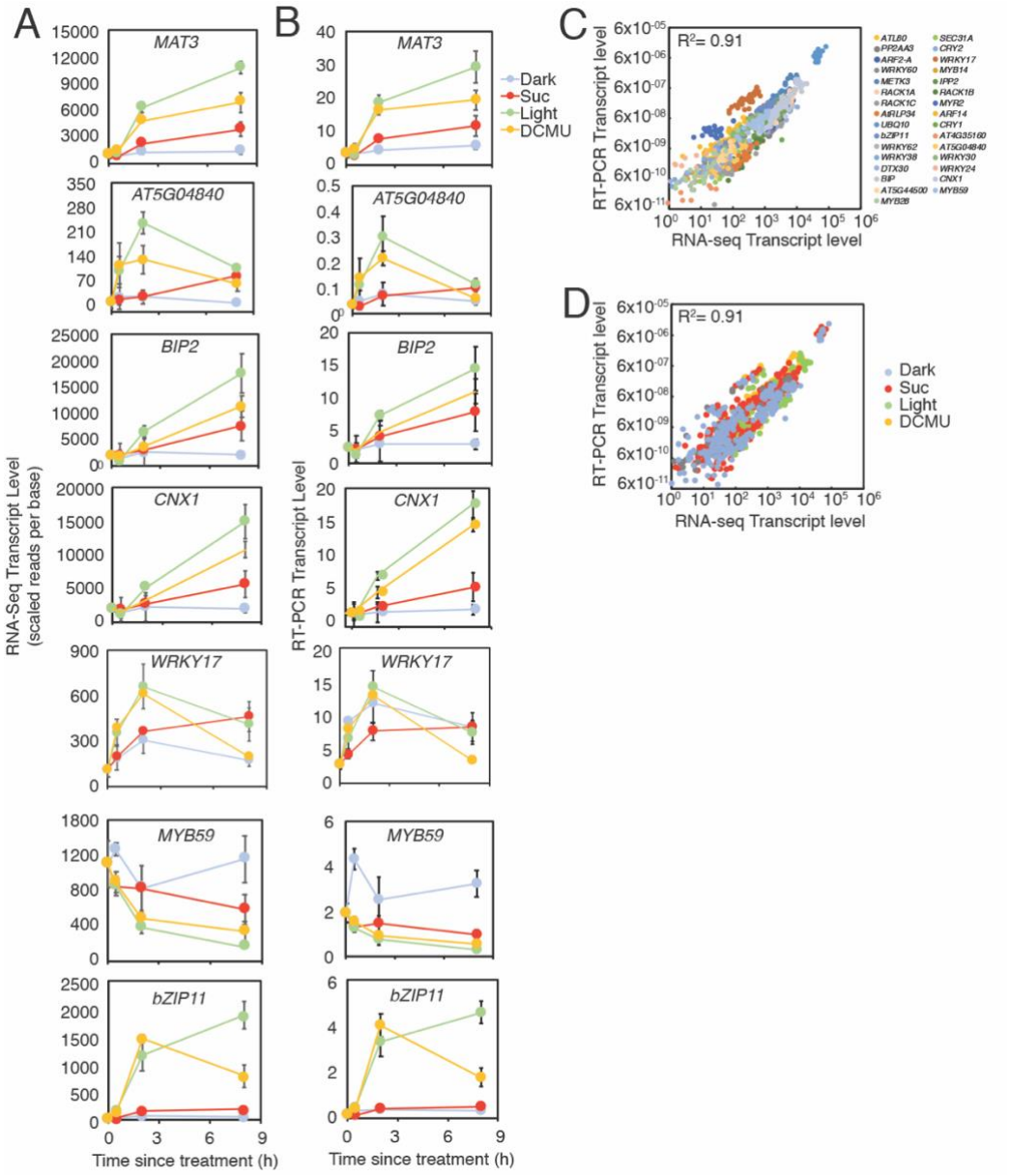

Fig. S1. Quality control of RNA-seq transcript data. (A) and (B) comparison of quantification of 8 representative marker genes determined by RNA-Seq (A) and qRT-PCR relative to geometric mean of *PP2AA3 and IPP2* (B) (means ± SD, *N* = 3). (C) and (D) comparison of quantification of 31 transcripts by qRT-PCR (PCR efficiency^{-Ct}) and RNA-seq (scaled reads per base). Plots are the same data coloured by transcript (C) or treatment (D). Values are individual biological replicates.

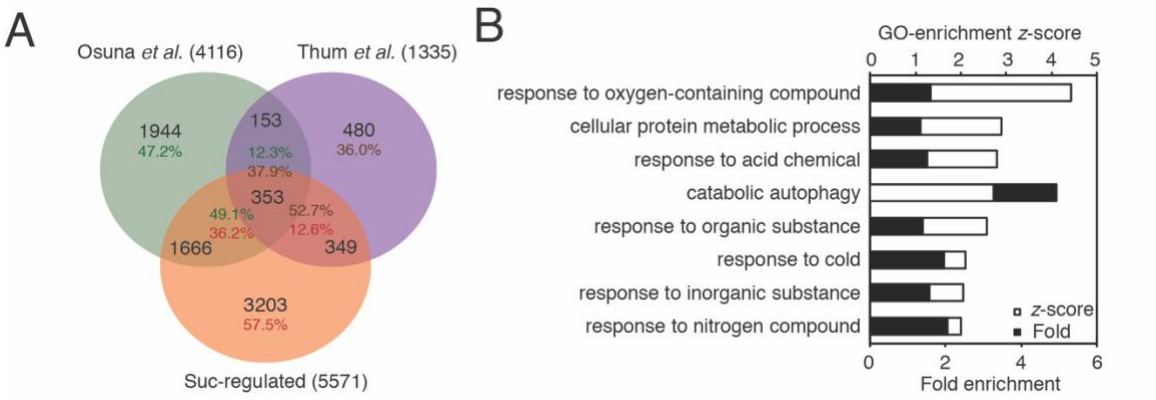

Fig. S2. Defining the light-independent sugar-regulated transcriptome in Arabidopsis shoots. (A) Comparison of genes identified as sugar-regulated in the dark in this study with two previous studies (17, 18). (B) Gene Ontology enrichment of 2772 differentially-expressed genes after 2 h treatment with mannitol or sucrose in the dark showing GO categories with a *z*-score > 2.

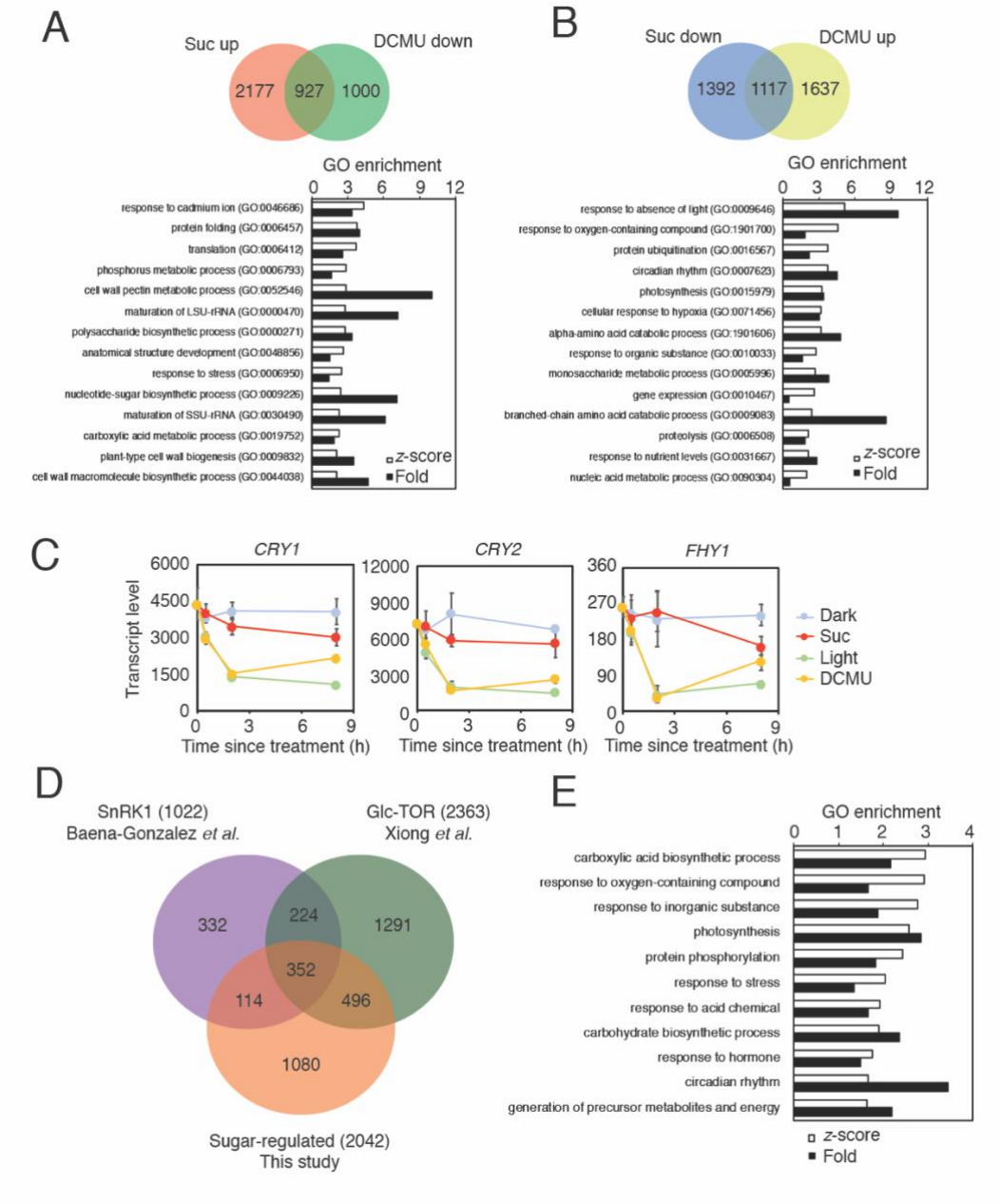

Fig. S3. Light-independent sugar-regulated genes in Arabidopsis. (A) Gene Ontology enrichment of 927 genes that are up-regulated by sucrose in the dark and down-regulated by DCMU in the light. (B) Gene Ontology enrichment of 1117 genes that are down-regulated by sucrose in the dark and up-regulated by DCMU in the light. Fold-enrichment and *z*-score are plotted on the same scale. (C) RNA-seq transcript level of light-signalling genes identified as down-regulated by sucrose and up-regulated by DCMU. (D) Comparison of 2042 genes identified as sugar-regulated in (A) and (B) to genes reported as regulated by SnRK1 (19) and TOR (20). (E) Gene Ontology enrichment of 1080 sugar-regulated genes not previously identified as SnRK1- or TOR-regulated showing GO categories with a *z*-score > 2. Fold-enrichment and *z*-score are plotted on the same scale.

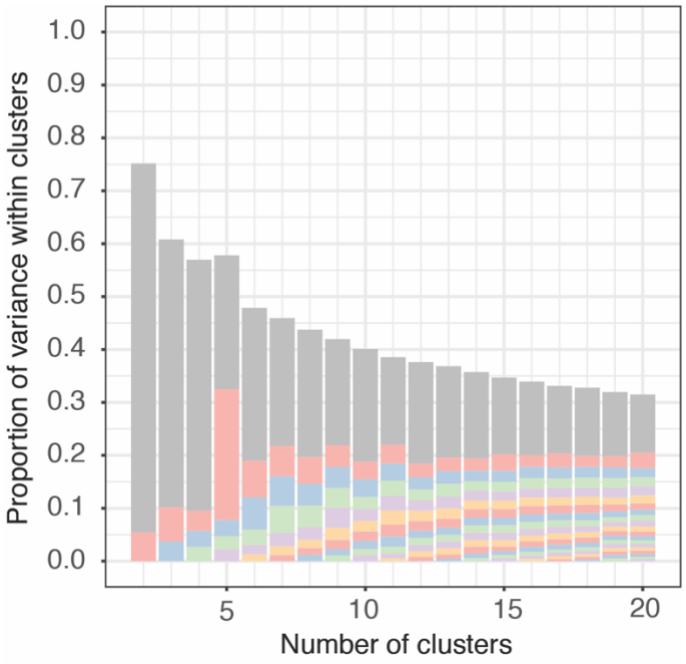

Fig. S4. Optimisation of gene clustering. Elbow plot of percentage of total variance within clusters for clustering runs with k=2 to k=20. Grey is cluster with largest variance, usually representing unclustered genes.

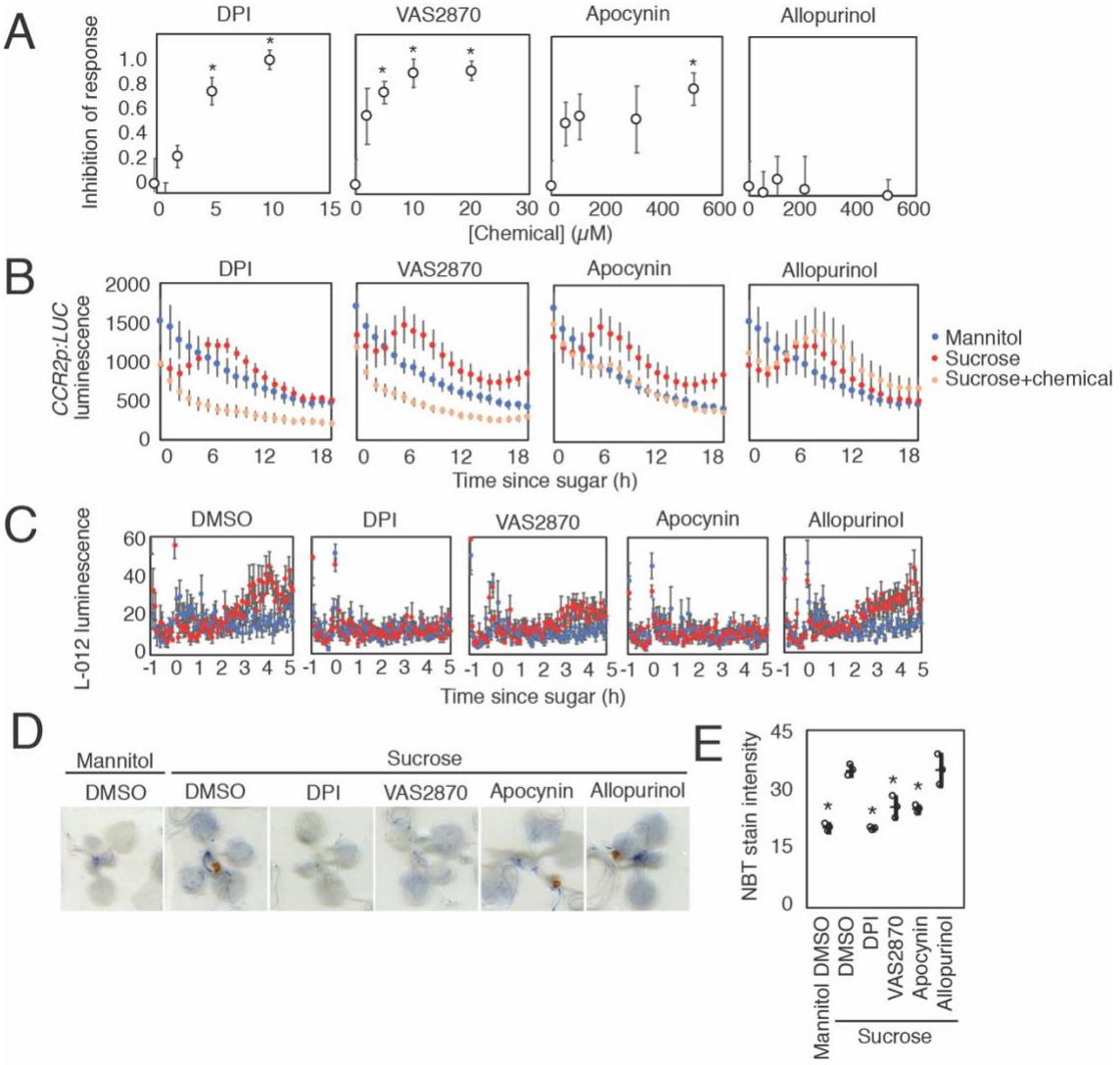

Fig S5. Effects of NADPH oxidase inhibitors. (A) Inhibition of response of luciferase luminescence to 30 mM sucrose in dark-adapted *CCR2p:LUC* seedlings by DPI, VAS2870, apocynin or allopurinol in the presence of four concentrations of each chemical inhibitor or DMSO (means ± SEM, *N* = 6; * *P* < 0.05 from DMSO; Bonferroni-corrected *t*-test). (B) Luciferase luminescence in dark-adapted *CCR2p:LUC* seedlings treated with 30 mM mannitol or sucrose in the presence of 0.1% DMSO, 10 µM DPI, 20 µM VAS2870, 500 µM apocynin or 500 µM allopurinol (means ± SEM, *N* = 6). (C) L-012 luminescence in dark-adapted Col-0 treated with 30 mM mannitol or sucrose in the presence of DMSO, 10 µM DPI, 20 µM VAS2870 or 500 µM apocynin or 500 µM allopurinol (means ± SEM, *N* = 12). (D) Representative images and (E) quantification of NBT stains in dark-adapted Col-0 seedlings 4 h after treatment with 30 mM mannitol or sucrose in presence of 0.1% DMSO, 10 µM DPI, 30 µM VAS2870, 500 µM Apocynin or 500 µM allopurinol (means ± SD, *N* = 3; * P < 0.05 from DMSO+Sucrose ; Bonferroni-corrected *t*-test).

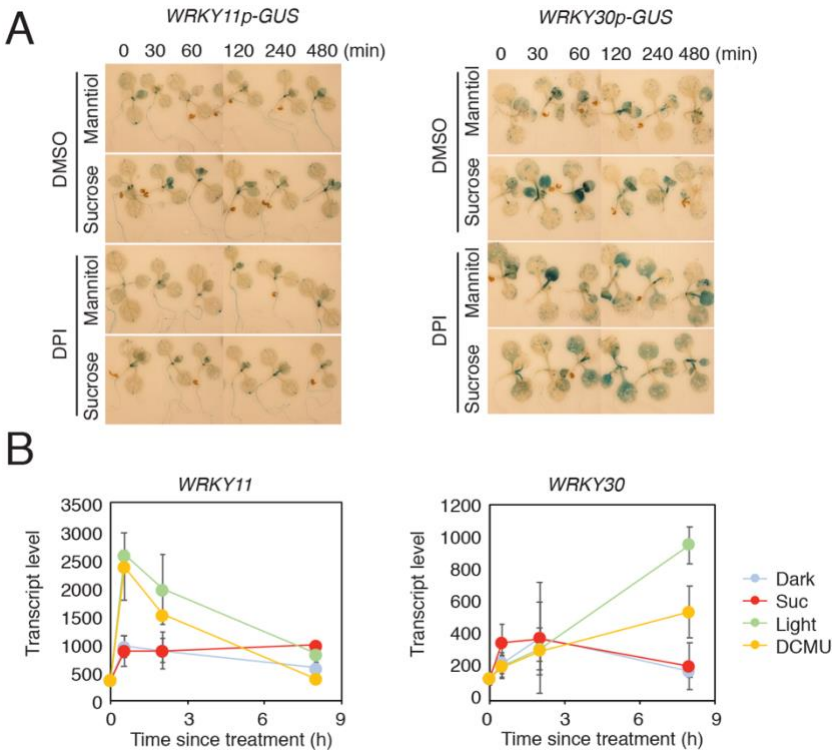

Fig. S6. Sugar and DPI affect *WRKY* promoter activity. (A) GUS stains of dark-adapted 10 d old *WRKY11p-GUS* and *WRKY30p-GUS* seedlings treated with 30 mM mannitol or sucrose, pre-treated for 30 min with DMSO or 10 µM DPI. (B) RNA-seq transcript levels of *WRKY11* and *WRKY30* (means ± SD, *N* = 3).

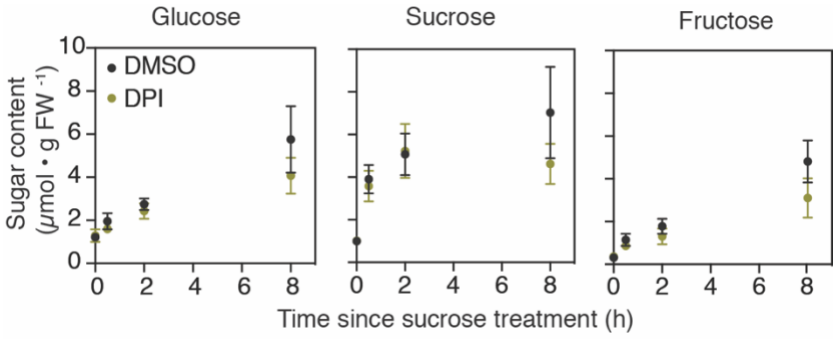

Fig. S7. Soluble sugar content in DPI-treated seedlings. Glucose, sucrose and fructose content in dark-adapted Col-0 seedlings treated with 30 mM sucrose in the presence of 0.1% DMSO or 10 µM DPI. Values are means ± SD, *N* = 4. No significant difference was identified between DMSO or DPI treated seedlings by *t*-test with Bonferroni correction, $P < 0.05$.

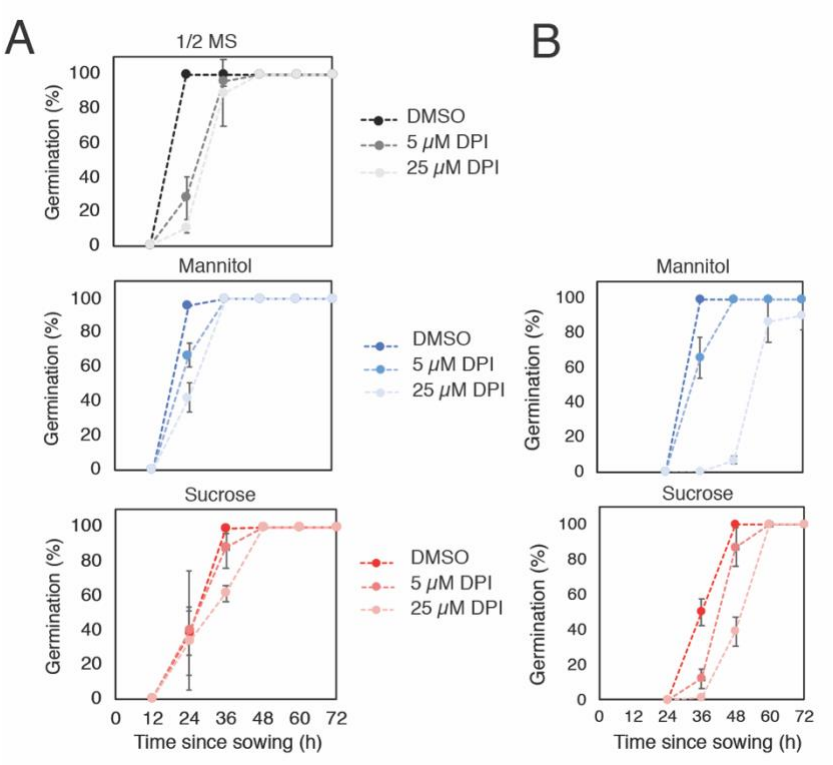

Fig. S8. Additive effects of DPI and sucrose on seed germination. (A) Percentage of germinated (A) non-dormant Col-0 seeds following 2 d chilling at 4ºC or (B) dormant seeds without chilling sown on ½ MS with or without 30 mM mannitol or sucrose and 0.1% DMSO or DPI. Values are mean ± SD of four independent seed populations.

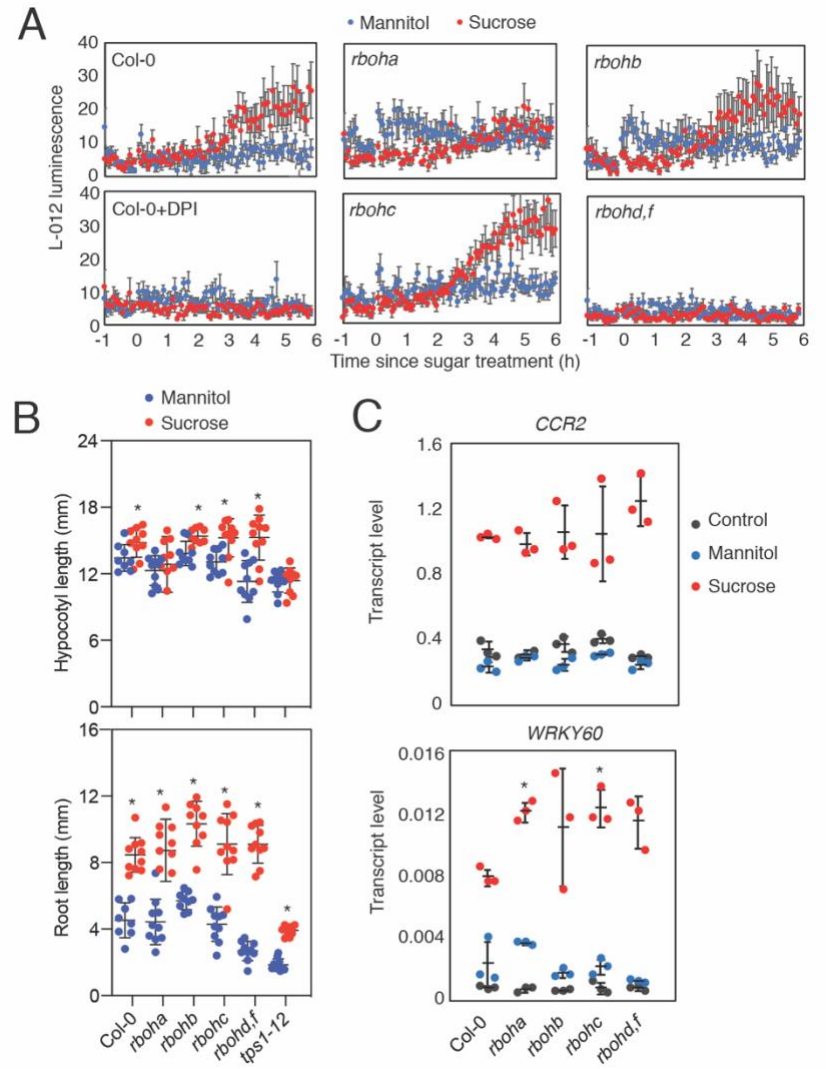

Fig. S9. NADPH oxidases contribute redundantly to sugar responses. (A) L-012 luminescence in dark-adapted Col-0 (with or without 10 µM DPI), *rboha, rbohb, rbohc* and *rbohd rbohf* seedlings after treatment with 30 mM mannitol or sucrose (means ± SEM, *N* = 6). (B) Hypocotyl length and root length of 5 d old dark-grown Col-0, *rboha, rboha, rbohb, rbohc, rbohd rbohf* and *tps1-12* seedlings grown on ½ MS with 30 mM mannitol or sucrose (means ± SD, *N* = 10; * *P* < 0.05 from mannitol, *t*-test). (C) Transcript level of *CCR2* and *WRKY60,* relative to *UBQ10* in dark-adapted Col-0 and *rboh* mutant seedlings (control) or 12 h after treatment with 30 mM mannitol or sucrose (means ± SD, *N* = 3; * *P* < 0.05 from Col-0; Bonferroni-corrected *t*-test).

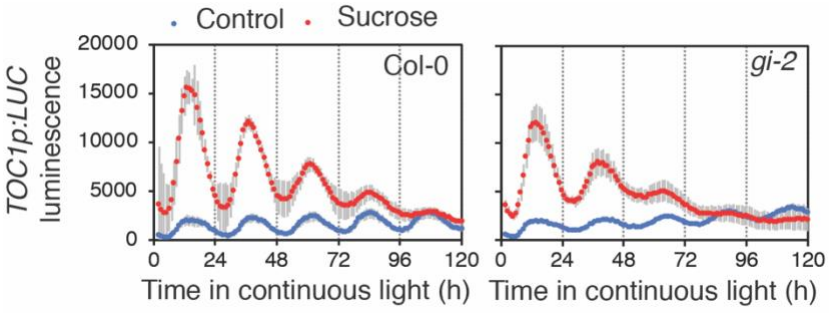

Fig. S10. Effects of ROS chemicals on circadian rhythms. Luciferase luminescence in Col-0 *TOC1p:LUC* and *gi-2 TOC1p:LUC* seedlings in continuous light with or without 90 mM sucrose (means ± SEM, *N* = 4).

Dataset 1 (separate file). Differentially expressed genes between Dark and Suc or Light and DCMU.

Dataset 2 (separate file). Lists of sugar-activated and sugar-repressed genes.

Dataset 3 (separate file). Gene lists and GO enrichment of 14 clusters.

Dataset 4 (separate file). Complete GO enrichment map of top 15 terms from 14 gene clusters.

Dataset 5 (separate file). Gene lists and phase analysis of ROS-regulated genes.

Dataset 6 (separate file). Primer sequences.

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