UNIVERSITY of York

This is a repository copy of *Superoxide* is promoted by sucrose and affects amplitude of circadian rhythms in the evening.

White Rose Research Online URL for this paper: <u>https://eprints.whiterose.ac.uk/171935/</u>

Version: Accepted Version

## Article:

Roman-Fernandez, Angela, Li, Xiang, Deng, Dongjing et al. (4 more authors) (2021) Superoxide is promoted by sucrose and affects amplitude of circadian rhythms in the evening. Proceedings of the National Academy of Sciences of the United States of America. e2020646118. ISSN 1091-6490

https://doi.org/10.1073/pnas.2020646118

#### Reuse

Items deposited in White Rose Research Online are protected by copyright, with all rights reserved unless indicated otherwise. They may be downloaded and/or printed for private study, or other acts as permitted by national copyright laws. The publisher or other rights holders may allow further reproduction and re-use of the full text version. This is indicated by the licence information on the White Rose Research Online record for the item.

#### Takedown

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.



eprints@whiterose.ac.uk https://eprints.whiterose.ac.uk/



1
л.
_

## 2 Main Manuscript for

- 3 Superoxide is promoted by sucrose and affects amplitude of circadian
- 4 rhythms in the evening
- Ángela Román<sup>a,b,1,2</sup>, Xiang Li<sup>a,1</sup>, Dongjing Deng<sup>a,1</sup>, John W. Davey<sup>b</sup>, Sally James<sup>b</sup>, Ian A.
   Graham<sup>b</sup>, Michael J. Haydon<sup>a,b,3</sup>
- 7 <sup>a</sup>School of BioSciences, The University of Melbourne, Parkville, 3010, Australia
- <sup>b</sup>Department of Biology, University of York, Wentworth Way, YO10 5DD, United Kingdom
- 9 <sup>1</sup> These authors contributed equally
- <sup>2</sup> Current address: Estación Experimental de Aula Dei CSIC, Zaragoza, E-50059, Spain.
- <sup>3</sup> Michael J. Haydon
- 12 Email: m.haydon@unimelb.edu.au
- 13 AR 0000-0003-3457-999X; XL 0000-0002-6325-6439; JWD 0000-0002-1017-9775; IAG 0000-14 0003-4007-1770; MJH 0000-0003-2486-9387
- 15 Classification
- 16 Biological Sciences; Plant Biology
- 17 Keywords
- 18 Circadian, superoxide, sugar, redox, ROS
- 19 Author Contributions
- MJH conceived the study; AR, XL, DD, MJH designed experiments; AR, XL, DD, JWD, SJ, MJH
- 21 performed experiments; AR, XL, DD, JWD, IAG, MJH analysed or interpreted data; MJH wrote 22 the manuscript; all authors edited the manuscript.
- 23 This PDF file includes:
- 24 Main Text 25 Figures 1 to 4

27

## 28 Abstract

29 Plants must coordinate photosynthetic metabolism with the daily environment and adapt rhythmic 30 physiology and development to match carbon availability. Circadian clocks drive biological 31 rhythms which adjust to environmental cues. Products of photosynthetic metabolism, including 32 sugars and reactive oxygen species (ROS), are closely associated with the plant circadian clock 33 and sugars have been shown to provide metabolic feedback to the circadian oscillator. Here, we 34 report a comprehensive sugar-regulated transcriptome of Arabidopsis and identify genes 35 associated with redox and ROS processes as a prominent feature of the transcriptional response. We show that sucrose increases levels of superoxide (O<sub>2</sub><sup>-</sup>) which is required for transcriptional 36 and growth responses to sugar. We identify circadian rhythms of O<sub>2</sub>-regulated transcripts which 37 38 are phased around dusk and find that  $O_2^{-}$  is required for sucrose to promote expression of TIMING OF CAB1 (TOC1) in the evening. Our data reveal a role for  $O_2^-$  as a metabolic signal 39 40 affecting transcriptional control of the circadian oscillator in Arabidopsis.

## 41 Significance Statement

42

43 Distinguishing the effects of light and sugars in photoautotrophic cells is challenging. The 44 circadian system is a regulatory network that integrates light and metabolic signals and controls 45 rhythmic physiology and growth. Our experimental approach has defined a light-independent, 46 sugar-regulated transcriptome in Arabidopsis and revealed reactive oxygen species (ROS) as a 47 prominent feature. ROS are by-products of photosynthetic metabolism and oscillate with circadian 48 rhythms but have not previously been demonstrated as inputs to the plant circadian oscillator. 49 Our data suggest a new role for superoxide as a rhythmic sugar signal which acts in the evening 50 and affects circadian gene expression and growth.

51

52

## 53

## 54 Main Text

# 56 Introduction

Plant metabolism is inextricably linked to daily photoperiodic cycles because of the requirement of light for photosynthesis. Anticipation and adaptation to changing light availability enables plants to optimise metabolism according to their immediate environment. Plant metabolism responds to environmental cues, such as light, temperature, biotic and abiotic stress by diverse mechanisms (1).

63

Plant cells require signalling mechanisms to sense carbon and energy status and adjust
 metabolism. Snf1 RELATED KINASE 1 (SnRK1) and TARGET OF RAPAMYCIN 1 (TOR1) are

66 counteracting signalling hubs which are activated under low and replete carbon status,

respectively (2, 3). Trehalose-6-phosphate (T6P) is an essential signalling sugar which indicates
 carbon status and acts through SnRK1 (4, 5).

- 69
- 70 Circadian clocks are an endogenous time-keeping mechanism which regulate rhythms of
- 71 physiology and metabolism and control responses to environmental signals according to the time
- of day (6). The core circadian oscillator in Arabidopsis is a network of transcription factors
- 73 comprised of Myb-like genes CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), LATE ELONGATED
- 74 HYPOCOTYL (LHY) and REVIELLE (RVE) expressed at dawn, PSEUDO RESPONSE
- 75 REGULATOR (PRR) genes expressed through the day including TIMING OF CAB 1 (TOC1) at

dusk, and the Evening Complex (EC) in the night. The phase and amplitude of gene expression
 and protein levels are responsive to environmental cues and they, in turn, coordinate the
 regulation of thousands of genes.

79

80 There is extensive transcriptional and post-transcriptional control of photosynthetic metabolism by 81 the circadian clock and there is metabolic feedback on the circadian oscillator. Elevated SnRK1 82 activity under carbon limitation lengthens circadian period and sucrose shortens period by T6P-SnRK1 acting on the oscillator gene PRR7 (7-9). Period also responds to glucose by a TOR-83 84 dependent mechanism (10). In continuous dark, circadian rhythms rapidly dampen, but can be 85 sustained by addition of sugars. This effect of sugar requires GIGANTEA (GI), a clock protein 86 which is stabilised by sucrose in the evening (11). Sugars can also reinitiate transcriptional 87 rhythms in dark-adapted seedlings, setting phase according to the time of sugar application (8, 88 12), but the mechanism in unknown.

89

90 Redox state and levels of reactive oxygen species (ROS), which are tightly linked to metabolism, 91 are also associated with circadian rhythms in plants. There are circadian rhythms of hydrogen 92 peroxide  $(H_2O_2)$  and NADP $(H)^+$  in Arabidopsis (13, 14). Circadian rhythms of peroxiredoxin 93 oxidation have been detected across Kingdoms (15). These rhythms of redox state and 94 associated ROS are generally considered as outputs of rhythmic metabolism controlled by the 95 circadian clock (13), or even independent of the circadian oscillator (15). The defence hormone 96 salicylic acid perturbs redox state and affects gating of immune response, dependent on the 97 redox-sensitive transcription factor NON-EXPRESSOR OF PATHOGENESIS 1 (NPR1) (14). But 98 there is presently no clear evidence of a role for redox signals as a mechanism of metabolic 99 feedback to the circadian oscillator in plants.

100

101 Distinguishing sugar and light signals can be challenging in photosynthetic cells since it is likely 102 that sugar signalling will be activated in the light. Recent advances in our understanding of the impact of metabolic signalling to the plant circadian clock have relied on experiments in low light 103 104 or darkness (7, 8, 10–12, 16). Here, we use an experimental approach based on the previous 105 observation that sugar can activate expression of circadian clock genes in dark-adapted 106 seedlings to define a light-independent, sugar-regulated transcriptome in Arabidopsis (8, 12). We 107 compare the response of the transcriptome to sucrose in the dark and inhibition of photosynthesis 108 in the light and identify redox and ROS processes as a prominent feature of transcriptional 109 responses to sugars. We demonstrate that superoxide  $(O_2^{-})$  can act as a signal to alter gene expression and growth in response to sucrose. This O2<sup>-</sup> signal acts to promote transcription of 110 111 circadian oscillator genes in the evening. These reveal that ROS can function as metabolic signals affecting circadian rhythms in Arabidopsis. 112 113

## 114 Results

115 116 To identify transcripts that are regulated by sugars in the presence and absence of light and 117 photosynthesis, we designed an RNA-seq experiment based on the previous observation that 118 sugars can reinitiate transcriptional circadian rhythms in dark-adapted Arabidopsis seedings (8, 119 12). Two-week old wild-type (Col-0) seedings were grown in the dark for 72 h to dampen 120 circadian rhythms and establish a stabilised C starvation state. At subjective dawn, dark-adapted 121 seedlings were transferred to media containing 10 mM mannitol (osmotic control) or sucrose and 122 maintained in the dark or transferred to media containing 10 mM mannitol with or without 3-(3,4-123 dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of photosynthesis, and grown in the light. 124 The four treatments provide conditions of no sugar/no light (Dark), sugar/no light (Suc), sugar/light (Light) and light/no sugar (DCMU) (Fig. 1A). We confirmed that seedling glucose 125 126 content increased in the Suc and Light treatments but not in the Dark or DCMU treatments (Fig. 1B). To capture both early and late transcriptional responses within the timeframe of a typical 127 128 photoperiod, shoot tissue was harvested at subjective dawn (0 h) and 0.5, 2 and 8 h after the 129 treatments and prepared for RNA-Seq.

131 We detected 5571 Suc-regulated genes that were differentially expressed between Dark and Suc 132 treatments and 4628 DCMU-regulated genes differentially expressed between Light and DCMU 133 (Fig. 1C, Dataset 1). The quantification of gene expression by RNA-seq was corroborated for 31 representative transcripts by qRT-PCR with a strong positive correlation ( $R^2$ =0.91) (Fig. S1). The 134 overlap of differentially expressed genes (DEGs) between time-points was relatively low (Fig. 135 136 1C), suggesting the sampling design captures a wide dynamic range of the transcriptional response. Comparison of our list of Suc-regulated genes to published microarray datasets (17, 137 138 18) indicated that we have captured a more extensive sugar-regulated transcriptome (Fig. S2A).

139

140 To identify genes that are regulated by sugar, independent of light availability, we generated a list 141 of genes that were upregulated by Suc in the dark and downregulated by DCMU in the light 142 (sugar-activated; 927) or downregulated by Suc in the dark and upregulated by DCMU in the light 143 (sugar-repressed; 1117) (Dataset 2; Fig. S3). The sugar-activated genes were enriched for Gene 144 Ontology (GO) terms related to protein and cell wall synthesis (Fig. S3A). Sugar-repressed genes 145 were enriched for GO terms related to light signalling, circadian rhythm and sugar metabolism 146 (Fig. S3B, S3C). We compared our list of all 2042 sugar-regulated genes to published lists of genes regulated by SnRK1 and TOR, which are two major energy signalling hubs (2, 3). There 147 148 was significant overlap with both datasets, but 1080 sugar-regulated genes were unique to this 149 study (Fig. S3D), including 929 genes represented on ATH1 microarrays. These unique genes 150 could represent responses either upstream or independent of SnRK1- and TOR-mediated 151 signalling. Among the most significantly enriched GO terms in this list was Response to oxygen 152 containing compound and Circadian rhythm (Fig. S3E).

153

154 To define the temporal characteristics of the complete transcriptome dataset, we performed 155 clustering analysis of expression of 18071 genes across all 53 samples using variational 156 Bayesian Gaussian mixture models (Fig. 1D, Dataset 3). We opted for 14 clusters as a tradeoff between maximizing the explained variance and producing meaningful clusters (Fig. S4, Fig. 1D). 157 158 Several clusters were associated with either sugar-repressed (clusters 1-4) or sugar-activated 159 (clusters 11-14) genes (Fig. 1D). We searched for enriched GO terms within each cluster 160 (Dataset 3) and summarised these using an enrichment map of the top 15 terms within each 161 cluster (Fig. 1E, Dataset 4). Some highly enriched GO term networks were specific to one or two 162 clusters such as inositol phosphate processes in cluster 13 or circadian rhythm and growth in clusters 8 and 13. Other enrichment GO term networks represent four or five clusters. The largest 163 of these networks included terms associated with metabolism of sugars, nucleotides and 164 phospholipids, chloroplast function and proteostasis. The second largest enrichment network 165 included terms associated with reactive oxygen species (ROS) metabolism and signalling, 166 167 metabolic stress and immune responses. 168

169 Since GO terms associated with ROS appear to be a strong feature of the complete dataset, we 170 hypothesised that ROS might be contributing to transcriptional responses to sugar. Indeed, Response to oxygen containing compound was the most significantly enriched GO term among 171 172 all 2042 sugar-regulated genes and among Suc-regulated genes at 2 h (Fig. S2B). Within the 173 former, 195 genes are associated with this GO term, including ANNEXIN 2 (ANN2) and six WRKY transcription factor genes (Fig. 2A, Dataset 5). We also identified 95 sugar-regulated 174 175 genes previously reported as ROS-responsive (19), including ASCORBATE PEROXIDASE 1 176 (APX1) and CATALASE 2 (CAT2) (Fig. 2B, Dataset 5).

177

To test whether treatment of Arabidopsis seedlings with sucrose affects production of ROS in dark-adapted seedlings, we used histochemical stains for hydrogen peroxide  $(H_2O_2)$  and superoxide  $(O_2^{-})$  (Fig. 2C,D). Treatment of dark-adapted seedlings with sucrose led to a decrease in staining for  $H_2O_2$  within 30 min. By contrast, sucrose treatment of dark-adapted seedlings increased stain for  $O_2^{-}$  within 2 h, compared to mannitol controls. The elevated NBT stain was observed throughout the shoot, including hypocotyl, cotyledons and leaves. To corroborate this 184 observation, we used a L-012 luminescence assay, which does not discriminate between H<sub>2</sub>O<sub>2</sub> 185 and O<sub>2</sub><sup>-</sup>, but provides better temporal resolution of ROS production than histochemical stains. Consistent with the NBT stains for O2<sup>-</sup>, we detected elevated L-012 luminescence within 2 h in 186 187 sucrose-treated seedlings compared to mannitol-treated controls (Fig. 2E). Presumably, this assay underestimates the difference in  $O_2^-$  production since the signal in sucrose-treated 188 seedlings will be the sum of the reduced  $H_2O_2$  and the increased  $O_2^-$  (Fig. 2C). The ROS-189 190 response detected in both the histochemical and luminescent assays is concomitant with the 191 timing of the transcriptional response associated with ROS-related genes that we detected after 2 192 h (Fig. 2A, 2B, S2B, Dataset 1).

193

194 The accumulation of O<sub>2</sub><sup>-</sup> in sucrose-treated seedlings might be a by-product of increased energy 195 metabolism or could be contributing as a signal to affect transcriptional changes. We looked for 196 chemicals that could inhibit the sucrose-induced production of O2-. Diphenyleneiodonium (DPI) is 197 an inhibitor of NADPH oxidases, which generate O2<sup>-</sup> at the plasma membrane. Methyl viologen (MV) interferes with electron transport from PS I and elevates O2-. 3-amino-1,2,4-triazole (3-AT) 198 199 is a catalase inhibitor which promotes  $H_2O_2$  accumulation. We tested the effect of these 200 chemicals on induction of a circadian-regulated luciferase reporter for COLD. CIRCADIAN 201 RHYTHM REGULATED 2 (CCR2). DPI strongly inhibited the increase of luciferase luminescence 202 in sucrose-treated, dark-adapted CCR2p:LUC seedlings, whereas MV and 3-AT did not (Fig. 3A). 203 Similarly, DPI, but not MV or 3-AT, also inhibited sucrose-induced L-012 luminescence (Fig. 3B) 204 and histochemical staining for  $O_2^-$  but did not affect sucrose-induced changes in staining for  $H_2O_2$ 205 (Fig. 3C, D).

207 We used the transcriptional response of CCR2p:LUC to generate a dose-response curve of 208 inhibition by DPI. This response was inhibited by 30% at 1  $\mu$ M DPI and by >70% at 209 concentrations above 5 µM (Fig. 3E). Similar dose-dependent effects were also observed for two 210 other NADPH oxidsase inhibitors, VAS2870 (20) and apocynin (21), but not for the xanthine 211 dehydrogenase inhibitor, allopurinol (22) (Fig S5). We confirmed that DPI also inhibited sucrose-212 induction of CCR2 and WRKY60 transcripts by qRT-PCR (Fig. 3F) as well as WRKY11p:B-213 GLUCURONIDASE (GUS) and WRKY30p:GUS reporters (Fig. S6). Thus, DPI effectively inhibits 214 transcriptional regulation of multiple sugar-regulated genes.

215

206

DPI could be inhibiting transcriptional responses to sugar in our assay by affecting uptake of sucrose, altered sugar metabolism, or inhibition of sugar sensing or signalling. We measured soluble sugars glucose, fructose and sucrose in sucrose-treated dark-adapted seedlings in the presence of DMSO or DPI. We did not detect a difference from controls for any sugar within 8 h of sucrose treatment (Fig. S7), suggesting that inhibition of sugar uptake or sucrose catabolism cannot account for the dramatic inhibition of the transcriptional response by DPI.

222

223 Since DPI can inhibit transcriptional responses to sugar, we sought to establish whether DPI also 224 affects other sugar-regulated processes in Arabidopsis. Seed germination in both dormant and 225 non-dormant seeds is inhibited by exogenous sugar, acting through abscisic acid-dependent 226 pathways (23). Similarly to sucrose, DPI also inhibits germination (24) (Fig. S8). If DPI inhibits 227 germination by the same pathway as sucrose, we expected that their effects would be non-228 additive. However, the effect of DPI on inhibition of germination was detected both with and 229 without sucrose in dormant and non-dormant seeds (Fig. S8). This suggests that DPI does not 230 affect the regulatory pathways through which sucrose inhibits seed germination.

231

Sugars promote growth. To test the effect of DPI on growth promotion by sucrose, we measured effects on hypocotyl elongation and root growth in dark-grown seedlings. This growth assay enables quantification of effects of sugar on cell elongation in the hypocotyl and cell division in the root in the absence of light signals. Seedlings growing on media containing DPI had slightly reduced hypocotyl length and root length in control media, and DPI strongly attenuated the positive effects of sucrose on both hypocotyl and root length (Fig 3G). These data suggest that
 DPI inhibits the signalling or metabolism of sucrose to promote cell elongation and cell division.

239

240 NADPH oxidases are encoded by a family of ten RESPIRATORY BURST OXIDASE HOMOLOG 241 (RBOH) genes in Arabidopsis. We tested whether rboh mutants had altered ROS production in 242 dark-adapted seedlings using L-012 luminescence assays. Both the rbohb and rbohc mutants 243 had similar response to sucrose as wild type, but rboha mutants and rbohd rbohf double mutants 244 had reduced L-012 luminescence (Fig. S9A), similar to wild type treated with DPI, VAS2890 or 245 apocynin (Fig. S5B). We also tested whether *rboh* mutants had altered growth responses to sucrose (Fig. S9B). The rbohd rbohf double mutant had reduced root and hypocotyl length on 246 247 control media compared to wild type but growth was still responsive to sucrose in the mutant. 248 Stimulation of hypocotyl growth by sucrose was reduced in the *rboha* mutant compared to wild 249 type, but stimulation of root growth was unaffected. Thus, although we detected small growth 250 effects in the mutants, none of those tested were able to phenocopy the effect of DPI. Similarly, 251 the transcriptional response of CCR2 or WRKY60 to sucrose in dark adapted seedlings was not 252 reduced in *rboh* mutants (Fig. S9C). These suggest that there is residual  $O_2^-$  accumulation in 253 these mutants sufficient to elicit a response and that there is genetic redundancy in the molecular 254 targets of DPI contributing to these sugar responses.

256 Sugars affect period of circadian rhythms (8) and the circadian clock contributes to rhythms of ROS homeostasis (13). We tested the effect of DPI, MV and 3-AT on circadian rhythms in media 257 258 with or without sucrose. We measured circadian rhythms of TOC1p:LUC in continuous low light 259 (10 µmol m<sup>-2</sup> s<sup>-1</sup>) because the effect of exogenous sucrose on circadian rhythms is more pronounced in these conditions (8). Circadian period was significantly shorter in seedlings grown 260 on sucrose compared to mannitol for all ROS modifiers, similar to the DMSO control (Fig. 4A, 261 262 4B). This suggests that these chemicals did not affect the adjustment of period by exogenous 263 sucrose.

264

255

265 Sugars also affect amplitude of circadian rhythms (11). Luciferase signal is dramatically elevated 266 in TOC1p:LUC seedlings transferred to media containing sucrose compared to mannitol (Fig 4A, 267 4C). This transcriptional response does not require GI (Fig. S10), a clock protein which is post-268 transcriptionally regulated by sucrose (11). The effect of sucrose in TOC1p:LUC seedlings was 269 strongly attenuated in the presence of DPI, elevated in the presence of MV and unaffected by 3-270 AT (Fig. 4C), which is consistent with the effects of these compounds on  $O_2^{-}$  levels. The effects of 271 DPI and MV were particularly pronounced during the night and were not observed in CCA1p:LUC 272 or *PRR7p:LUC* seedlings (Fig. 4C), suggesting O<sub>2</sub><sup>-</sup> acts on specific components of the oscillator.

273

274 Since the effects of DPI and MV differed between the morning-phased CCA1p:LUC and 275 PRR7p:LUC and evening-phased TOC1p:LUC, we wondered whether this might reflect a global 276 pattern of  $O_2^-$  on transcriptional rhythms. We used a set of previously reported  $O_2^-$  and  $H_2O_2^-$ 277 responsive transcripts (19) to determine their phases in continuous light from a published RNA-278 seq dataset (25). The distribution of phases of transcripts up- and down-regulated by  $O_2^-$  or  $H_2O_2$ deviated significantly from expectations (Fig. 4D, Dataset 5). The phase of transcripts 279 280 upregulated by H<sub>2</sub>O<sub>2</sub> were enriched several hours after subjective dawn and downregulated 281 transcripts were enriched before subjective dawn. This is consistent with the reported role of 282 CCA1 in driving rhythms of H<sub>2</sub>O<sub>2</sub> which peak in the early morning (13). By contrast, the phase of 283 transcripts upregulated by  $O_2^-$ , which included TOC1, GI, PRR5 and LUX, were enriched around 284 subjective dusk. About 20% of these genes are direct TOC1 targets (26) (Dataset 5). Transcripts 285 down-regulated by O<sub>2</sub><sup>-</sup>, including LHY and RVE8, were enriched around subjective dawn. This 286 suggests that  $H_2O_2$  and  $O_2^-$  production or signalling are antiphased and is consistent with a role 287 of O<sub>2</sub><sup>-</sup> contributing to promoting oscillations of circadian transcripts in the evening.

288

## 292 Discussion

293 294 We have identified ROS-regulated genes as a prominent feature in the response of the 295 Arabidopsis transcriptome to sugars in both dark and light (Fig.1). The transcriptional response to 296 sucrose in dark-adapted seedlings coincides with an increase in ROS levels, including O<sub>2</sub><sup>-</sup> (Fig. 297 2). Both the accumulation of  $O_2^-$  and transcriptional response to sucrose were strongly attenuated 298 in seedlings treated with DPI, a chemical inhibitor of flavoenzymes including NADPH oxidases 299 (Fig. 3). DPI also inhibited the promotion of hypocotyl elongation and root growth by sucrose, 300 demonstrating a broader impact of the ROS signal in sugar responses. Finally, we found that DPI 301 inhibited the effect of sucrose on the evening expressed TOC1 and identified a highly significant 302 anti-phasing of rhythmic transcripts that are up- and down-regulated by O2<sup>-</sup> to dusk and dawn, 303 respectively (Fig. 4). This is different to the redox effects of salicylic acid on both morning and evening genes (14). Thus, we propose that  $O_2^{-1}$  functions as a metabolic signal associated with 304 305 sugar levels which acts positively on the circadian oscillator in the evening. An association 306 between cellular sugar status and redox state has been long recognised in the context of 307 metabolism and oxidative stress (27), but our data provide evidence of a role for  $O_2^-$  as a 308 dynamic sugar signal affecting daily rhythms of gene expression. This effect of sugar on the 309 oscillator appears to be distinct from the T6P/SnRK1-mediated effect on period via transcriptional 310 regulation of *PRR7* (7) (Fig. 4) and the post-transcriptional control of GI (11) (Fig. S9) revealing 311 an additional layer of metabolic control of circadian rhythms in plants. 312

313 DPI is a potent inhibitor of NADPH oxidases which generate extracellular  $O_2^-$  at the plasma 314 membrane activated by intracellular signals (28). We observed reduced sucrose-activated ROS 315 production and modest growth phenotypes in *rboha* and *rbohd rbohf* mutants, but the 316 transcriptional response to sucrose was similar to wild type (Fig. S8). Notwithstanding that the 317 five *rboh* mutants examined here represent over 90% of total *RBOH* gene expression (Dataset 1), 318 the subtle phenotypes in the *rboh* mutants compared to DPI-treated seedlings probably reflects 319 functional redundancy within this gene family. This will be challenging to verify, since higher order 320 mutants would be expected to be lethal. It is possible that effects of DPI on O2--mediated 321 responses to sugar can be attributed to inhibition of other flavoenzymes. For example, in 322 photosynthetic organisms DPI inhibits O<sub>2</sub> production from xanthine dehydrogenases, glutathione 323 reductases and mitochondrial NAD(P)H dehydrogenases (29-31). However, the similar effects of 324 VAS2890 and apocynin, but not allopurinol, on sugar responses support the role of NADPH 325 oxidases (Fig. S5). 326

MV interferes with electron transport from PSI, as well as in mitochondria (32), and leads to accumulation of  $O_2^-$ , so the opposite effects on transcriptional responses might be expected compared to DPI. MV was unable to induce a transcriptional response in *CCR2p:LUC* seedlings without sucrose (Fig. 3A), which suggests that  $O_2^-$  alone does not activate circadian gene expression or that the site of  $O_2^-$  accumulation in MV-treated seedlings is not sufficient to act as the signal. However, MV elevated the response to sucrose in *TOC1p:LUC* seedlings (Fig. 4C) suggesting that  $O_2^-$  and sucrose might act synergistically.

334

335  $O_2^-$  is generated in mitochondria, chloroplasts, peroxisomes and the apoplast (28).  $O_2^-$  is typically 336 scavenged quickly by superoxide dismutases. Elevation of O2<sup>-</sup> could be due to increased 337 production or reduced scavenging. The increase in O<sub>2</sub><sup>-</sup> triggered by sucrose in dark-adapted 338 seedlings by histochemical stain and L-012 assay was relatively low and slow compared to 339 elicitor-induced respiratory burst (33) but faster than a ROS effect reported for cell-wall damage 340 (34). It might be that sucrose generates  $O_2^-$  in specific cell-types or subcellular locations and the 341 signal might be diluted in bulk tissues or our detection methods might have insufficient sensitivity. 342 This might explain why we couldn't detect L-012 luminescence in rbohd rbohf double mutants (Fig 343 S8A). Thus, it will be useful to map the cellular and subcellular location of the  $O_2^-$  signal using the expanding toolset of available redox probes (35–37). This will also provide clearer identity of
 candidate proteins producing the signal.

346

Reversible oxidation of redox-sensitive proteins by ROS can alter their activity. In Arabidopsis, redox-sensitive proteins that are oxidised by  $H_2O_2$  have been identified in most cellular compartments (38). These include plasma membrane receptors (39), alvcolvtic enzymes (38, 40)

which can localise in the nucleus and associate with DNA (41, 42) and transcription factors (43).

Thus, localised changes in redox state could affect signalling pathways and gene expression by various mechanisms. Changes in localised  $O_2^-$  concentration could modify protein function

indirectly after dismutation to  $H_2O_2$  or directly by affecting Fe-S proteins (28).

354

It is experimentally difficult to separate the effects of  $H_2O_2$ ,  $O_2^-$  or other ROS on protein oxidation. Differences in target specificity for ROS might depend on their redox dynamics or subcellular location.  $H_2O_2$  is regarded as the most likely ROS signal because it is relatively stable compared to the more reactive  $O_2^-$  (28). However, our phase analyses of  $H_2O_2$  and  $O_2^-$  regulated transcripts indicates clear temporal separation of their effects (Fig. 4). This might reflect differences in spatial organisation of oxidative metabolism at different times of day. The mechanism by which sugaractivated  $O_2^-$  production affects gene regulation will depend on its cellular location.

362

363 By examining the effects of sugar on the Arabidopsis transcriptome independently of light, we have uncovered a role for redox status, exemplified by accumulation of  $O_2^-$ , that promotes 364 365 responses to sugar including growth and circadian rhythms. In contrast to the previously reported 366 association of circadian rhythms of H<sub>2</sub>O<sub>2</sub>, which are phased in the morning (13), the O<sub>2</sub><sup>-</sup>-activated transcriptome peaks in the evening and includes core genes within the circadian oscillator. Sugar 367 368 promotes O<sub>2</sub><sup>-</sup> which alters gene expression by either an extracellular or intracellular redox signal 369 which could transmit to the nucleus via signalling or protein localisation. We propose that this 370 metabolic signal functions to coordinate rhythmic physiology and growth in response to 371 environmental conditions that affect photosynthetic metabolism. 372

## 373 Materials and Methods

374

Details of plant materials and growth conditions, RNA-Seq and clustering, qRT-PCR,
histochemical stains, luminescence assays and sugar quantification are described in SI
Appendix. Primers are listed in Dataset 6.

# 378379 Acknowledgments

380

We thank Ms. Heather Eastmond (University of York) for technical support and Prof Alex Webb (University of Cambridge) for useful comments on the manuscript. This research was funded by BBSRC grant (BB/L021188/1) to MJH and IAG, Royal Society Research Grant (RG150144) to MJH and by The University of Melbourne through the Research Grants Support Scheme to MJH and a Melbourne Research Scholarship to XL. This research was not funded by the Australian Research Council.

## 387388 References

- 388 389
- H. A. Herrmann, J. M. Schwartz, G. N. Johnson, Metabolic acclimation A key to enhancing photosynthesis in changing environments? *J. Exp. Bot.* **70**, 3043–3056 (2019).
- E. Baena-González, F. Rolland, J. M. Thevelein, J. Sheen, A central integrator of transcription networks in plant stress and energy signalling. *Nature* 448, 938–42 (2007).
- Y. Xiong, *et al.*, Glucose-TOR signalling reprograms the transcriptome and activates meristems. *Nature* **496**, 181–6 (2013).

- H. Schluepmann, T. Pellny, A. van Dijken, S. Smeekens, M. Paul, Trehalose 6-phosphate is indispensable for carbohydrate utilization and growth in Arabidopsis thaliana. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 6849–6854 (2003).
- 5. C. Nunes, *et al.*, The Trehalose-6-phosphate / SnRK1 signaling pathway primes growth recovery following relief of sink limitation. *Plant Phsyiology* **162**, 1720–1732 (2013).
- 401 6. M. J. Haydon, X. Li, M. K. Y. Ting, Temporal control of plant-environment interactions by
  402 the circadian clock. *Annu. Plant Rev. online* 2, 1–32 (2019).
- 403 7. A. Frank, *et al.*, Circadian Entrainment in Arabidopsis by the Sugar-Responsive
  404 Transcription Factor bZIP63. *Curr. Biol.* 28, 2597-2606.e6 (2018).
- 405 8. M. J. Haydon, O. Mielzcarek, F. C. Robertson, K. E. Hubbard, A. a. R. Webb,
  406 Photosynthetic entrainment of the Arabidopsis circadian clock. *Nature* 502, 689–692
  407 (2013).
- 4089.J. Shin, *et al.*, The metabolic sensor AKIN10 modulates the Arabidopsis circadian clock in<br/>a light-dependent manner. *Plant Cell Environ.* **40**, 997–1008 (2017).
- N. Zhang, *et al.*, Metabolite-mediated TOR signaling regulates the circadian clock in
  Arabidopsis. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 25395–25397 (2019).
- 412 11. M. J. Haydon, O. Mielczarek, A. Frank, Á. Román, A. A. R. Webb, Sucrose and ethylene
  413 signaling interact to modulate the circadian clock. *Plant Physiol.* **175**, pp.00592.2017
  414 (2017).
- 12. N. Dalchau, *et al.*, The circadian oscillator gene GIGANTEA mediates a long-term
  response of the Arabidopsis thaliana circadian clock to sucrose. *Proc. Natl. Acad. Sci. U. S. A.* 108, 5104–5109 (2011).
- A. G. Lai, *et al.*, CIRCADIAN CLOCK-ASSOCIATED 1 regulates ROS homeostasis and oxidative stress responses. *Proc. Natl. Acad. Sci.* **109**, 17129–17134 (2012).
- 420 14. M. Zhou, *et al.*, Redox rhythm reinforces the circadian clock to gate immune response.
  421 Nature 523, 472–476 (2015).
- 422 15. R. Edgar, *et al.*, Peroxiredoxins are conserved markers of circadian rhythms. *Nature* 485, 459–464 (2012).
- 424 16. E. Shor, I. Paik, S. Kangisser, R. Green, E. Huq, PHYTOCHROME INTERACTING
  425 FACTORS mediate metabolic control of the circadian system in Arabidopsis. *New Phytol.*426 215, 217–228 (2017).
- 427 17. K. E. Thum, M. J. Shin, P. M. Palenchar, A. Kouranov, G. M. Coruzzi, Genome-wide
  428 investigation of light and carbon signaling interactions in Arabidopsis. *Genome Biol.* 5,
  429 R10 (2004).
- 430 18. D. Osuna, *et al.*, Temporal responses of transcripts , enzyme activities and metabolites after adding sucrose to carbon-deprived Arabidopsis seedlings. *Plant J.* 49, 463–491 (2007).
- 433 19. I. Gadjev, *et al.*, Transcriptomic footprints disclose specificity of reactive oxygen species signaling in Arabidopsis. *Plant Physiol.* 141, 436–445 (2006).
- 435 20. S. Mangano, et al., Molecular link between auxin and ROS-mediated polar growth. Proc.

- 436 Natl. Acad. Sci. U. S. A. 114, 5289–5294 (2017).
- 437 21. J. Stolk, T. J. Hiltermann, J. H. Dijkman, A. J. Verhoeven, Characteristics of the inhibition
  438 of NADPH oxidase activation in neutrophils by apocynin, a methoxy-substituted catechol.
  439 Am. J. Respir. Cell Mol. Biol. 11, 95–102 (1994).
- 440 22. C. Hesberg, R. Hänsch, R. R. Mendel, F. Bittner, Tandem orientation of duplicated
  441 xanthine dehydrogenase genes from Arabidopsis thaliana: Differential gene expression
  442 and enzyme activities. *J. Biol. Chem.* 279, 13547–13554 (2004).
- 443 23. J. Price, T. C. Li, S. G. Kang, J. K. Na, J. C. Jang, Mechanisms of glucose signaling 444 during germination of Arabidopsis. *Plant Physiol.* **132**, 1424–1438 (2003).
- 445 24. K. Müller, A. C. Carstens, A. Linkies, M. A. Torres, G. Leubner-metzger, The NADPH446 oxidase AtrbohB plays a role in Arabidopsis seed after-ripening. *New Phytol.* 184, 885–
  447 897 (2009).
- 448 25. A. Romanowski, R. G. Schlaen, S. Perez-Santangelo, E. Mancini, M. J. Yanovsky, Global transcriptome analysis reveals circadian control of splicing events in Arabidopsis thaliana.
  450 *Plant J.* 103, 889–902 (2020).
- 451 26. W. Huang, *et al.*, Mapping the core of the Arabidopsis circadian clock defines the network 452 structure of the oscillator. *Science (80-. ).* **336**, 75–79 (2012).
- 453 27. I. Couée, C. Sulmon, G. Gouesbet, A. El Amrani, Involvement of soluble sugars in reactive oxygen species balance and responses to oxidative stress in plants. *J. Exp. Bot.* 57, 449–459 (2006).
- 456 28. N. Smirnoff, D. Arnaud, Hydrogen peroxide metabolism and functions in plants. *New* 457 *Phytol.* 221, 1197–1214 (2019).
- 458 29. M. Zarepour, *et al.*, Xanthine dehydrogenase AtXDH1 from Arabidopsis thaliana is a
  459 potent producer of superoxide anions via its NADH oxidase activity. *Plant Mol. Biol.* 72, 301–310 (2010).
- 461 30. J. M. Diaz, *et al.*, NADPH-dependent extracellular superoxide production is vital to
  462 photophysiology in the marine diatom Thalassiosira oceanica. *Proc. Natl. Acad. Sci. U. S.*463 *A.* 116, 16448–16453 (2019).
- 464 31. T. H. Roberts, K. M. Fredlund, I. M. Møller, Direct evidence for the presence of two
  465 external NAD(P)H dehydrogenases coupled to the electron transport chain in plant
  466 mitochondria. *FEBS Lett.* 373, 307–309 (1995).
- 467 32. F. Cui, *et al.*, Interaction of methyl viologen-induced chloroplast and mitochondrial signalling in Arabidopsis. *Free Radic. Biol. Med.* **134**, 555–566 (2019).
- 469 33. J. M. Smith, A. Heese, Rapid bioassay to measure early reactive oxygen species
  470 production in Arabidopsis leave tissue in response to living Pseudomonas syringae. *Plan*471 *Methods* 10, 6 (2014).
- 472 34. L. Denness, *et al.*, Cell Wall Damage-Induced Lignin Biosynthesis Is Regulated by a
  473 Reactive Oxygen Species- and Jasmonic Acid-dependent Process in Arabidopsis. *Plant*474 *Physiol.* 156, 1364–1374 (2011).
- 475 35. A. J. Meyer, *et al.*, Redox-sensitive GFP in Arabidopsis thaliana is a quantitative biosensor
  476 for the redox potential of the cellular glutathione redox buffer. *Plant J.* 52, 973–986 (2007).

- 477 36. T. Nietzel, *et al.*, The fluorescent protein sensor roGFP2-Orp1 monitors in vivo H 2 O 2
  478 and thiol redox integration and elucidates intracellular H 2 O 2 dynamics during elicitor479 induced oxidative burst in Arabidopsis. *New Phytol.* 221, 1649–1664 (2019).
- 480 37. J. Steinbeck, *et al.*, In vivo NADH/NAD+ biosensing reveals the dynamics of cytosolic
  481 redox metabolism in plants. *Plant Cell* 32, 3324–3345 (2020).
- 482 38. P. Liu, H. Zhang, H. Wang, Y. Xia, Identification of redox-sensitive cysteines in the
  483 arabidopsis proteome using OxiTRAQ, a quantitative redox proteomics method.
  484 *Proteomics* 14, 750–762 (2014).
- 485 39. F. Wu, *et al.*, Hydrogen peroxide sensor HPCA1 is an LRR receptor kinase in Arabidopsis.
  486 Nature **578**, 577–581 (2020).
- 487 40. C. H. Marchand, *et al.*, Thioredoxin targets in Arabidopsis roots. *Proteomics* **10**, 2418–2428 (2010).
- 489 41. Y. H. Cho, S. D. Yoo, J. Sheen, Regulatory Functions of Nuclear Hexokinase1 Complex in Glucose Signaling. *Cell* 127, 579–589 (2006).
- 491 42. S. C. Kim, L. Guo, X. Wang, Nuclear moonlighting of cytosolic glyceraldehyde-3492 phosphate dehydrogenase regulates Arabidopsis response to heat stress. *Nat. Commun.*493 11, 1–15 (2020).
- 494 43. Y. Li, W. Liu, H. Zhong, H. L. Zhang, Y. Xia, Redox-sensitive bZIP68 plays a role in 495 balancing stress tolerance with growth in Arabidopsis. *Plant J.* **100**, 768–783 (2019).
- 496

## 498

## 499 Figures legends500

501 Figure 1. A light-independent sugar-regulated transcriptome of Arabidopsis. (A) Two week old 502 seedlings were grown in the dark for 72 h, then transferred to 10 mM mannitol (Dark) or sucrose 503 (Suc) in the dark, or into the light with 10 mM mannitol (Light) or 20 µM DCMU and 10 mM mannitol (DCMU). Shoot tissue was collected at 0, 0.5, 2 and 8 h for RNA-Seq. (B) Leaf glucose 504 content in seedlings grown as in (A) (means  $\pm$  SD, N = 3; \* P < 0.05 from Dark; Bonferroni-505 506 corrected *t*-test). (C) Venn diagrams of differentially expressed genes at each time-point in samples collected in the dark (left) or light (right). (D) Expression trajectories of 14 clusters of co-507 508 expressed genes identified by variational Bayesian Gaussian mixture model. Pink and blue lines 509 indicate genes identified as up/down or down/up regulated by sucrose/DCMU, respectively. The 510 number of genes within each cluster are in parentheses. (E) Gene Ontology enrichment maps of the top 15 terms in each cluster in (D). Node colours correspond to the cluster(s) represented in 511 512 (D). Node sizes are proportional to the number of genes. Selected nodes are labelled with 513 significantly enriched, representative GO terms for each network. See Dataset 4 for the fully 514 annotated networks.

515

**Figure 2.** Sucrose promotes superoxide production and ROS-regulated transcripts in darkadapted seedlings. Transcript levels of representative ROS-associated genes identified as sugarregulated from RNA-seq that are (A) from the GO class 'responsive to oxygen-containing compound' or (B) identified from a previous study (19) (means  $\pm$  SD, N = 3). (C) Histochemical stains for hydrogen peroxide (DAB) and superoxide (NBT) in 10 d old, dark-adapted Col-0 seedlings treated with 30 mM mannitol or sucrose. (D) DAB and NBT stain intensity in seedlings grown as in (C) (means  $\pm$  SD, N = 6; P < 0.05 from mannitol; Bonferroni-corrected *t*-test). (E) L- 523 012 luminescence in dark-adapted Col-0 treated with 30 mM mannitol or sucrose (means  $\pm$  SEM, 524 N = 6).

525 Figure 3. Modifiers of superoxide inhibit responses to sucrose. (A) Luciferase luminescence in dark-adapted CCR2p:LUC seedlings treated with 30 mM mannitol or sucrose in the presence of 526 DMSO, 10  $\mu$ M DPI, 2  $\mu$ M MV or 200  $\mu$ M 3-AT (means ± SEM, N = 6). (B) L-012 luminescence in 527 dark-adapted Col-0 treated as in (A) (means  $\pm$  SEM, N = 6). (C) Histochemical NBT stain for  $O_2^{-1}$ 528 529 and DAB stains for H<sub>2</sub>O<sub>2</sub> in dark-adapted Col-0 seedlings treated with 30 mM mannitol or sucrose in the presence of 0.1% DMSO or 10 µM DPI. (D) Stain intensity in Col-0 seedlings 4 h (NBT) or 530 0.5 h (DAB) after treatment as in (A) (N = 6; \* P < 0.05; t-test). (E) Inhibition of response of 531 532 luciferase luminescence to 30 mM sucrose in dark-adapted CCR2p:LUC seedlings in the presence of 0 (0.1% DMSO), 1, 5 or 25  $\mu$ M DPI. (means ± SEM, N = 3; \* P < 0.05 from DMSO; 533 Bonferroni-corrected t-test). (F) Transcript level of CCR2 and WRKY60, relative to UBQ10 in 534 535 dark-adapted Col-0 seedlings 8 h after treatment with 30 mM mannitol, sucrose or sucrose with 10  $\mu$ M DPI (means ± SD, N = 4; \* P < 0.05 from mannitol; Bonferroni-corrected *t*-test. (G) 536 Hypocotyl length and root length of 5 d old dark-grown Col-0 seedlings grown on 1/2 MS with or 537 538 without 30 mM mannitol or sucrose, 0.1% DMSO or 1  $\mu$ M DPI (means ± SD, N = 10; \* P < 0.05 539 from 1/2 MS; Bonferroni-corrected t-test).

540 Figure 4. Modifiers of superoxide affect modulation of circadian rhythms by sucrose. (A) 541 Normalised luciferase luminescence in TOC1p:LUC seedlings in continuous low light with 30 mM 542 mannitol (blue) or sucrose (red) in the presence of 0.1% DMSO or 10 µM DPI, 2 µM MV or 200 543  $\mu$ M 3-AT (means ± SD, N = 4). (B) Circadian period estimates of luciferase luminescence in 544 TOC1p:LUC seedlings in (A) (means  $\pm$  SD, N = 4; \* P < 0.05 from mannitol; Bonferroni-corrected 545 t-test). (C) Luciferase luminescence in TOC1p:LUC, PRR7p:LUC and CCA1p:LUC seedlings for 24 h in light/dark treated as in (A) (means  $\pm$  SD, N = 4). (D) Phase of rhythmic O<sub>2</sub><sup>-</sup>- and H<sub>2</sub>O<sub>2</sub>-546 responsive transcripts in continuous light. Values are enrichment (observed/expected) of up- and 547 down-regulated genes in each 4-h phase window (\* P < 0.01;  $\chi^2$ ). 548









# PNAS www.pnas.org

1	
2	
3	
4	
5	
6	
7	Supplementary Information for
/	Supplementary mormation for
ð	
9	Superoxide is promoted by sucrose and affects amplitude of circadian rhythms in the evening
10	
11	Ángela Román, Xiang Li, Dongjing Deng, John W. Davey, Sally James, Ian A Graham, Michael J
12	Havdon
13	
1/	Michael L Haydon
15	Emoil: m baydon@unimolb.odu.ou
15	Email. m.naydon@unimeid.edu.au
10	
1/	
18	This PDF file includes:
19	
20	Supplementary text
21	Figures S1 to S10
22	Legends for Datasets 1 to 6
$\frac{1}{23}$	SI Beferences
$\frac{23}{24}$	
$\frac{2}{25}$	Other supplementary metarials for this menuagrint include the following.
25	Other supplementary materials for this manuscript include the following.
20	
27	Datasets 1 to 6
28	
29	
30	
31	
01	
37	
52	
22	
33	
34	
35	
~~	

## 37 Supplementary Information Text

## 38 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 4°C, seedlings were grown at 20°C in 12 h light/12 dark cycles (LD) under 100-140
µmol m<sup>-2</sup> s<sup>-1</sup> light. Concentrations of DPI, MV and 3-AT were based on a previous study (4)

50

51 For dark growth assays, seeds were germinated on ½ MS in LD for 48 h. Within 1 h of dawn 52 before photomorphogenesis, germinated seeds were transferred to ½ MS with 1% (w/v) agar 53 containing treatments, wrapped in foil and grown vertically for 3 d. Plates were photographed and 54 root and hypocotyl lengths were quantified with ImageJ (NIH). 55

56 **RNA-seq.** Col-0 seeds were sown on nylon membrane on modified Hoagland's solution and 57 grown at 45° angle. Two week old seedlings were wrapped in aluminium foil before dawn and 58 grown in the dark for 72 h. Under dim green light, dark-adapted seedlings were transferred to 59 Hoagland's media containing 10 mM mannitol or 10 mM sucrose and maintained in the dark or 10 60 mM mannitol with or without 20 µM DCMU and returned to the light. Shoots of 40 seedlings were 61 collected at 0, 0.5, 2 and 8 h after treatments, snap-frozen in liquid nitrogen and stored at -80°C 62 until processing. The RNA-seg samples were taken from two independent experiments; the first 63 produced three biological replicates for all conditions, and the second, three further replicates for 64 the dark-grown 0, 2 and 8 h conditions. RNA was extracted with RNeasy Plant Mini Kit including 65 on-column DNase treatment (Qiagen). RNA quantity and purity were confirmed using a 66 Nanodrop spectrophotometer (ThermoScientific), and samples were run on an Agilent 2100 67 Bioanalyzer, with RNA 6000 Nano kit, to confirm RNA integrity (all samples displayed RINs of > 68 7). mRNA sequencing libraries were prepared from 1 µg total RNA using the NEBNext RNA Ultra 69 Directional Library preparation kit for Illumina (New England BioLabs Inc.), in conjunction with the 70 NEBNext Poly(A) mRNA Magnetic Isolation Module and NEBNext multiplex oligos for Illumina 71 (dual 8 bp indexing primers set 1), according to the manufacturer's instructions. Libraries were 72 pooled at equimolar ratios, and the pool was sent for 2 x 150 base paired end sequencing on a 73 HiSeq 3000 at the University of Leeds Next Generation Sequencing Facility. Each sample was 74 sequenced twice on two separate lanes, except replicate 3 of the light 2 h condition, which failed 75 and was resequenced on one lane only, and replicate 1 of the 0 h condition in experiment 2, 76 which also failed and was not resequenced. Raw reads have been uploaded to the European 77 Nucleotide Archive, ENA accession PRJEB40453 [these will be made public on acceptance].

78

RNA-seq samples were quantified with Salmon v0.8.2 (5) using options -I 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

83 https://www.arabidopsis.org/download/index-

auto.jsp?dir=%2Fdownload\_files%2FGenes%2FAraport11\_genome\_release on 26 April 2017
 (included in Dryad repository []). A map of transcript names to gene names to use with Salmon
 option -g was created with the following Unix one liner:

- 87 cut -f9 Araport11\_GFF3\_genes\_transposons.201606.gtf | sort | uniq | 88 perl -ne 'print "\$1\t\$2\n" if /transcript\_id "(.+)"; gene\_id "(.+)";/'
- 89 > Araport11\_GFF3\_gene\_transposons.201606.salmon.geneMap.tsv

- 90 Salmon output was converted to sleuth-compatible format with wasabi
- 91 (https://github.com/COMBINE-lab/wasabi, commit f31c73e). These files will be included in a
- 92 Dryad repository (https:datadryad.org) on acceptance but can be accessed during peer review
- 93 here https://drive.google.com/drive/folders/18zc1PCFvZaRTnxTce3IVdhwPFZ11inCm/.
- 94 95

96 Differential expression was analysed with Sleuth v0.29.0 (6) with multiple testing correction by 97 stageR v0.1.0, commit 59af4d7 (7), against the Araport11 gene annotation (8) imported from 98 Ensembl Genomes release 36 (9) with biomaRt (10). Models were run with a log2 transformation 99 function on the counts (log2(x+0.5)). A Sleuth model was built for each pairwise comparison 100 (Dark vs Sucrose 0.5 h. Dark vs Sucrose 2 h. Dark vs Sucrose 8 h. Light vs DCMU 0.5 h. Light vs 101 DCMU 2 h, Light vs DCMU 8 h) with differentially expressed genes detected with a Wald test for 102 each comparison. A full model was run on all samples including control 0 h samples with 103 differentially expressed genes detected with a likelihood ratio test. Screening p-values for stageR 104 were taken from the full model's likelihood ratio test and confirmation p-values from the pairwise 105 models' Wald tests. stageR results targeted a 10% overall false discovery rate using the Holm 106 method for family-wise error rate correction. R code to run Sleuth and stageR analyses is 107 provided in our Dryad repository (run\_sleuth.R, run\_stageR.R). Comparisons between gene lists 108 were made using a Venn diagram tool http://bioinformatics.psb.ugent.be/webtools/Venn/. Gene 109 ontology (GO) enrichment of these lists used PANTHER Classification System (11) accessed 110 through The Arabidopsis Information Resource (TAIR).

111

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 (BioRad). 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)<sup>-Ct</sup>.
Primer sequences are listed in Dataset 5.

118

143

**Transcriptome Clustering.** Genes were clustered based on Sleuth scaled\_reads\_per\_base abundance values for each sample, using scikit-learn's BayesianGaussianMixture (13) <u>https://scikit-learn.org/stable/modules/generated/sklearn.mixture.BayesianGaussianMixture.html</u>) 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.ht
 ml). R code for clustering is provided in the Dryad repository (cluster\_analysis.R).

128 Histochemical stains. Seeds were sown on ½ MS and grown in LD and 11 d old seedlings were 129 wrapped in aluminium foil at dusk. After 72 h, at subjective dusk under dim green light, seedlings 130 were transferred into 0.5 ml liquid 1/2 MS containing 0.1% (v/v) DMSO or chemical treatments in 131 48-well plates. At the following subjective dawn in dim green light, 0.5 ml of 60 mM mannitol or 132 sucrose was added (30 mM final sugar concentration). For H2O2 stains, 1 mg/ml (w/v) 3'3-133 diaminobenzidine tetrahydrochloride hydrate was dissolved in 50 mM Tris acetate (pH 5.0). For 134  $O_2^{-}$  stains, 2 mg/ml (w/v) nitroblue tetrazolium was dissolved in 10 mM potassium phosphate 135 buffer (pH 7.8), 10 mM NaN<sub>3</sub>. Seedlings were vacuum infiltrated for 1 min in freshly prepared 136 staining solutions and incubated in the dark for 24 h. Samples were cleared by boiling for 5 min in 137 1:1:4 lactic acid:glycerol:ethanol then transferred to 1:4 glycerol:ethanol. GUS-stains of 138 transgenic lines was performed overnight as previously (15). Stained seedlings were mounted 139 under coverslips on microscope slides and imaged immediately with a SMZ800 stereomicroscope 140 (Nikon) or a V370 Photo flatbed scanner (Epson). DAB and NBT stain intensity were quantified in 141 whole shoots by dividing integrated density by area of individual seedlings and subtracting 142 background signal in ImageJ (NIH).

144 **L-012 luminescence assay.** Clusters of 7 d old seedlings grown on ½ MS or 6 mm leaf discs 145 from 4 week old plants grown in LD were transferred to 96-well luminescence plates (Greiner) 146 containing 250  $\mu$ l liquid ½ MS before dusk (ZT12), wrapped in aluminium foil and placed in the 147 dark for 72 h. At subjective dawn under dim green light, media was replaced with 100  $\mu$ l 100  $\mu$ M 148 L-012, 20  $\mu$ g/ml horseradish peroxidase containing 0.01% DMSO, 10  $\mu$ M DPI, 2  $\mu$ M MV, 0.2 mM 149 3-AT, 20  $\mu$ M VAS2870, 500  $\mu$ M apocynin or 500  $\mu$ M allopurinol. After 1 h of chemical pre-150 treatment 100  $\mu$ l of 60 mM sucrose or mannitol was added to each well (final sugar concentration 151 30 mM). Luminescence was measured in the dark at 90 s intervals in a Lumistar Omega plater 152 reader (BMG) using a 4 mm orbital well scan.

153

154 Luciferase luminescence assays. For sugar-response assays, CCR2p:LUC seeds were sown 155 on 1/2 MS and grown in LD. Pairs of 10 d old seedlings were transferred into 96-well luminescence 156 plates (Greiner) containing 200 µl ½ MS with agar at dusk, wrapped in foil and grown in the dark. 157 1 mM D-luciferin, K-salt (Promega) was applied twice under dim green light. After 84 h in the dark 158 (subjective dawn). 20 µl of 0.5% (v/v) DMSO. 50 µM DPI. 10 µM MV or 1 mM 3-AT was applied to 159 seedlings under dim green light, 1 h before addition of 30 µl of 30 mM mannitol or sucrose. For 160 the dose response curves, seedlings were transferred under dim green light to 1/2 MS media 161 containing DMSO, DPI, VAS2870, apocynin or allopurinol 12 h before application of sugar at 162 subjective dawn. Luminescence was measured in the dark at 1 h intervals in a Lumistar Omega 163 plate reader (BMG) using a 4 mm orbital well scan.

164

165 To measure circadian rhythms, clusters of 5 seeds were sown on 1/2 MS and grown in LD. 166 Clusters of 7 d old seedlings were transferred at dawn to 1/2 MS containing 30 mM mannitol or 167 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 168 (Promega) was applied to seedlings twice prior to imaging. Luciferase was imaged in 10 min 169 integrations following 120 s of dark at 1 hr intervals with an HRPCS5 intensified CCD camera 170 (Photek) fitted with LB3 red (640 nm) and blue (470 nm) LED arrays providing light at 60 µmol m<sup>-2</sup> 171 s<sup>-1</sup> for 1 LD followed by continuous low light at 10 μmol m<sup>-2</sup> s<sup>-1</sup>. Luminescence counts were 172 extracted from ROIs using Image32 software (Photek) and circadian rhythms were analysed by 173 Fast Fourier Transform Non-linear Least Squares using Biodare2 (16).

174

175 Sugar quantification. Seedlings were grown as for the RNA-Seq experiment or pairs of seeds 176 were sown on 1/2 MS and grown in LD. Seven d old seedlings were wrapped in foil at dusk and 177 grown in the dark. After 72 h, seedlings were transferred under dim green light into 96 well plates 178 containing ½ MS with 0.1% DMSO or 10 µM DPI. At subjective dawn, seedlings were treated with 179 30 µL 30 mM mannitol or sucrose. 30 seedlings were harvested per biological replicate, frozen in 180 liquid N and stored at -80°C until processing. Soluble sugars were extracted in 80% (v/v) ethanol 181 measured using a Sucrose/Glucose/Fructose calorimetric assay kit (Megazyme) scaled down for 182 96-well plates.



186

**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  $\pm$  SD, N = 3). (C) and (D) comparison of quantification of 31 transcripts by qRT-PCR (PCR efficiency<sup>-Ct</sup>) and RNA-seq (scaled reads per base). Plots are the same data coloured by transcript (C) or treatment (D). Values are individual biological replicates.

- 193
- 194



- 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
- 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.



204

205 Fig. S3. Light-independent sugar-regulated genes in Arabidopsis. (A) Gene Ontology enrichment 206 of 927 genes that are up-regulated by sucrose in the dark and down-regulated by DCMU in the 207 light. (B) Gene Ontology enrichment of 1117 genes that are down-regulated by sucrose in the 208 dark and up-regulated by DCMU in the light. Fold-enrichment and z-score are plotted on the 209 same scale. (C) RNA-seq transcript level of light-signalling genes identified as down-regulated by 210 sucrose and up-regulated by DCMU. (D) Comparison of 2042 genes identified as sugar-regulated 211 in (A) and (B) to genes reported as regulated by SnRK1 (19) and TOR (20). (E) Gene Ontology 212 enrichment of 1080 sugar-regulated genes not previously identified as SnRK1- or TOR-regulated 213 showing GO categories with a z-score > 2. Fold-enrichment and z-score are plotted on the same 214 scale.



Fig. S4. Optimisation of gene clustering. Elbow plot of percentage of total variance within clusters

- 217 218 219 for clustering runs with k=2 to k=20. Grey is cluster with largest variance, usually representing unclustered genes.
- 220 221



222

225 Fig S5. Effects of NADPH oxidase inhibitors. (A) Inhibition of response of luciferase 226 luminescence to 30 mM sucrose in dark-adapted CCR2p:LUC seedlings by DPI, VAS2870, 227 apocynin or allopurinol in the presence of four concentrations of each chemical inhibitor or DMSO 228 (means  $\pm$  SEM, N = 6; \* P < 0.05 from DMSO; Bonferroni-corrected *t*-test). (B) Luciferase 229 luminescence in dark-adapted CCR2p:LUC seedlings treated with 30 mM mannitol or sucrose in 230 the presence of 0.1% DMSO, 10 μM DPI, 20 μM VAS2870, 500 μM apocynin or 500 μM 231 allopurinol (means  $\pm$  SEM, N = 6). (C) L-012 luminescence in dark-adapted Col-0 treated with 30 232 mM mannitol or sucrose in the presence of DMSO, 10 µM DPI, 20 µM VAS2870 or 500 µM 233 apocynin or 500  $\mu$ M allopurinol (means ± SEM, N = 12). (D) Representative images and (E) 234 quantification of NBT stains in dark-adapted Col-0 seedlings 4 h after treatment with 30 mM 235 mannitol or sucrose in presence of 0.1% DMSO, 10 µM DPI, 30 µM VAS2870, 500 µM Apocynin 236 or 500  $\mu$ M allopurinol (means ± SD, N = 3; \* P < 0.05 from DMSO+Sucrose ; Bonferroni-corrected 237 t-test). 238



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, pretreated for 30 min with DMSO or 10  $\mu$ M DPI. (B) RNA-seq transcript levels of *WRKY11* and *WRKY30* (means ± SD, *N* = 3).



251 252 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 10 253  $\mu$ M DPI. Values are means ± SD, N = 4. No significant difference was identified between DMSO 254 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 4°C 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  $\pm$  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  $\frac{1}{2}$  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  $\pm$  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 280 (means ± SEM, N = 4).

284

292

- Dataset 1 (separate file). Differentially expressed genes between Dark and Suc or Light and
   DCMU.
- 287 **Dataset 2 (separate file).** Lists of sugar-activated and sugar-repressed genes.
- 288 Dataset 3 (separate file). Gene lists and GO enrichment of 14 clusters.
- 289 **Dataset 4 (separate file).** Complete GO enrichment map of top 15 terms from 14 gene clusters.
- 290 **Dataset 5 (separate file).** Gene lists and phase analysis of ROS-regulated genes.
- 291 Dataset 6 (separate file). Primer sequences.

## 293 SI References294

- 2951.M. J. Haydon, O. Mielzcarek, F. C. Robertson, K. E. Hubbard, A. a. R. Webb,296Photosynthetic entrainment of the Arabidopsis circadian clock. Nature 502, 689–692297(2013).
- L. D. Gómez, A. Gilday, R. Feil, J. E. Lunn, I. A. Graham, AtTPS1-mediated trehalose 6 phosphate synthesis is essential for embryogenic and vegetative growth and
   responsiveness to ABA in germinating seeds and stomatal guard cells. *Plant J.* 64, 1–13
   (2010).
- 302 3. M. J. Haydon, *et al.*, Vacuolar nicotianamine has critical and distinct roles under iron deficiency and for zinc sequestration in Arabidopsis. *Plant Cell* **24**, 724–37 (2012).
- 3044.A. G. Lai, et al., CIRCADIAN CLOCK-ASSOCIATED 1 regulates ROS homeostasis and<br/>oxidative stress responses. Proc. Natl. Acad. Sci. 109, 17129–17134 (2012).
- 3065.R. Patro, G. Duggal, M. I. Love, R. A. Irizarry, C. Kingsford, Salmon provides fast and<br/>bias-aware quantification of transcript expression. *Nat. Methods* 14, 417–419 (2017).
- 3086.H. Pimentel, N. L. Bray, S. Puente, P. Melsted, L. Pachter, Differential analysis of RNA-<br/>seq incorporating quantification uncertainty. *Nat. Methods* 14, 687–690 (2017).
- K. Van den Berge, C. Soneson, M. D. Robinson, L. Clement, stageR: A general stagewise method for controlling the gene-level false discovery rate in differential expression and differential transcript usage. *Genome Biol.* 18, 1–14 (2017).
- 3138.C. Y. Cheng, *et al.*, Araport11: a complete reannotation of the Arabidopsis thaliana314reference genome. *Plant J.* **89**, 789–804 (2017).
- 315 9. R. J. Kinsella, *et al.*, Ensembl BioMarts: A hub for data retrieval across taxonomic space.
  316 Database 2011, 1–9 (2011).
- S. Durinck, P. T. Spellman, E. Birney, W. Huber, Mapping identifiers for the integration of genomic datasets with the R/ Bioconductor package biomaRt. *Nat. Protoc.* 4, 1184–1191 (2009).
- 32011.H. Mi, *et al.*, Protocol Update for large-scale genome and gene function analysis with the<br/>PANTHER classification system (v.14.0). *Nat. Protoc.* 14, 703–721 (2019).
- 12. J. M. Ruijter, *et al.*, Amplification efficiency: Linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res.* **37** (2009).
- 13. F. Pedregosa, *et al.*, Scikit-learn: Machine learning in Python. *J. Mach. Learn. Res.* 12, 2825–2830 (2011).
- 32614.G. Yu, L. G. Wang, Y. Han, Q. Y. He, ClusterProfiler: An R package for comparing<br/>biological themes among gene clusters. *OMICS* 16, 284–287 (2012).
- A. Román, J. F. Golz, A. A. R. Webb, I. A. Graham, M. J. Haydon, Combining GAL4 GFP
  enhancer trap with split luciferase to measure spatiotemporal promoter activity in
  Arabidopsis. *Plant J.* **102**, 187–198 (2020).
- 331 16. T. Zielinski, A. M. Moore, E. Troup, K. J. Halliday, A. J. Millar, Strengths and Limitations of

332		Period Estimation Methods for Circadian Data. <i>PLoS One</i> <b>9</b> , e96462 (2014).
333	17.	K. E. Thum, M. J. Shin, P. M. Palenchar, A. Kouranov, G. M. Coruzzi, Genome-wide
334		investigation of light and carbon signaling interactions in Arabidopsis. Genome Biol. 5,
335		R10 (2004).
336	18.	D. Osuna, et al., Temporal responses of transcripts, enzyme activities and metabolites
337		after adding sucrose to carbon-deprived Arabidopsis seedlings. Plant J. 49, 463-491
338		(2007).
339	19.	E. Baena-González, F. Rolland, J. M. Thevelein, J. Sheen, A central integrator of
340		transcription networks in plant stress and energy signalling. Nature 448, 938-42 (2007).
341	20.	Y. Xiong, et al., Glucose-TOR signalling reprograms the transcriptome and activates
342		meristems. Nature 496, 181–6 (2013).
343		