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**Coordinated transcriptional regulation between a
reactive oxygen species-responsive gene network
and the circadian clock in *Arabidopsis thaliana***

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Abstract

Most organisms have evolved endogenous biological clocks as internal timekeepers to fine-tune physiological processes to the external environment. Energetic cycles such as photosynthesis and glycolytic cycles are physiological processes that have been shown to be under clock control. This work sought to understand the mechanism of the synchrony between the circadian oscillator and products of energetic cycles. The fact that plants rely on photosynthesis for survival, and that photosynthesis relies on the sun, this would have meant that oxygen levels would have fluctuated across the day. A common by-product of oxygen metabolism and photosynthesis is the Reactive Oxygen Species (ROS). Evidence has proposed ROS as regulators of cellular signaling and plant development. However, if ROS levels are left unmanaged, it may cause oxidative stress in organisms, which could damage cellular components and disrupt normal mechanisms of cellular signaling. Therefore, it is advantageous for plants to be able to anticipate such periodic burst in ROS. My research investigates the role of the circadian clock in regulating ROS homeostasis in the model plant *Arabidopsis thaliana*. I found that ROS production and scavenging wax and wane in a periodic manner under diurnal and circadian conditions. Not only that, at the transcriptional level, ROS-responsive genes exhibited time-of-day specific phases under diurnal and circadian conditions, suggesting the role of the circadian clock in ROS signaling. Mutations in the core-clock regulator, *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)*, affect both the transcriptional regulation of ROS genes and ROS homeostasis. Furthermore, mis-expressions of other clock genes such as *EARLY FLOWERING 3 (ELF3)*, *LUX ARRHYTHMO (LUX)* and *TIMING OF CAB EXPRESSION 1 (TOC1)* also have profound effects on ROS signaling and homeostasis, thus suggesting a global clock effect on ROS networks. Taken together, *CCA1* is proposed as a master regulator of ROS signaling where the response to oxidative stress is dependent on the time of *CCA1* expression. Plants exhibit the strongest response at dawn, the time when *CCA1* peaks. Moreover, *CCA1* can associate to the Evening Element or *CCA1*-Binding Site on promoters of ROS genes *in vivo* to coordinate transcription. A common feature of circadian clocks is the presence of multiple interlocked transcriptional feedback loops. It is shown here that

the oscillator incorporates ROS as a component of the loop where ROS signals could feed back to affect circadian behavior by changing *CCA1* and *TOC1* transcription. The clock regulates a plethora of output pathways; particularly the transcription of an output gene *FLAVIN BINDING KELCH REPEAT F-BOX 1 (FKF1)* is affected by ROS signals. Temporal coordination of ROS signaling by *CCA1* and the reciprocal control of circadian behavior by ROS revealed a mechanistic link of which plants match their physiology to the environment to confer fitness.

Preface

Additional published work undertaken during the course of study, as stated below, is included in section 7. Publication.

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Authors' contributions:

AGL carried out sample preparations, LR-PCR, sequence analysis, data interpretation and validation experiments. MD-G participated in sequence analysis. JHMS and BM-R participated in research design and data interpretation. AGL and PPD conceived and designed the project. AGL drafted the manuscript. PPD critically reviewed the manuscript. All authors contributed to the draft, read and approved the final manuscript.

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Abbreviations

3-AT	3-aminotriazole
ABA	Absciscic acid
ACT2	<i>ACTIN 2</i>
AGI	Arabidopsis Gene Identifier
AOX	Alternative oxidase
APX	Ascorbate peroxidase
APX4	<i>ASCORBATE PEROXIDASE 4</i>
AsA	Ascorbate
ASN1	<i>ASPARAGINE SYNTHASE 1</i>
AtCP1	<i>Ca²⁺ BINDING PROTEIN 1</i>
ATPase	Adenosine triphosphatase
<i>bHLH128</i>	<i>Basic helix-loop-helix DNA-binding superfamily</i>
<i>BMAL1</i>	<i>Brain and muscle ARNT-like 1</i>
BME	β-mercaptoethanol
BOA	<i>BROTHER OF LUX ARRHYTHMO</i>
°C	Degrees celcius
<i>CAB2</i>	<i>CHLOROPHYLL A/B BINDING PROTEIN 2</i>
cADPR	Cyclic adenosine diphosphate ribose
<i>CAT</i>	<i>CATALASE</i>
<i>CBF</i>	<i>CRT/DRE BINDING FACTORS</i>
CBS	CCA1-binding site
<i>CCA1</i>	<i>CIRCADIAN CLOCK ASSOCIATED 1</i>
CCD	Charged couple device
<i>CCR2</i>	<i>COLD CIRCADIAN RHYTHM RNA-BINDING 2</i>
CCT	CONSTANS (CO), CO-like, TOC1
cDNA	Complementary DNA
<i>CHE</i>	<i>CCA1 HIKING EXPEDITION</i>
ChIP	Chromatin immunoprecipitation
<i>CLOCK</i>	<i>Circadian locomotor output cycles kaput</i>

cm	Centimeter
<i>CRY</i>	<i>CRYPTOCHROME</i>
<i>CSD2</i>	<i>Cd/Zn superoxide dismutase</i>
Cytb6f	Cytochrome b6f
DAB	3,3-diaminobenzidine
DD	Constant darkness
DHA	Dehydroascorbate
DHAR	DHA reductase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
DNase	Deoxyribonuclease
DPI	Diphenylene iodonium
EC	Evening complex
EE	Evening element
<i>ELF</i>	<i>EARLY FLOWERING</i>
<i>ERF2</i>	<i>ETHYLENE RESPONSE FACTOR 2</i>
Fd	Ferredoxin
<i>FeSOD</i>	<i>Fe superoxide dismutases</i>
FFT-NLLS	Fast fourier transform-nonlinear least squares
<i>FIO1</i>	<i>FIONA 1</i>
<i>FKF1</i>	<i>FLAVIN BINDING KELCH REPEAT F-BOX 1</i>
FNR	Ferredoxin NADPH reductase
FW	Fresh weight
g	Gram
GA	Gibberellin
GCL	Glutamate cysteine ligase
GAST1	GIBBERELIC ACID STIMULATED TRANSCRIPT1
<i>GDH1</i>	<i>GLUTAMINE DEHYDROGENASE</i>
GFP	Green fluorescent protein
<i>GI</i>	<i>GIGANTEA</i>
<i>GLN 1.3</i>	<i>GLUTAMINE SUNTHASE</i>

GLR	Glutaredoxin
Glu	Glutamate
GO	Gene ontology
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Oxidized glutathione
h	Hour(s)
H ₂ O	Water
H ₂ O ₂	Hydrogen peroxide
HRP	Horseradish peroxidase
<i>HSFA4A</i>	<i>HEAT SHOCK TRANSCRIPTION FACTOR A4A</i>
<i>IPP2</i>	<i>ISOPENTENYL PYROPHOSPHATE 2</i>
<i>JMJD5</i>	<i>JUMONJI DOMAIN PROTEIN 5</i>
KI	Potassium iodide
krpm	Kilo-revolutions per minute
LD	12 h light 12 h dark
<i>LHCB</i>	<i>LIGHT-HARVESTING CHLOROPHYLL A/B-BINDING PROTEIN</i>
<i>LHY</i>	<i>LATE ELONGATED HYPOCOTYL</i>
LKP2	LOV KELCH PROTEIN 2
LL	Constant light
LUC	Luciferase
<i>LUX</i>	<i>LUX ARRHYTHMO</i>
<i>LWD</i>	<i>LIGHT-REGULATED WD 1</i>
M	Molar
MAPK	Mitogen-activated protein kinase
MDA	Monodehydroascorbate
MDAR	Monodehydroascorbate reductase
<i>MES18</i>	<i>METHYL ESTERASE 18</i>
μE	Micro-Einstein
μg	Micro-gram

μl	Micro-litre
μM	Micro-molar
mg	Milli-gram
mL	Milli-liter
min	Minute(s)
mM	Milli-molar
mRNA	Messenger ribonucleic acid
MS	Murashige and Skoog
MV	Methyl viologen
<i>MYB59</i>	<i>MYB DOMAIN PROTEIN 59</i>
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
ng	Nano-gram
nm	Nano-meter
NOX	NADPH oxidase
NTR	NADPH thioredoxin reductase
NTRX	NADP-linked thioredoxin
$^1\text{O}_2$	Singlet oxygen
O_2	Oxygen
O^{2-}	Superoxide anion
OH^\cdot	Hydroxyl radical
<i>PAL1</i>	<i>PHENYLALANINE AMMONIA-LYASE 1</i>
PC	Plastocyanin
PCR	Polymerase chain reaction
<i>PDX1</i>	<i>PYRIDOXINE BIOSYNTHESIS 1</i>
<i>PER</i>	<i>PERIOD</i>
<i>PHY</i>	<i>PHYTOCHROME</i>
<i>PIF7</i>	<i>PHYTOCHROME INTERACTING FACTOR 7</i>
PIN	PIN-FORMED
PQ	Plastoquinone

<i>PRMT5</i>	<i>PROTEIN ARGININE METHYL TRANSFERASE 5</i>
<i>PRR</i>	<i>PSEUDO-RESPONSE REGULATOR</i>
PRX	Peroxiredoxin
PrxR	Peroxireductase
PSI	Photosystem I
PSII	Photosystem II
<i>PUP1</i>	<i>PURINE PERMEASE 1</i>
qPCR	Quantitative polymerase chain reaction
<i>RAV1</i>	<i>RAV family transcription factor</i>
RBOH	Respiratory burst oxidase homologue
<i>RBOHC</i>	<i>RESPIRATORY BURST OXIDASE HOMOLOG C</i>
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RuBisCO	Ribulose-1,5-bisphosphate carboxylase oxygenase
<i>RVE</i>	<i>REVEILLE</i>
s.d.	Standard deviation
s.e.m	Standard error of mean
SA	Salicylic acid
<i>SAND</i>	<i>GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C2</i>
SCF	Skp1-Cullin-F-box
sec	Second(s)
SHAM	Salicylhydroxamic acid
SOD	Superoxide dismutase
TCA	Tricarboxylic acid
TCP	TB1, CYC and PCF
TE	Tris-ethylene diamine tetra-acetic acid
TF	Transcription factor
<i>TIC</i>	<i>TIME FOR COFFEE</i>
<i>TOC1</i>	<i>TIMING OF CAB EXPRESSION 1</i>
Trx	Thioredoxin

TTFL	Transcriptional-translational feedback loop
<i>TUB2</i>	<i>TUBULIN BETA-2</i>
U	Unit(s)
<i>UPL7</i>	<i>UBIQUITIN-PROTEIN LIGASE 7</i>
VIM	Video-intensified microscopy
<i>VTC2</i>	<i>VITAMIN C 2</i>
WT	Wild-type
<i>XCT</i>	<i>XAP5 CIRCADIAN TIME-KEEPER</i>
ZAT	Zinc-finger transcription factor
<i>ZAT10</i>	<i>SALT TOLERANCE ZINC FINGER</i>
ZT	Zeitgeber time
<i>ZTL</i>	<i>ZEITLUPE</i>

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