Functional Genomic Characterization of the *Arabidopsis* Circadian Circuit in the Plant Osmoregulatory and Nutrient Stress Responses

by

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in

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Abstract

Plants require periods of light and darkness to grow and develop properly. In plants, the timing of daily events is facilitated by the circadian clock. Most eukaryotes possess a circadian circuit that is entrained by different inputs such as light and temperature. Here, I show that the only elucidated activator of the circadian clock, RVE8, and its two homologs, RVE4 and RVE6 are required for plants to confer osmotolerance. I show that wild-type (WT) plants perform better than *rve 4 6 8* plants by examining primary roots under osmotic and salt stress. Subsequent total proteome analyses between WT and *rve 4 6 8* whole seedlings at zeitgeber (ZT)11 and ZT23 illustrate that WT plants have differentially abundant proteins which aid in osmoprotection. Next, I surveyed the circadian clock for its role in regulating nutrient acquisition by utilizing a series of plant lines deficient in different core circadian clock transcription factors. Here, I show that the circadian clock has disparate roles in the regulation of nitrogen (N), phosphorus (P), and sulfur (S) nutrition, through the observation of nutrient-dependent primary root and hypocotyl etiolation phenotypes. After screening a compendium of circadian clock deficient mutants, I undertook a focused characterization of the *prr5-11 prr7-11*, *prr5-11*, and *prr7-11* mutant plants at zeitgebers ZT0, ZT4, ZT8, and ZT12, under control (CTL), -N, -P, and -S conditions, as these mutant lines exhibited the greatest phenotypic differences under nutrient starvation. Using gas chromatography mass spectrometry (GC-MS), I found that the metabolite pool largely differs within each genotype and across different nutrient regiments, implicating both PRR5 and PRR7 proteins in the regulation of nutrient-mediated outputs in *Arabidopsis thaliana*. Research conducted throughout my thesis finds that the circadian clock has wide-ranging roles in regulating the osmoregulatory and nutrient stress responses, laying a foundation for future experimentation aimed at further exploring the interplay between the circadian clock and drought or plant nutrition.

Preface

This thesis is an original work by Ibrahim Khodabocus. No part of this thesis has been previously published.

Chapter 2

Khodabocus I., Mehta D., Rodriguez M., Campkin C., Uhrig R.G. Quantitative proteomic analysis shows that *REVIELLE* clock genes are involved in the salt and drought stress responses. Manuscript in progress.

I will be primary author on this manuscript

Chapter 3

Khodabocus I., Scandola S., Uhrig R.G. Quantitative multi-omic analysis unveils that *PSEUDO-RESPONSE REGULATORS 5* and *7* clock genes are involved in the nitrogen, sulfur, and phosphorus nutrient stress responses. Manuscript in progress.

I will be primary author on this manuscript

Other notable contributions during MSc. thesis

Khodabocus I*., Li Q*., Mehta D., Uhrig R.G. (2022) A Road Map for Undertaking Quantitative Proteomics in Plants: New Opportunities for Cereal Crops. In: Bilichak A.,Laurie J.D. (eds) Accelerated Breeding of Cereal Crops. Springer Protocols Handbooks. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-1526-3_14

I was a co-primary author on this manuscript

Mehta D., Scandola S., **Khodabocus I**., Uhrig R.G. Systems-level nutrient stress analysis of soil grown *Brassica napus* shoots and roots by quantitative proteomics. Manuscript in progress.

I will be a contributing author on this manuscript

Dedication

To my father and sisters for a lifetime of unwavering support and encouragement.

Acknowledgments

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Chapter 3: Exploring the *Arabidopsis* circadian responses to nitrogen, phosphorus, and sulfur nutrient stress responses through phenomics

List of Abbreviations

Abbreviations	Descriptions
ABA	abscisic acid
ABI5	ABSCISIC ACID INSENSITIVE 5
ABA3	ABA DEFICIENT3
Arabidopsis	*Arabidopsis thaliana*
ASA1	A-METHYL TRYPTOPHAN RESISTANT 1
ASB1	ANTHRANILATE SYNTHASE BETA SUBUNIT 1
ATG8D	AUTOPHAGY-RELATED PROTEIN 8D
ATP	adenosine triphosphate
AZI1	AZELAIC ACID INDUCED 1
CA	citric acid
CAX3	CATION EXCHANGER 3
CCA1	CIRCADIAN CLOCK-ASSOCIATED 1
CDF1	CYCLING DOF FACTOR 1
CO	CONSTANS
CTL	control
DHAR	DEHYDROASCORBATE REDUCTASE
DIA	data independent acquisition
EARLI1	EARLY ARABIDOPSIS ALUMINUM INDUCED 1
EC	evening complex
ED	end-of-day
EDS1	ENHANCED DISEASE SUSCEPTIBILITY
ELF	EARLY FLOWERING
EN	end-of-night
FA	fumaric acid
FAB2	FATTY ACID BIOSYNTHESIS 2
FT	FLOWERING LOCUS T
GC-MS	gas chromatography mass spectrometry
GI	GIGANTEA
GMD	Golm Metabolome Database
GO	gene ontology
GR	glutathione reductase
GST	glutathione transferase
GSTF	GLUTATHIONE S-TRANSFERASE PHI
GSTU	GLUTATHIONE S-TRANSFERASE TAU
JA	jasmonic acid
JAZ5	JASMONATE-ZIM-DOMAIN 5
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-MS	liquid chromatography mass spectrometry
LD	long-day
LHY	LATE ELONGATED HYPOCOTYL
Log2FC	log base 2-fold change
LUX	LUX ARRHYTHMO
MPK3	MITOGEN-ACTIVATED PROTEIN KINASE 3

MYB	MYB DOMAIN PROTEIN
MYC2	JASMONATE INSENSITIVE 1
MV	methyl viologen
N	nitrogen
NIST20	National Institute of Standards and Technology library
OA	oleic acid
OX	overexpressing
P	phosphorus
PAI1	PHOSPHORIBOSYLANTHRANILATE ISOMERASE 1
PAP1	ARABIDOPSIS THALIANA PRODUCTION OF ANTHOCYANIN PIGMENT 1
P5CS1	DELTA1-PYRROLINE-5-CARBOXYLATE SYNTHASE 1
PEG	polyethylene glycol
PHT	PHOSPHATE TRANSPORTER
PIF	PHYTOCHROME INTERACTING FACTOR
PRR	PSEUDO-RESPONSE REGULATOR
PTM	post-translational modification
RD20	ARABIDOPSIS THALIANA CALEOSIN 3
ROS	reactive oxygen species
RVE	REVEILLE
S	sulfur
SA	salicylic acid
SD	short-day
SID2	SALICYLIC ACID INDUCTION DEFICIENT 2
SNAT1	SEROTONIN N-ACETYLTRANSFERASE 1
SOT12	ARABIDOPSIS THALIANA SULFOTRANSFERASE 1
SUS	SUCROSE SYNTHASE
TCA	tricarboxylic acid
TSB1	TRYPTOPHAN SYNTHASE BETA-SUBUNIT 1
TSB2	TRYPTOPHAN SYNTHASE BETA-SUBUNIT 2
TCP2	CYCLOIDEA AND PCF TRANSCRIPTION FACTOR 2
TOC1	TIMING OF CAB EXPRESSION1
WT	wild-type
WUE	water use efficiency
ZT	Zeitgeber time

Data Availability

All raw proteomics (BoxCar DIA LC–MS/MS) data will be deposited to the Proteomics IDEntifications Database (PRIDE; https://www.ebi.ac.uk/pride/) upon publication (**Chapter 2**). All processed proteomics data will be included in the publications once submitted (**Chapter 2**).

All raw GC-MS data will be provided in the supplemental data of the publications once submitted (**Chapter 3**).

Chapter 1:
Introduction

1.1.1 Circadian Circuitry in Arabidopsis

Plants require periods of light and dark to grow and develop properly (Jabbur & Johnson, 2022; Steed et al., 2021; Webb et al., 2019). In plants, the precise timing of daily events is facilitated by the circadian clock (Dodd et al., 2005; Gottlieb, 2019). Most eukaryotes possess a circadian circuit (Ambesh et al., 2018; Dunlap, 1999; Jabbur & Johnson, 2022; Krahmer et al., 2022; Phillips, 2005; McClung, 2006; Ruben et al., 2019; Salomé et al., 2008; Spoelstra et al., 2015; Young & Kay, 2001) that is entrained by different inputs such as light (**Figure 1**; Czeisler et al., 1986; Haydon et al., 2013) and temperature (**Figure 1**; Boothroyd et al., 2007; Eckardt, 2005; Somers et al., 2000). The circadian clock increases in complexity along the tree of life from the older cyanobacteria and photosynthetic algal specimens to the more contemporary flowering angiosperms (Jabbur & Johnson, 2022; Maeda & Nakamichi, 2022; Ouyang et al., 1998; Sartor et al., 2019).

Arabidopsis thaliana (Arabidopsis) is a eudicotyledonous angiosperm that is a member of the Brassicaceae family. Arabidopsis has been described as an ideal model for studying plant molecular biology (Meinke, 1998; Van Norman & Benfey, 2009), due to its short life cycle of 6-8 weeks (Koornneef & Scheres, 2001; Passardi et al., 2007; Woodward & Bartel, 2018) and subjectively low-maintenance characteristics such as the ability to be propagated cheaply indoors (Gelvin, 2003; Gelvin, 2012; Chen et al., 2004; Koornneef & Scheres, 2001; Tzfira et al., 2004). Arabidopsis is the first plant to have its genome fully sequenced (Kaul et al., 2000) with 11 revisions since the first iteration (Bevan & Walsh, 2005; Provart et al., 2020). The fact that the genome of Arabidopsis has been elucidated and re-annotated multiple times allows plant biochemists to apply several technologies derived from analytical chemistry and biochemistry to study the molecular changes in Arabidopsis when exposed to different treatment conditions (Mergner et al., 2020).

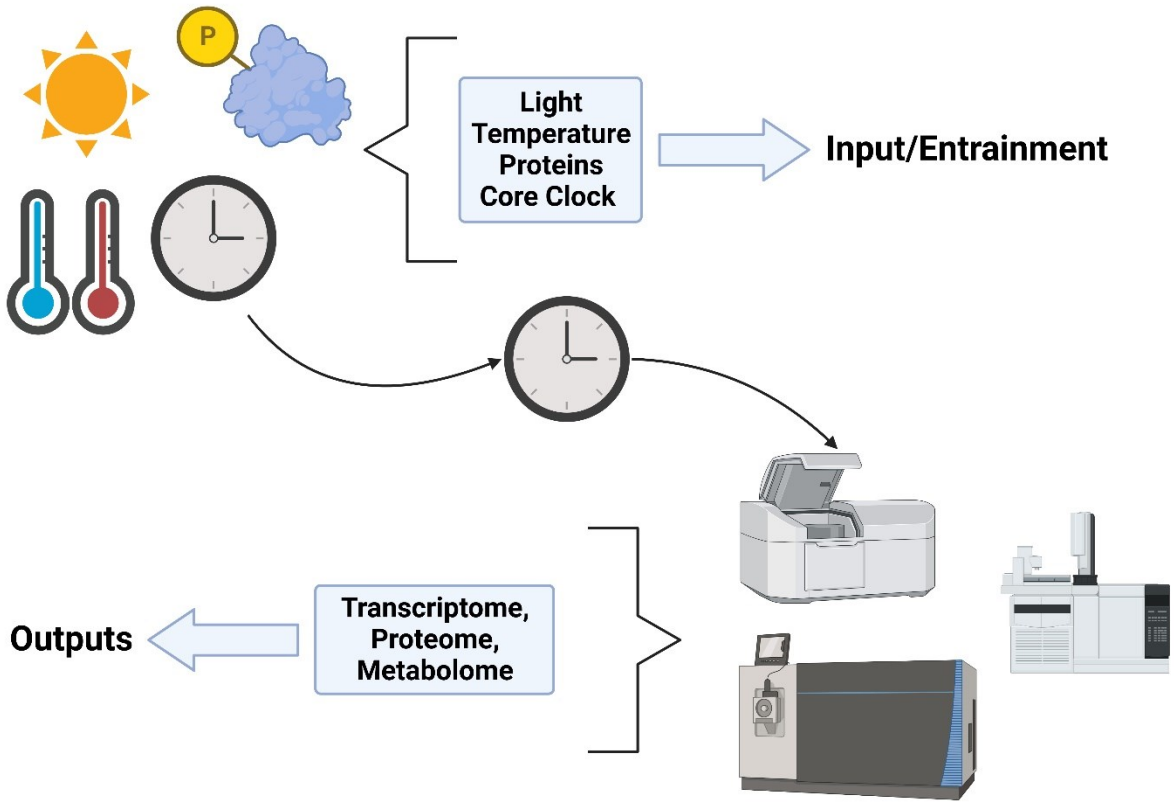

Figure 1: Principle behind the circadian circuitry. The circadian circuit in Arabidopsis and other eukaryotes is entrained by different factors such as light and temperature, which function as inputs into the core clock. The core circuit regulates the expression of downstream genes. This output by the clock has been monitored through a series of omics technologies derived from analytical chemistry ranging from transcriptomic (Covington et al., 2008; Romanowski et al., 2020; Yang et al., 2020), to proteomic (Graf et al., 2010; Krahmer et al., 2022; Uhrig et al., 2019), to metabolomic (Annunziata et al., 2018; Choudhary et al., 2016; Flis et al., 2019; Moraes et al., 2019) analyses.

Most of the research examining the plant circadian circuitry has been conducted using the model plant organism, Arabidopsis (Maeda & Nakamichi, 2022; Mehta et al., 2021; Millar et al., 1995; Shalit-Kaneh et al., 2018; Steed et al., 2021). In Arabidopsis, the core clock consists of morning, afternoon, midday, and evening expressed proteins (**Figure 2**; Kamioka et al., 2016) that form a series of negative feedback loops (**Figure 2**; Shalit-Kaneh et al., 2018). Correspondingly, the circadian clock proteins are transcription factors that are expressed at specific times throughout the photoperiod to induce the expression of clock-output genes, while simultaneously repressing the expression of the other circadian clock transcription factors (**Figure 2**; Covington et al., 2008). It has been estimated that as much as 40% of the genes in Arabidopsis are under circadian control (Romanowski et al., 2020).

1.1.2 Morning-Expressed Transcription Factors

The morning expressed transcription factors consist of two MYB-like transcription factors CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1; AT2G46830) and LATE ELONGATED HYPOCOTYL (LHY; AT1G01060) (Gong et al., 2008; Mizoguchi et al., 2002). LHY and CCA1 are activated by light in anticipation of the dawn (Green & Tobin, 2002). LHY and CCA1 directly interact with one-another (Lu et al., 2009). LHY and CCA1 have partially redundant activities and additively control the timing of flowering (**Figure 3**; Mizoguchi et al., 2002). LHY and CCA1 mediate the timing of starch metabolism (**Figure 3**; Shor et al., 2017; van Hoogdalem et al., 2021), where *lhy cca1* knockout lines exhaust all carbon stores by the onset of dawn (Graf et al., 2010). Plants deficient in *lhy* or *cca1* flower later than wild-type (WT) plants (Fujiwara et al., 2008), which suggests that LHY and CCA1 proteins regulate flowering time (**Figure 3**; He et al., 2021). CCA1 and LHY modulate thermoregulatory responses (**Figure 3**; Gould et al., 2006; Phan et al., 2022; van Hoogdalem et al., 2021) and callus developmental processes (**Figure 3**; Shim et al., 2021), while also directly influencing phytohormone pools in Arabidopsis (**Figure 3**; Martínez-GarcíA et al., 2000).

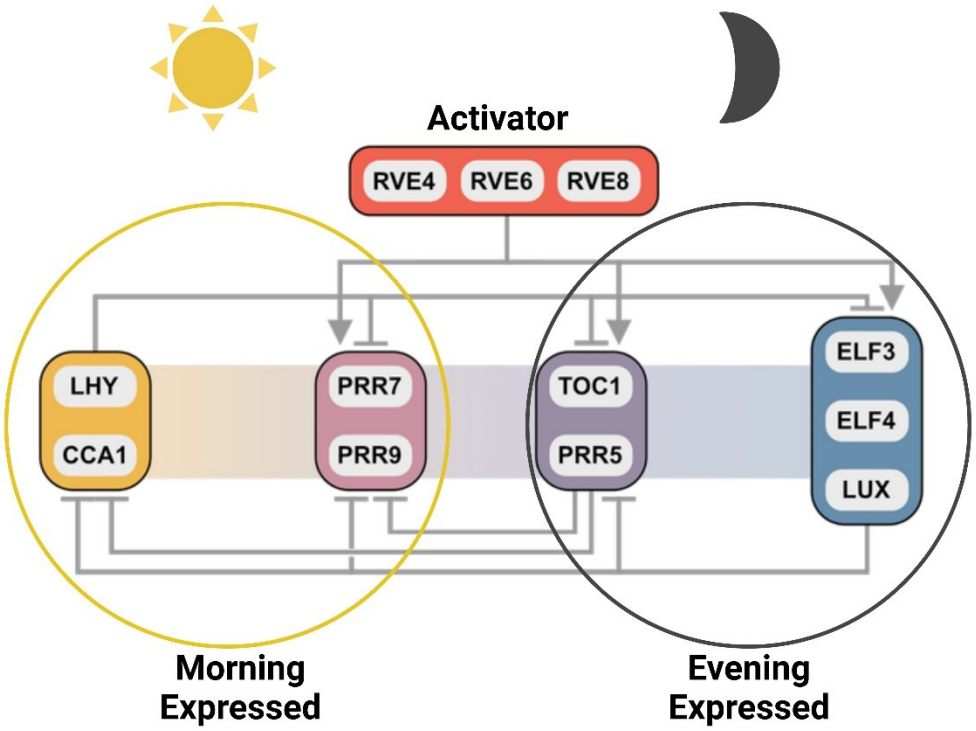

Figure 2: Transcription regulation within the core clock. Schematic depicting the transcription regulatory activity of the core clock in Arabidopsis consisting of LATE ELONGATED HYPOCOTYL (LHY; AT1G01060), CIRCADIAN CLOCK (CCA1; AT2G46830), PSEUDO-RESPONSE REGULATOR 9 (PRR9; AT2G46790), PSEUDO-RESPONSE REGULATOR 7 (PRR7; AT5G02810), PSEUDO-RESPONSE REGULATOR 5 (PRR5; AT5G24470), TIMING OF CAB EXPRESSION 1 (TOC1; AT5G61380), EARLY FLOWERING 3 (ELF3; AT2G25930), EARLY FLOWERING 4 (ELF4; AT2G40080), and LUX ARRHYTHMO (LUX; AT3G46640), as well as the only elucidated activators of the circadian clock REVEILLE 8 (RVE8; AT3G09600), REVEILLE 4 (RVE4; AT5G02840), and REVEILLE 6 (RVE6; AT5G52660) (Adapted from Mehta et al., 2021).

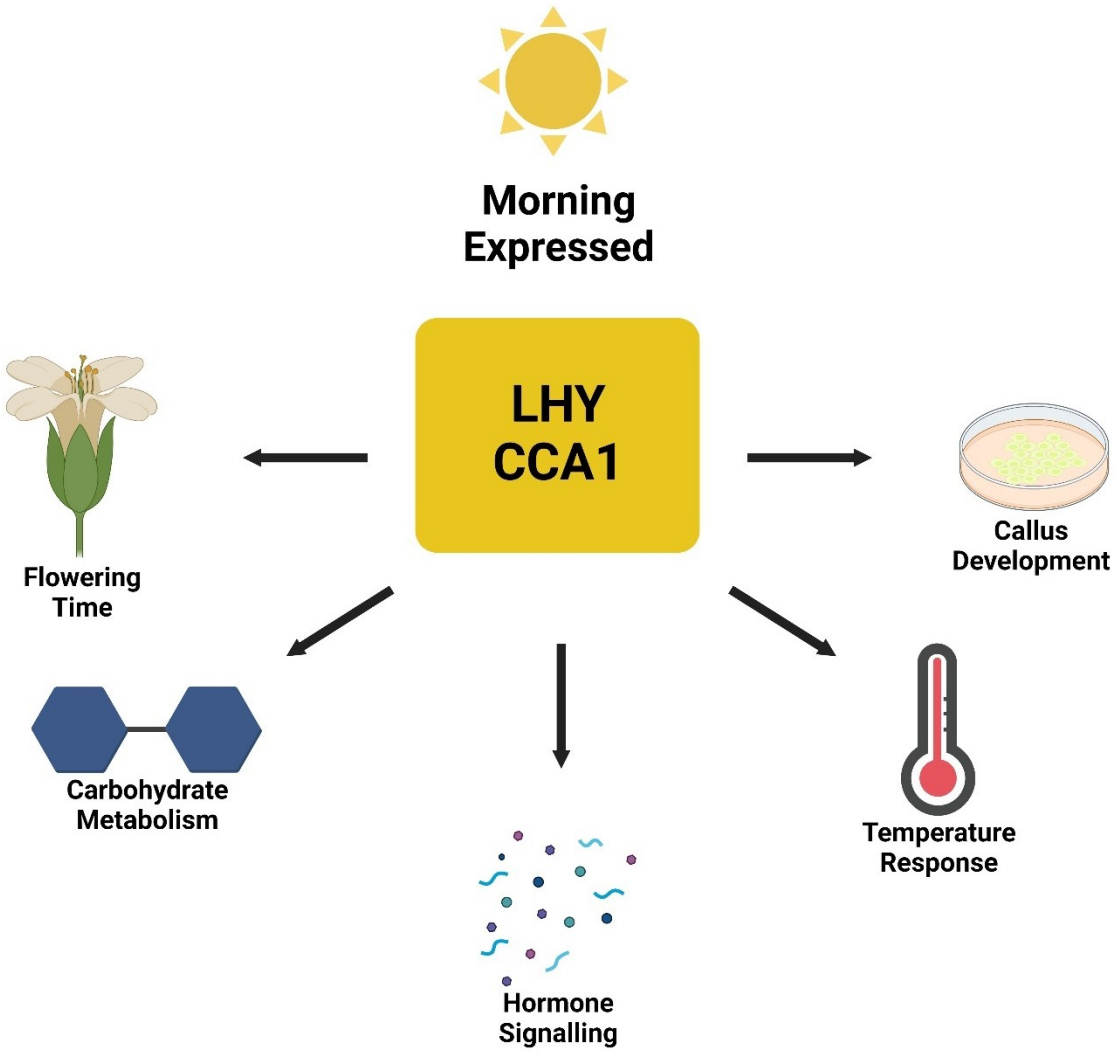

Figure 3: Outputs of CCA1 and LHY proteins. The morning regulated CCA1 and LHY have been shown to mediate the timing of flowering, carbohydrate metabolism, hormone signaling, thermoregulation, and callus development in Arabidopsis.

1.1.3 Mid-Day-Expressed Transcription Factors

The mid-day expressed transcription factors of the circadian clock consist of the PSEUDO-RESPONSE REGULATOR (PRR) proteins: PRR5 (AT5G24470), PRR7 (AT5G02810), PRR9 (AT2G46790), as well as PRR1/TIMING OF CAB EXPRESSION1 (TOC1; AT5G61380) (Ito et al., 2008; Nakamichi et al., 2005; Nimmo & Laird, 2021; Wang et al., 2010). The expression of the *PRRs* occurs in a stepwise fashion, where *PRR9* is expressed just after the dawn, followed by *PRR7*, *PRR5*, and lastly by *TOC1* (Farré & Liu, 2013). LHY and CCA1 represses TOC1 (**Figure 2**; Alabadí et al., 2001; Pruneda-Paz et al., 2009) and TOC1 represses LHY and CCA1 (**Figure 2**; Gendron et al., 2012; Huang et al., 2012; Pokhilko et al., 2012) in a double negative feedback loop. TOC1-OX (overexpressing) plants have lower *LHY* and *CCA1* expression (Makino et al., 2002; MáS et al., 2003) by way of repressing *LHY* and *CCA1* expression. LHY and CCA1 also represses PRRs 5, 7, and 9 (**Figure 2**; Farré et al., 2005; Kamioka et al., 2016) and are negatively regulated by the earlier-expressed PRR5, 7, and 9 proteins (**Figure 2**; Creux & Harmer, 2019; Joanito et al., 2018; Nakamichi et al., 2010; Yuan et al., 2021).

PRRs 5, 7, and 9 regulate flowering under long-day (LD) conditions, where *prr7 prr9*, *prr5 prr7*, and *prr5 prr7 prr9* plants develop higher leaf counts than WT plants, in addition to delayed flowering (**Figure 4**; Nakamichi et al., 2007). PRR5 and PRR7 specifically regulate flowering time under LD, as flowering time in *prr5 prr7* plants under short-day (SD) conditions is not delayed (**Figure 4**; Nakamichi et al., 2005). Plants deficient in PRR5 and PRR7 produce less leaves than WT plants before flowering under SD conditions, while the opposite was observed in *prr7 prr9*, *prr5 prr7*, and *prr5 prr7 prr9* plants (**Figure 4**; Nakamichi et al., 2007). TOC1 acts with PRR5 to regulate flowering time, where *toc1* flowers early in SD conditions, while *toc1 prr5* flowers late in long day conditions by failing to negatively regulate the expression of *CYCLING DOF FACTOR 1* (*CDF1*; AT5G62430) (**Figure 4**; Ito et al., 2008). PRR5, PRR7, PRR9, and TOC1 all regulate flowering by increasing the stability of the CONSTANS (CO; AT5G15840) transcription factor (**Figure 4**; Hayama et al., 2017). Triple knockouts for *PRR5*, *PRR7*, and *PRR9* have delayed flower opening and closing times, relative to WT (**Figure 4**; Muroya et al., 2021). Quadruple knockout *prr5 prr7 prr9 toc1* plants flower later due to a lowered *CO* expression profile, which decreases the binding between CO and the promoter of *FLOWERING LOCUS T* (FT; AT1G65480) (**Figure 4**; Hayama et al., 2017).

TOC1-OX and *PRR5-OX* have shorter hypocotyls than WT when subjected to a 29ºC for 3 days, which suggests that TOC1 (**Figure 4**; Phan et al., 2022) and PRR5 could regulate temperature-related responses in Arabidopsis (**Figure 4**; Zhu et al., 2016). PRR7 and PRR9 are also involved in thermoregulation (**Figure 4**; Blair et al., 2019; Li et al., 2020), where *prr7 prr9* plants have a temperature sensitive phenotype which simultaneously causes the clock to reset after discrete cold pulses (Salomé & McClung, 2005). PRR5 and TOC1 regulate hypocotyl length (**Figure 4**; Zhu et al., 2016), as *prr5 toc1* plants have longer hypocotyls under SD and LD conditions, relative to *prr5* or *toc1* plants (Li et al., 2020). WT, *prr5*, *toc1*, and *prr5 toc1* plants all have sequentially longer hypocotyls, further, *prr5* or *toc1* deficient plants exhibit distinct phenotypes from one another (**Figure 4**; Yan et al., 2021). WT, *prr5*, *toc1*, and *prr5 toc1* plants have different photoperiod times when transferred to free-running conditions, suggesting that PRR5 and TOC1 regulate hypocotyl elongation by different mechanisms (**Figure 4**; Yan et al., 2021). TOC1 controls plant development, where *TOC1-OX* plants exhibit a reduction in plant and leaf size (Fung-Uceda et al., 2018; Zhu et al., 2016). PRR5, PRR7, PRR9, and TOC1 could all modulate hypocotyl length, as *prr5 prr7 prr9 toc1* plants appear to have longer hypocotyl lengths than WT under LD and SD conditions (**Figure 4**; Hayama et al., 2017).

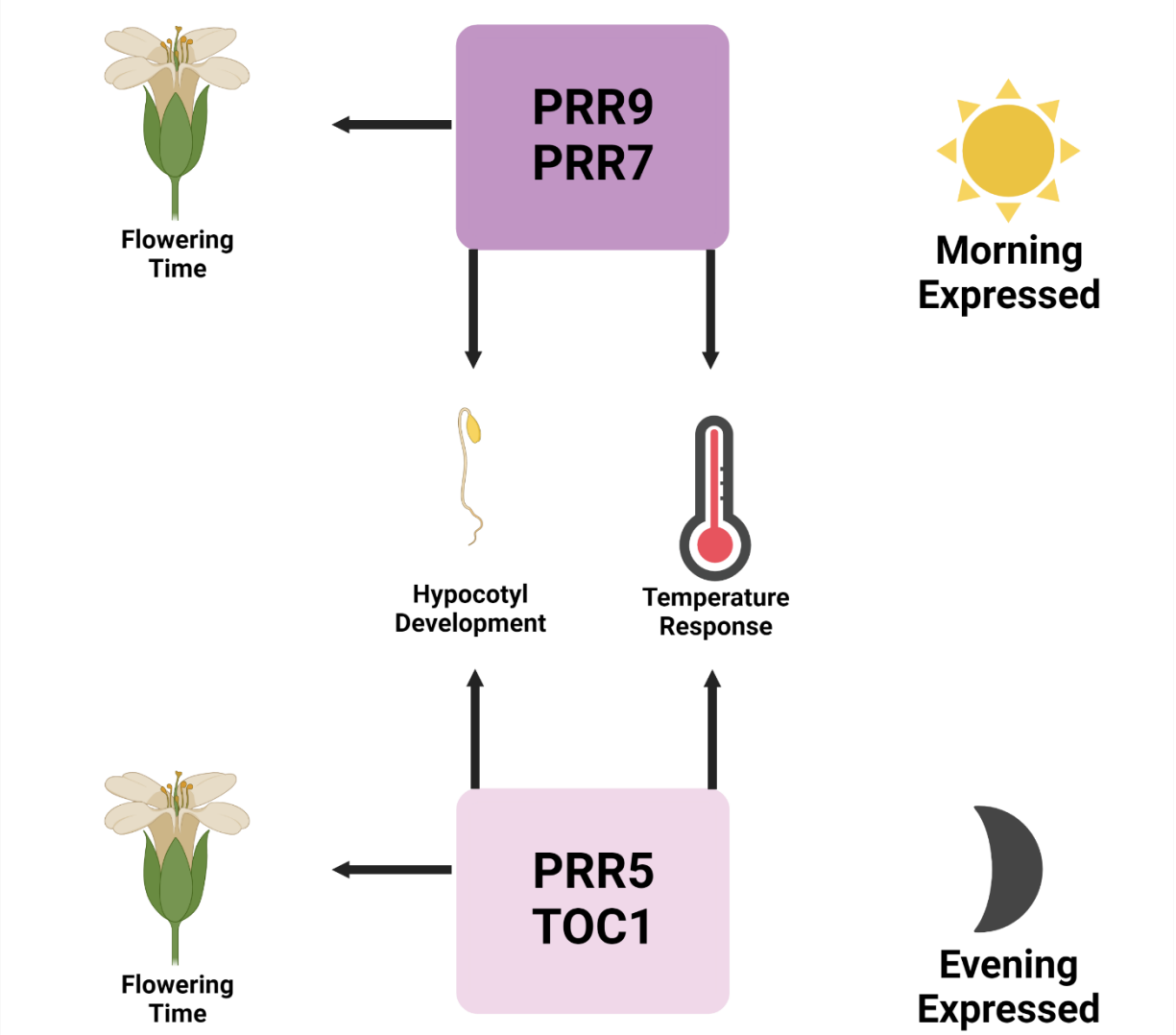

Figure 4: Outputs of PRR proteins. The morning regulated PRR9 and PRR7, as well as the evening activated PRR5 and TOC1 have been documented to modulate flowering time, hypocotyl development, and thermoregulation in Arabidopsis.

1.1.4 Afternoon-Expressed Transcription Factors

To signal the end-of-day, the MYB-like afternoon expressed REVEILLE proteins consisting of RVE8 (AT3G09600) and its two homologs, RVE4 (AT5G02840) and RVE6 (AT5G52660) are expressed as the sole activators of the circadian clock (**Figure 2**; Hsu et al., 2013; Xie et al., 2014). Research around the RVE proteins has arguably been slower than the other members of the core clock due to their recent discovery (Creux & Harmer, 2019; Farinas & Mas, 2011; Rawat et al., 2011). Hsu et al., (2013) showed that the circadian period of *rve4* and *rve6* single mutant plants was statistically indifferent from WT, while *rve4 6 8* plants have a 4 hr longer period than WT. This suggests that RVE8, RVE4, and RVE6 maintain the pace of the clock by having partially redundant roles, as eliminating all three *REVEILLE* genes alters the pace of the clock more significantly than in *rve8* specimens (Hsu et al., 2013).

RVE8 forms a negative feedback loop with PRR5 (**Figure 2**; Craigon, 2004; Rawat et al., 2011), where it directly binds to the promoter of *PRR5*. *RVE8-OX* plants have an increased expression of *PRR5*, while the expression of *RVE8* decreases in *PRR5-OX* plants (Craigon, 2004; Rawat et al., 2011). RVE8 regulates the activity of TOC1 by directly binding to the promoter of *TOC1* (**Figure 2**; Farinas & Mas, 2011). *RVE8-OX* and *rve8* plants increase and decrease the expression of *TOC1*, respectively (Farinas & Mas, 2011). RVE proteins activate the expression of evening-expressed transcription factors in Arabidopsis, alluding to the role of the RVE8-like proteins in the crucial transition between the morning and the evening, while further cementing their roles as activators of the circadian clock (**Figure 2**; Creux & Harmer, 2019; Harmer & Kay, 2005; Nohales, 2021; Rawat et al., 2011).

RVE8 regulates diel production of anthocyanin, with pigment biosynthesis repressed at midday and elevated in the evening (**Figure 5**; Pérez-García et al., 2015). Plants deficient in RVE proteins (*rve4 6 8* and *rve3 4 5 6 8*) have larger biomass than WT plants, suggesting that RVE proteins could play a role in the regulation of plant architecture and morphology by modulating cell size (**Figure 5**; Gray et al., 2017). RVE8-like proteins regulate thermotolerance (**Figure 5**; Sorkin et al., 2022) by modulating the expression of ethylene-synthesizing genes (Li et al., 2019; Kidokoro et al., 2021). REVEILLE proteins have recently been shown to regulate carbohydrate metabolism and proteosome function, where *rve4 6 8* plants display with starch excess at ZT0 and a reduction in proteosome activity at ZT11 and ZT23 (**Figure 5**; Scandola et al., 2022).

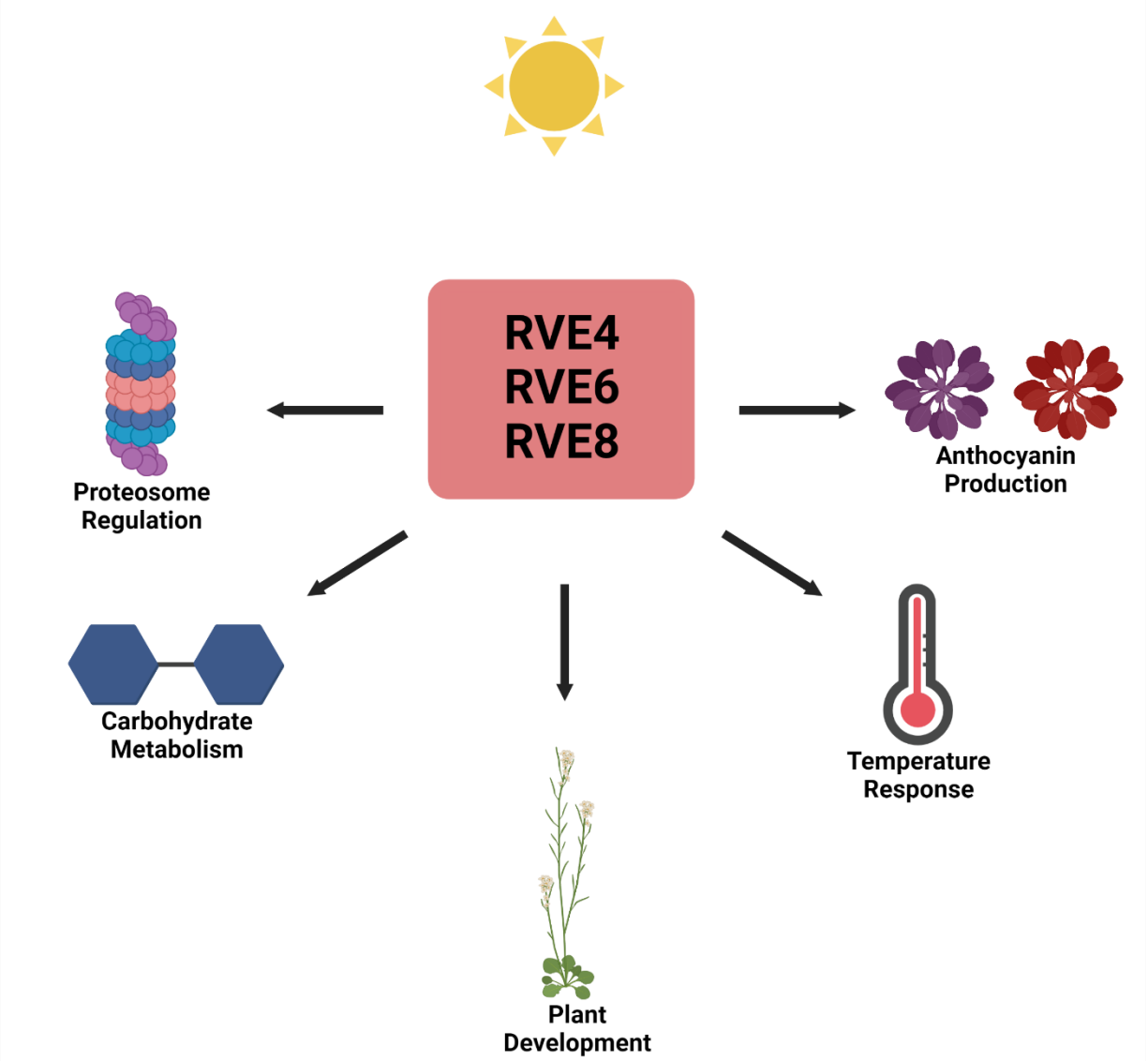

Figure 5: Outputs of RVE8-like proteins. RVE8, RVE4, and RVE6 have been shown to regulate proteasomal activity, carbohydrate metabolism, plant development, thermoregulation, and anthocyanin production in Arabidopsis.

1.1.5 Evening-Expressed Transcription Factors

The evening complex (EC) consists of three proteins: LUX ARRHYTHMO (LUX; AT3G46640, a MYB-like transcription factor), EARLY FLOWERING 3 (ELF3; AT2G25930) and EARLY FLOWERING 4 (ELF4; AT2G40080) (two unrelated nuclear proteins) (Doyle et al., 2002; Ezer et al., 2017; Hazen et al., 2005; Hicks et al., 2001; Huang & Nusinow, 2016). The EC is active at night, where ELF3 acts as a scaffold between ELF4 and LUX (Herrero et al., 2012; Nusinow et al., 2011; Silva et al., 2020). LHY and CCA1 are repressed by the EC in the evening (**Figure 2**; Li et al., 2011; Nusinow et al., 2011). The PRR proteins are also repressed by the EC in the evening (**Figure 2**; Lee et al., 2019; Mizuno et al., 2014; Li et al., 2020).

The EC regulates thermo-related responses in the evening (**Figure 6**; Box et al., 2015; Jung et al., 2020; Li et al., 2022; Mizuno et al., 2014; Zhu et al., 2021). Plants deficient in any one of the members of the EC have been shown to be arrhythmic (Dixon et al., 2011; Helfer et al., 2011; Herrero et al., 2012; Hsu & Harmer, 2014; Nusinow et al., 2011). The EC cooperatively regulates hypocotyl morphology by regulating the expression of *PHYTOCHROME INTERACTING FACTOR* (*PIF*) *4* (AT2G43010) and *PIF5* (AT3G59060) at night (**Figure 6**; Nusinow et al., 2011). ELF3 also acts alone to solely repress the downstream activity of PIF4, where *ELF3-OX* plants have shorter hypocotyls (Nieto et al., 2015). ELF3 may also regulate flowering time in Arabidopsis, through its interaction with CYCLOIDEA AND PCF TRANSCRIPTION FACTOR 2 (TCP2; AT4G18390) (**Figure 6**; He et al., 2021).

The EC has also been implicated in biotic stress responses by altering salicylic acid (SA) and jasmonic acid (JA) biosynthesis in Arabidopsis (**Figure 6**; He et al., 2021; Zhang et al., 2019). *JASMONATE INSENSITIVE 1* (*MYC2*; AT1G32640) is an activator of JA-promoted leaf senescence (Zhang et al., 2018). The interaction between ELF3 and MYC2 likely alters JA pools, as *elf3-1 myc2* plants display with delayed JA-mediated senescence (Zhang et al., 2018). Alternatively, LUX binds to the promoters of *ENHANCED DISEASE SUSCEPTIBILITY* (*EDS1*; AT3G48090.1) and *JASMONATE-ZIM-DOMAIN 5* (*JAZ5*; AT1G17380) to regulate SA and JA-controlled defense-related mechanisms, respectively (He et al., 2021).

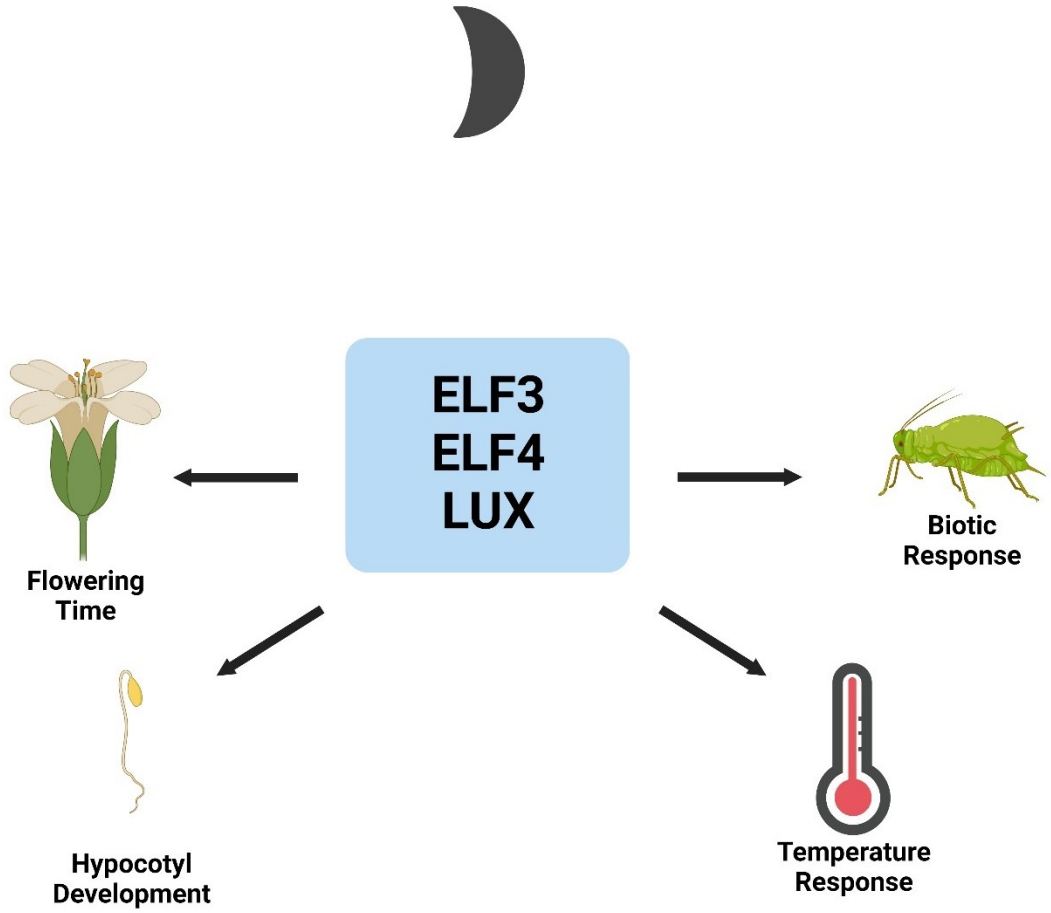

Figure 6: Outputs of the EC proteins. The evening activated ELF3, ELF4, and LUX have been shown to regulate flowering time, hypocotyl development, thermoregulation, and biotic responses in Arabidopsis.

1.2.1 Osmoregulation in Plants

Climate change is having a significant impact on food production worldwide and is one of the main contributors of global food insecurity, requiring the need for more resilient crop cultivars (Dhankher & Foyer, 2018; Long et al., 2015; Mahajan & Tuteja, 2005; Zhu, 2016). Plants exhibit both molecular and phenotypic changes when exposed to abiotic stress conditions, such as alterations in stomatal aperture and differential changes in metabolite profiles (Abdullah et al., 2021), in addition to changes in the primary root architecture (Smolko et al., 2021). Oxidative stress results in the accumulation of reactive oxygen species (ROS) (Hossain et al., 2015) such as hydrogen peroxide (Zwiewka et al., 2019) within the plant cell, directly impeding plant root growth by altering phytohormone profiles at the cellular level (Huang et al., 2018). In response to osmoregulatory stress, plants upregulate the biosynthesis of the phytohormone abscisic acid (ABA) (Wang et al., 2020). ABA mediates plant germination (Lopez-Molina et al., 2001) and stomatal pore perforation diameter (Yoshida & Fernie, 2018) to elicit adaptive changes in plants (Bartels & Sunkar, 2005; Guo et al., 2020; He et al., 2021; Shinozaki & Yamaguchi-Shinozaki, 2006). Endogenous ABA is utilized for proper growth and development by interlinking external inputs with internal regulatory mechanisms to confer biological homeostasis (Humplík et al., 2017).

To induce osmoregulatory stress in plants, a proxy for osmotic stress or salinity stress is often exogenously supplied (Perez-Alfocea et al., 1993). Polyethylene glycol (PEG) and mannitol have both been utilized to simulate osmotic stress in plants by conferring a hyperosmotic plant cell environment (Lawlor, 1970; van den Broeck et al., 2017). However, PEG has been shown to cause the premature differentiation of root stem cells, resulting in increased lateral root growth coupled with a cessation in primary root growth (Ji et al., 2014). PEG has also been shown to promote heavy metal toxicity as well as osmotic stress in plants, preventing biochemical botanists from parsing out the effects of two different stressors on plant homeostasis (Plaut & Federman, 1985). Alternatively, mannitol exposure inhibits cell division and thus leaf growth in plants exposed to osmotic stress (Kalve et al., 2020; Claeys et al., 2014). It has also been shown to impact ribosome function (Skirycz et al., 2011) and decrease metabolite and redox-related gene expression (Nikonorova et al., 2018). Arabidopsis plants exposed to either salt or mannitol stress over 24 hrs have been found to exhibit significant changes in protein phosphorylation (245 phosphosites), lysine acetylation (35 acetylation sites), and protein abundance (107 proteins), illustrating that

osmoregulatory-mediated abiotic stress elicits multiple protein-level changes (Rodriguez et al., 2021). With rising sea levels, increasing saline concentrations in coastal areas is becoming a pervasive issue (Guha & Panday, 2012). Salt stress has been shown to have deleterious effects on plant growth and development (Munns & Tester, 2008) by decreasing germination rates (Li et al., 2021) and delaying flowering time (Li et al., 2007; Lutts et al., 1995; Hongqiao et al., 2021). The bioengineering of crop cultivars with enhanced salt tolerance is of profound importance, however due to the multigenetic nature of salt stress tolerance, this has been a challenging endeavor (Zhu, 2016).

1.2.2 Osmoregulation and the Circadian Clock

Intersections between abiotic stress and the clock have been resolved, with several circadian clock genes suggested to mediate drought-like responses in Arabidopsis (Covington et al., 2008; Kamrani et al., 2022; Wilkins et al., 2010). The pool of ABA in leaves as well as its biosynthesis is under circadian control (Seung et al., 2011). For example, LHY has been shown to directly affect the rate-limiting step of ABA production by binding to the promoter of ABA-synthesizing genes, while simultaneously repressing the synthesis of 9-cis-epoxycarotenoid dioxygenase enzymes (Adams et al., 2018). *LHY-OX* and *lhy* plants decrease and alter the synthesis of ABA, respectively, which further suggests that LHY could be involved in the ABA-regulated osmoregulatory stress response (Adams et al., 2018). When methyl viologen (MV is a known proxy for osmotic stress that increases superoxides in plants) is added to *CCA1-OX* plants, genes that were shown to be down-regulated in WT plants are upregulated *CCA1-OX* plants (Ding et al., 2018; Lai et al., 2012). When subjected to MV, the number of wilted leaves was also found to increase in *cca1*, *lhy*, and *lhy cca1* plants, while the number of compromised leaves decreased significantly for *CCA1-OX* plants, suggesting that CCA1 and LHY could be cooperatively involved in the osmoregulatory process (Lai et al., 2012). Beyond Arabidopsis, when rice plants deficient in CCA1 (*oscca1*) were exposed to NaCl or mannitol, they were found to maintain a survival rate of 10%–30%, relative to the much higher survival rates of 50% to 70% found in WT plants (Wei et al., 2022).

Further, PRR5, 7, and 9 have also been implicated in ABA-dependent abiotic stress responses (Yang et al., 2021). ABSCISIC ACID INSENSITIVE 5 (ABI5; AT2G36270) has been suggested to be a key regulator of ABA-dependent germination through ABA (*PYR/PYL/RCAR*)

synthesizing genes (Zhao et al., 2020). ABI5 is also involved in ABA-induced anthocyanin biosynthesis (An et al., 2020), ABA signaling (Bhagat et al., 2021), drought responses (Li et al., 2021), amongst other regulatory processes (Collin et al., 2021). ABI5 directly interacts with PRR5 and PRR7, suggesting that PRR5 and PRR7 could have a role in the ABI5-modulated synthesis of ABA (Yang et al., 2021). Further, *prr5 prr7 prr9* plants have a higher germination rate than WT, *prr5 prr7*, or *prr5 prr9* plants (Yang et al., 2021). Taken together, the observations of Yang et al., (2021) suggests that PRR5, PRR7, and PRR9 could have different roles in ABA signaling. TOC1 binds to the promoter of *ABAR/CHLHGUN5* to regulate the endogenous pools of ABA in plants (Legnaioli et al., 2009). ABA further induces the binding between TOC1 and ABAR/CHLH/GUN5, which also possibly implicates TOC1 in the ABA-dependent osmoregulatory process (Legnaioli et al., 2009).

Scandola et al., (2022) recently suggested that RVE8-like transcription factors could be involved in the abiotic-stress response through the regulation of multiple salt-induced, osmotic-mediated, and sulfur-assimilating (which have been observed to increase in response to persistent osmotic stress) proteins (Rodriguez et al., 2021; Torres-Franklin et al., 2009). Further, ELF3 exhibits salt tolerance by modulating the expression of osmoregulatory protein GIGANTEA (GI; AT1G22770) (Sakuraba et al., 2017; Yu et al., 2008). Here, ELF3 interacts with CO to form a complex, which destabilizes and represses GI (Yu et al., 2008). *ELF3-OX* plants perform better than WT when exposed to NaCl, where *ELF3-OX* plants have higher chlorophyll concentrations, elevated shoot fresh weight, and longer primary root lengths (Sakuraba et al., 2017).

Prior work has suggested that the RVE8-like proteins could have a role to play in drought-like responses. Namely, Scandola et al., (2022) has shown that proteins regulating ABA and sulfur metabolism are enriched between *rve 4 6 8* and WT plants. Simon et al., (2020) has shown that there is a non-significant difference between WT and *rve8* and *rve4* in water use efficiency (WUE; another proxy for drought-like stress looking at stomatal aperture parameters), wherein plants deficient in *rve8* showed a slight non-significant increase in WUE relative to WT, while *rve4* plants showed the converse (Simon et al., 2020). RVE8-like proteins activate PRR and EC complex proteins (**Figure 2**; Creux & Harmer, 2019; Harmer & Kay, 2005; Nohales, 2021; Rawat et al., 2011) and *prr9* and *elf3* plants have been observed to have significantly lower WUE than WT (Simon et al., 2020). Thus, it could be that plants lacking in *rve 4 6 8* fare worse than WT, as opposed to plants deficient in any one of RVE8, RVE4, or RVE6 proteins (Scandola et al., 2022;

Simon et al., 2020). It is also plausible that RVE8-like proteins could also be affecting osmoregulatory homeostasis through its interaction with PRR9 and ELF3 (Simon et al., 2020). As such, elucidating the role of the sole activator of the circadian clock in the osmoregulatory response, and unraveling the precise plant cell metabolic processes and pathways that are implicated is of paramount importance in contemporary botanical chronobiology.

1.3.1 Nutrient Stress in Plants

Nitrogen (N), phosphorus (P), and sulfur (S) are essential macronutrients required for plant growth, development, and effective crop production (Kopriva et al., 2012; Zenda et al., 2021). The deprivation of essential nutrients can limit growth, by driving extensive molecular changes (Forieri et al., 2016). For example, elevated levels of ethylene (a stress-related phytohormone) has been shown to develop in response to N, P, and S related nutrient deprivation, indicating a partially shared response to macronutrient deprivation in plants (García et al., 2015). Further, transcriptomic analysis of N, P, and S starved plants have demonstrated a common molecular senescence response under nutrient deprivation, irrespective of the type of nutrient that is lacking (Watanabe et al., 2010).

N, which is utilized *in vivo* as bioavailable nitrate is ubiquitous with proper plant growth, development, homeostasis, and metabolism due to its presence in all 20 naturally occurring amino acids (Flis et al., 2019; Miller et al., 2007; Zhu et al., 2018) and critical plant pigments such as chlorophyll (Allison et al., 1997; Bassi et al., 2018) and anthocyanin (Ibrahim et al., 2011; Soubeyrand et al., 2014). Plants preferentially increase the abundance of nitrates within the source cells to promote the biosynthesis of amino acids within the leaves (Nunes-Nesi et al., 2010). In response to low N pools, characteristic changes in root architecture have also been observed in Arabidopsis (Gruber et al., 2013). In response to N stress during development, plants have been found to recycle the existing N pools by breaking down N-containing compounds in older leaves and transporting the components to younger leaves (Fan et al., 2009). Amino acids and proteins also get transported to reproductive organs (Nunes-Nesi et al., 2010) or are unloaded into sinks, such as plant seeds (Tegeder et al., 2000). Nitrogen remobilization and transportation occurs across all stages of plant development from seeds to mature plant specimens (Fan et al., 2017).

P, which is utilized *in vivo* as bioavailable phosphate is critical to multiple metabolic processes, representing ~2% of the total dry biomass in Arabidopsis (Kumar et al., 2018). Its

importance is highlighted by its requirement for adenosine triphosphate (ATP) biosynthesis (Igamberdiev & Kleczkowski, 2015). ATP is an energy molecule that drives many universal eukaryotic processes such as gene expression (Lim et al., 2014; Zhu et al., 2019), metabolism (Hong et al., 2022; Liang et al., 2015), protein transport (Kim et al., 2006; Thomas et al., 2000), and key plant cellular mechanisms including gravitropism (Tang et al., 2003), pollen-tube growth (Reichler et al., 2009), root hair growth (Kim et al., 2006), amongst others (Hao et al., 2012; Liang et al., 2015). ATP is also required for reversible protein phosphorylation, which has been estimated to regulate the function of approximately 75% of all proteins in eukaryotes (Sharma et al., 2014). Phosphorylation is a key post-translational modification (PTM) which alters the function and activity of a substrate protein (Haubrich & Swinney, 2016; Uhrig et al., 2019), amongst other roles (e.g., subcellular localization). In response to P deprivation, root architecture modification at the cellular level occurs due to cortical root cell alterations (Janes et al., 2018). In response to P stress, proteomic changes and differential protein profiles (Chevalier & Rossignol, 2011; Mehta et al., 2020) as well as gene expression alterations (Scheible et al., 2022) have been found, emphasizing the important role that P plays in the maintenance of plant homeostasis.

S, which is utilized *in vivo* as bioavailable sulfate has been described as a growth-limiting macronutrient due to the utilization of sulfur in essential amino acids cysteine and methionine in plants (Dietzen et al., 2020; Koprivova & Kopriva, 2014). Cysteine amino acids are required for ABA biosynthesis, whereby ABA DEFICIENT3 (ABA3; AT1G16540) utilizes cysteine to synthesize ABA in response to low-water conditions (Batool et al., 2018). Furthermore, the application of exogenous cysteine was met with an increased amount of endogenous ABA, further coupling cysteine amino acids to ABA biosynthesis (Batool et al., 2018). Glucosinolates are S-containing secondary metabolites that regulate plant homeostasis in response to abiotic stress inputs (Chowdhury, 2022). More specifically, under salinity conditions (López-Berenguer et al., 2008; Steinbrenner et al., 2012) and water-limiting conditions (Radovich et al., 2005), glucosinolate pools were shown to increase. Plants deprived of S demonstrate with a 23-fold decrease in glucosinolate metabolites (Forieri et al., 2016), directly tying S metabolism to Arabidopsis homeostasis (Wittstock & Halkier, 2002). Further, S has also been shown to be an essential component in protein persulfidation (PTM where hydrogen sulfide is utilized as a signalling molecule (Filipovic & Jovanović, 2017), where it has a function in protein protection from excess oxidation (Filipovic & Jovanović, 2017). At least 5% of the Arabidopsis proteome is

capable of being persulfidated (Aroca et al., 2017). S also comprises ~2% of the dry biomass in Arabidopsis (Kumar et al., 2018). Under S depletion, a 2-fold decrease in root biomass and alterations in root architecture has been observed (Forieri et al., 2016; Joshi et al., 2018), illustrating that S pools have a role to play in proper plant development.

1.3.2 Nutrient Stress and the Circadian Clock

Given the intimate tie between N, P, and S stress and deviations from plant homeostasis, greater analyses into the effects of N, P, and S deprivation in plants are required. The systemic role of the Arabidopsis circadian circuitry in the nutrient stress response is yet to be elucidated, unlike the preliminary interplay between the clock and other abiotic stressors. Currently, it is known that in response to diel light regiments global amino acid profiles differentially change in *prr7 prr9* and *elf3* plants, relative to WT, suggesting that the clock could have a role in the regulation of N pools in Arabidopsis (Flis et al., 2019). Further, TOC1 decreases the expression of *AUTOPHAGY-RELATED PROTEIN 8D* (*ATG8D*; AT2G05630) when exposed to N-deficient media (Chen et al., 2022), with *ATG8D* expression observed to be higher in *toc1* plants, suggesting that TOC1 likely represses N deficiency-mediated autophagy (Chen et al., 2022). CCA1 has also been shown to directly bind to N-assimilating proteins, which suggests that N-transporter proteins could be under circadian control (Gutiérrez et al., 2008). CCA1 could also regulate P assimilation by regulating the rhythmic expression of PHOSPHATE TRANSPORTER 4;1 (PHT 4;1; AT2G29650), as the expression of *PHT4;1* was observed to be largely dysregulated in *CCA1-OX* plants (Wang et al., 2014). Under P stress, it seems that *LHY*, *CCA1*, *PRR9*, and *RVE8* expression is induced, further implicating the clock in the mitigation of nutrient-dependent homeostasis in Arabidopsis (Scheible et al., 2022). Approximately 20 sulfur-related genes are differentially regulated when exposed to sulfur deficient conditions, such as RVE2 (an RVE8-like protein; AT5G37260), which is down-regulated at the end-of-night in root tissue subjected to sulfur stress (Forieri et al., 2016; Peixoto et al., 2021).

Although there is some evidence suggesting that the circadian clock could play a role in the N, P, and S stress responses, a systematic analysis of the interplay between the clock and nutrient stress has not been pursued. Further, evidence linking the clock to nutrient stress mediated responses has been largely transcriptomic, with few studies looking into the proteomic or metabolomic landscape of plants deprived of N, P, or S. This distinction is important because

changes in protein levels largely deviate from alterations in transcriptomic profiles (Graf et al., 2017), thus elucidating the role of the clock in the nutrient stress response at the proteomic level remains of paramount importance and is largely unexplored in contemporary plant chronobiology.

1.4 Project Significance, Potential Applications, and Research Objectives

Contemporary agricultural practices aim to minimize inputs (e.g., nutrients) and maximize yield (e.g., biomass) to ensure sustainable crop production and improve food security (Fan et al., 2011). Given that the circadian clock regulates the expression of approximately 30-40% of the genes in Arabidopsis, botanical researchers can utilize the features of the clock in order to uncover potential targets for agricultural improvement (Romanowski et al., 2020; Steed et al., 2021). Arabidopsis plants deficient in ELF3 flower faster than WT, as *elf3* plants have approximately half the number of rosette leaves of WT plants in LD conditions (Zagotta et al., 1996). Loss of ELF3 causes the accumulation of CO and GI which causes early flowering in plants (Hicks et al., 2001; Yu et al., 2008; Zhao et al., 2021), such that plants *ELF3-OX* plants exhibit late flowering in LD (Liu et al., 2001). Recently, it has been reported that breeders have indirectly been selecting for knockouts of wheat (*Triticum turgidum*) homologues of ELF3 (*TtELF3*), which increases the heading (flowering time in wheat) date (Wittern et al., 2022). With rising global temperatures in the wake of climate change, especially within the harsh summer months, accelerating the flowering time of crop cultivars would allow for the benefit of increased yield by avoiding crop loss due to heat stress (Suraweera et al., 2020).

Drought and water limitations due to climate change is another constraint on contemporary agriculture, requiring the breeding of more water-efficient crop cultivars (Kijne et al., 2003). Water loss throughout the day occurs primarily through the stomata, where water is lost via transpiration (Bertolino et al., 2019). Select members of the core clock, including *PRR5* and *ELF3* have higher expression levels in the guard cell versus the whole plant leaf organ, suggesting that these genes could have especially large effects in the regulation of stomatal aperture and perhaps WUE (Hassidim et al., 2017). Plants where stomatal aperture is no longer under circadian control fare worse under significant drought stress (50% of field capacity) by having a smaller plant biomass than WT (Hassidim et al., 2017). Recent reports have shown that plants deficient in ELF4 and LUX have a slight increase in WUE relative to WT, while plants deficient in PRR5 and ELF3 show a decrease in WUE (Simon et al., 2020), which illustrates the disparate roles of the EC in

regulating water loss in the evening. Agricultural specialists could utilize the features of the clock to increase WUE in crops, which would decrease the input of water in crop production (Steed et al., 2021).

In my project, I aim to further elucidate the role of the RVE8-like proteins in the osmoregulatory response and systematically evaluate the N, P, or S nutrition responses by using a wide-range of circadian clock-deficient mutants (**Chapter 2**; **3**). In **Chapter 2**, I show that plants deficient in RVE8-like proteins fare worse than WT plants when subjected to salt and osmotic stress. I also show that the total proteome of WT and *rve 4 6 8* plants differ at key circadian time-points when subjected to drought-like conditions (**Figure 7**). In **Chapter 3**, I subject a series of well characterized circadian clock deficient plants to nutrient stress. Here, I was able to show that several members of the clock show statistically significant alterations in key plant phenotypes (primary root length and hypocotyl length) when subjected to N, P, or S stress. I then characterized the changing metabolome of a specific subset of circadian clock mutants at ZT0, ZT4, ZT8, and ZT12 when subjected to nutrient stress conditions (**Figure 8**). Collectively, these systems level results provide new insights into the circadian clock and its role in drought-like and nutrient stress responses, providing a clear foundation for future endeavors in these areas within the field of botanical chronobiochemistry. Within the context of agricultural application, my project could provide targets for improving osmoregulatory tolerance and nutrient use efficiency in crops.

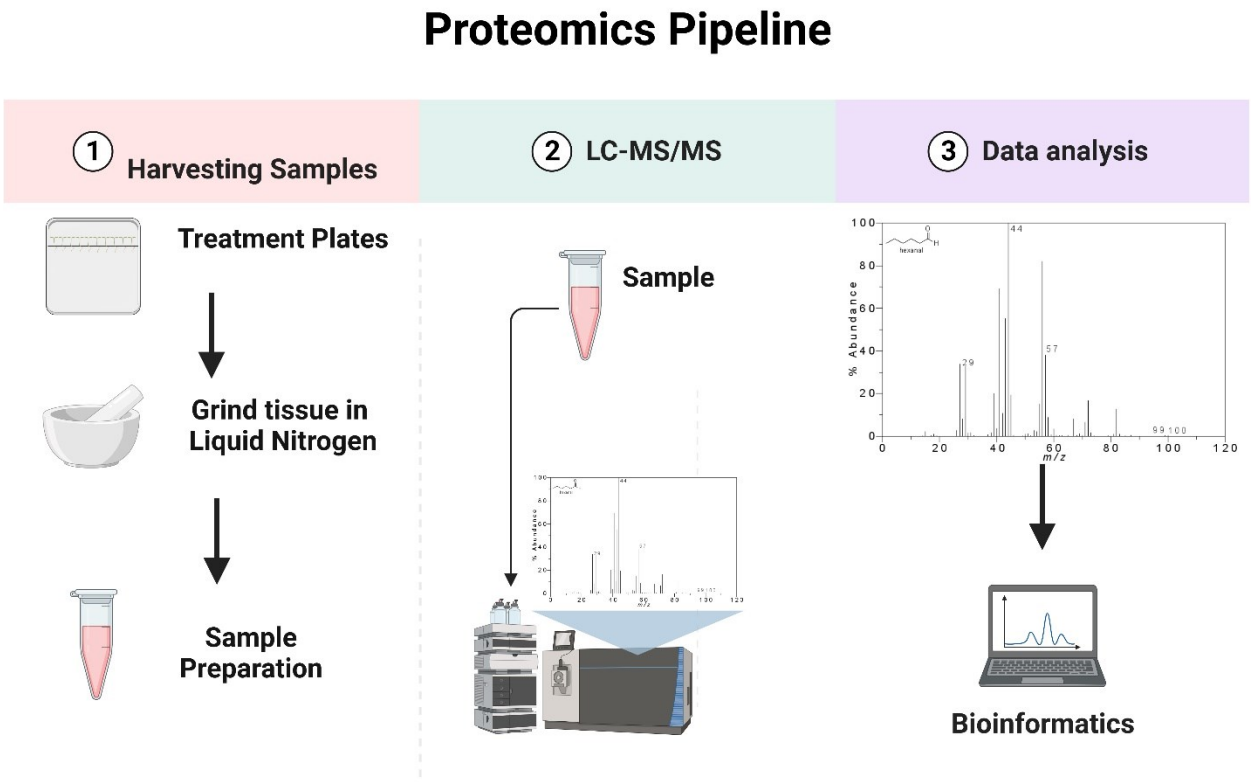

Figure 7: Proteomic workflow schematic.

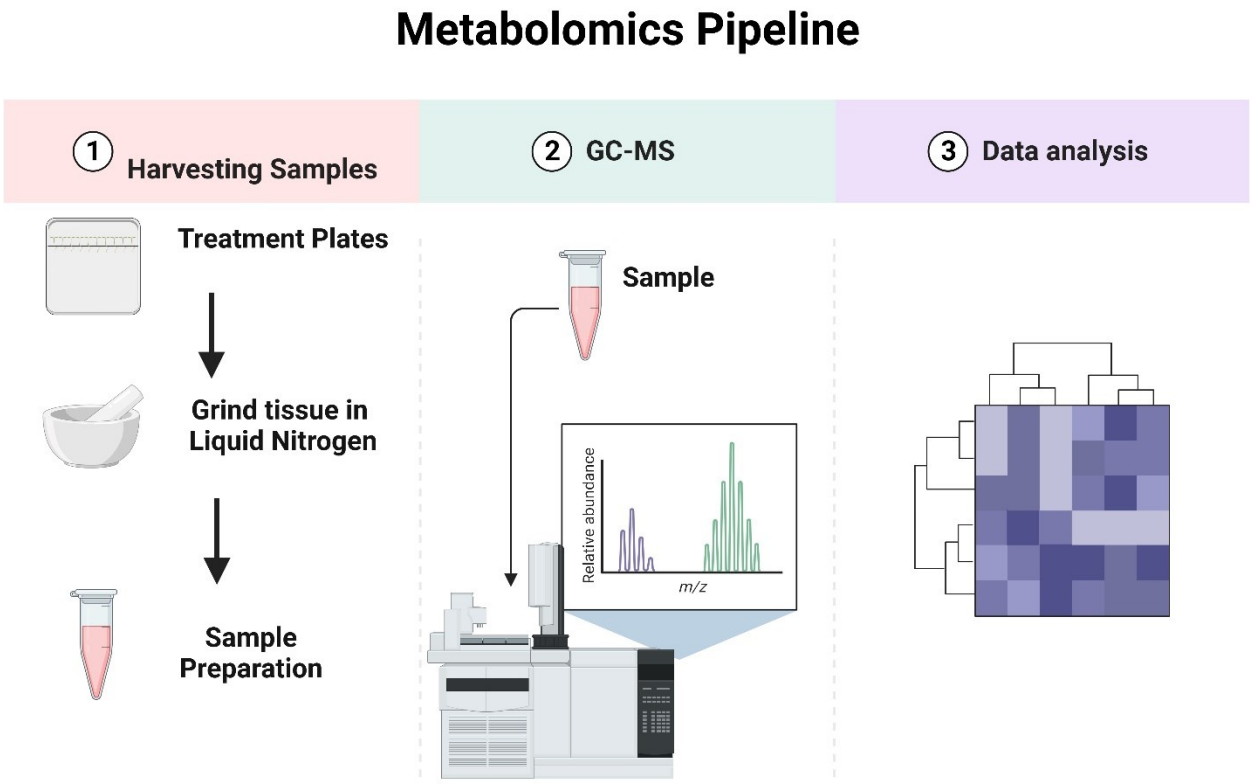

Figure 8: Metabolomic workflow schematic.

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Chapter 2:

Quantitative proteomic analysis illustrates that *REVIELLE* clock genes are involved in the salt and osmotic stress response

2.1 INTRODUCTION

Plants are entrained by environmental cues such as light and temperature to grow and develop properly (Webb et al., 2019). In plants, the precise timing of daily events is facilitated by the circadian clock (Dodd et al., 2005; Gottlieb, 2019). The circadian clock transcription factors are expressed at specific times in the day-night cycle to activate the expression of key genes, while simultaneously repressing the expression of the other circadian clock transcription factors in order to precisely time diel molecular cell processes (Covington et al., 2008; Kamioka et al., 2016; Shalit-Kaneh et al., 2018). The MYB-like REVEILLE transcription factor proteins consisting of RVE8, RVE4, and RVE6 act as activators of the core circadian clock genes comprising of either the morning or evening loop (Farinas & Mas, 2011; Hsu et al., 2013; Rawat et al., 2011; Xie et al., 2014). RVE8, RVE4, and RVE6 function to cooperatively pace the end-of-day (Li et al., 2019; Hsu et al., 2013; Gray et al., 2017), with plants lacking in *rve 4 6 8* possessing a longer circadian period with smaller oscillation amplitudes (Hsu et al., 2013). Compared to *rve 4 6 8* plants, WT plants possess shorter hypocotyls, have smaller leaf size, and a reduced leaf biomass (Gray et al., 2017), while both Gray et al., (2017) and Scandola et al., (2022) have shown that *rve 4 6 8* plants possess a delay in flowering relative to WT.

Upon examining proteome changes between WT and *rve 4 6 8* at the end-of-day (ED; ZT11) and the end-of-night (EN; ZT23), gene ontology (GO) clusters for ABA and S metabolism were shown to be enriched suggesting that RVE8-like proteins could play a role in the regulation of osmoregulatory stress (Scandola et al., 2022). Further, proteomic analysis of Arabidopsis plants subjected to osmotic and salt stress also found enriched GO categories for S metabolism, suggesting that S assimilation and metabolic processes could play a role in the mitigation of drought-like stress (Rodriguez et al., 2021). Bioavailable sulfate is needed for the synthesis of the amino acid cysteine *in vivo* (Kopriva, 2004). Cysteine is incorporated into ABA by ABA3 (Batool et al., 2018). Pools of ABA are increased in response to drought-like conditions (Batool et al., 2018). Under salt and osmotic stress, ABA has been shown to mitigate water loss by regulating the stomatal aperture (Guo et al., 2020). Upon supplying exogenous cysteine to plants, greater

pools of ABA has been reported, further tying ABA production to sulfur metabolism via the production of cysteine.

Interestingly, in soybean (*Glycine max*) crops, an RVE8-like ortholog (GmMYB133) has been implicated in osmoregulatory stress regulation (Shan et al., 2021). Here, *GmMYB133-OX* plants fared better under salt stress by increasing the expression of *CATION EXCHANGER 3* (*CAX3*; AT3G51860), *EARLY ARABIDOPSIS ALUMINUM INDUCED 1* (*EARLI1*; AT4G12480), *AZELAIC ACID INDUCED 1* (*AZI1*; AT4G12470), and *MITOGEN-ACTIVATED PROTEIN KINASE 3* (*MPK3*; AT3G45640) (Shan et al., 2021). Moreover, *GmMYB133-OX* plants exhibited an altered rhythmic expression of *PRR5*, suggesting a clock connection to RVE8-like proteins (Shan et al., 2021). In Arabidopsis, PRR5 has been shown to directly modulate the expression of *ABI5* (Yang et al., 2021), which directly influences ABA-dependent germination through the regulation of ABA (*PYR/PYL/RCAR*) synthesizing genes (Li et al., 2021; Zhao et al., 2020). RVE8 has been shown to directly regulate the activity of PRR5 in Arabidopsis by binding to the promoter of *PRR5* (Craigon, 2004; Rawat et al., 2011), as *RVE8-OX* plants have elevated gene expression of *PRR5*. Conversely, *PRR5-OX* plants have lowered levels of *RVE8* (Craigon, 2004; Rawat et al., 2011), illustrating a tightly regulated feedback loop between RVE8 and PRR5.

In chapter 2, I investigate whether RVE8-like proteins are directly involved in the osmoregulatory stress response in Arabidopsis by undertaking a quantitative proteomic analysis of WT versus *rve 4 6 8* plants at ZT11 and ZT23 when exposed to drought-like stress conditions. Given that RVE8-like proteins are associated with multiple agronomically important traits such as plant biomass (Gray et al., 2017), carbohydrate metabolism (Scandola et al., 2022), and thermotolerance (Li et al., 2019; Chen et al., 2020; Kidokoro et al., 2021), elucidating if plants deficient in RVE8-like proteins are more or less susceptible to osmotic and/or salt stress conditions is prudent for the bioengineering of climate change resilient crops (Rodriguez et al., 2021; Shan et al., 2021).

2.2 MATERIALS AND METHODS

2.2.1 Plant Growth and Preliminary Phenomics

WT (wild-type, Columbia ecotype) and *rve 4 6 8* (in the Columbia background; Hsu et al., 2013) seeds were rinsed firstly in a 70% (v/v) ethanol solution for 2 minutes, followed by a 30% (v/v) bleach (Clorox® 7.5%) wash for 7 minutes, and lastly with three sequential washes with

distilled water. The seeds were then immediately imbibed on 0.5x MS media (Caisson Labs MS Media with macronutrients and micronutrients; MSP01) and 7 g/L of agar (control; CTL) at pH 5.8 (with KOH). All seeds were stratified for 3 days at 4ºC in the dark and then exposed to 12h light and 12h dark photoperiod of 100 µmol/m^2/s of florescent light for 5 days at 22ºC, before being transferred onto experimental plates. Seedlings were transferred to either CTL, 50 mM mannitol, 100 mM mannitol, 200 mM mannitol, 25 mM NaCl, 50 mM NaCl, or 100 mM NaCl experimental plates for a subsequent 8 days. Primary Root measurements were obtained every 24 hours over the course of an 8-day time course. Photographs of the seedlings were taken on the last day of the time course, along with hypocotyl length, number of lateral roots, and plate-wise wet biomass measurements.

2.2.2 Plant Harvesting and Storage for Proteomics

WT and *rve 4 6 8* seeds were sterilized, stratified, and germinated on CTL plates under a 12h light and 12h dark photoperiod of 100 µmol/m^2/s of florescent light for 5 days at 22ºC. WT and *rve 4 6 8* seedlings were then transferred onto and grown on CTL, 50 mM mannitol, or 100 mM NaCl experimental plates for another subsequent 8 days. Whole seedlings were harvested at ZT11 and ZT23, and were immediately snap frozen in liquid N_2. Samples were stored at -80ºC until they were ready to be grounded in liquid N_2 to be used for quantitative proteomics and subsequent downstream data analyses. Ground samples were aliquoted into ~50 mg fractions for proteomic experiments.

2.2.3 Quantitative Proteomics Sample Preparation - LC–MS/MS

Aliquoted samples were extracted at a 1:2 (w/v) ratio with a solution of 50 mM HEPES-KOH pH 8.0, 50 mM NaCl, and 4% (w/v) SDS. Samples were then vortexed and placed in a 95ºC table-top shaking incubator (Eppendorf) at 1100 xg for 15 mins, followed by an additional 15 mins shaking at room temperature. All samples were then spun at 20,000 xg for 5 min to clarify extractions, with the supernatant retained in fresh 1.5 mL Eppendorf tubes. Sample protein concentrations were measured by bicinchoninic acid (BCA) assay (23225; Thermo Scientific). Samples were then reduced with 10 mM dithiothreitol (DTT) at 95ºC for 5 mins, cooled, then alkylated with 30 mM iodoacetamide (IA) for 30 min in the dark without shaking at room temperature. Subsequently, 10 mM DTT was added to each sample, followed by a quick vortex,

and incubation for 10 min at room temperature without shaking. Total proteome peptide pools were then generated using a KingFisher Apex (5400910; Thermo Scientific) automated sample preparation device as outlined by Leutert et al. (2019) without deviation. Sample digestion was performed using sequencing grade trypsin (V5113; Promega), with generated peptide pools quantified by Nanodrop and acidified with formic acid (FA) to a final concentration of 5% (v/v) prior to being desalted using 1cc tC18 Sep Pak cartridges (WAT036820; Waters) as previously described (Scandola et al., 2022; Uhrig et al., 2019). All peptides were then dried and re-suspended in 3% (v/v) ACN / 0.1% (v/v) FA immediately prior to MS analysis.

2.2.4 BoxCarDIA Mass Spectrometry (BoxCar DIA LC–MS/MS)

Changes in protein abundance was assessed using a FAIMS mounted Fusion Lumos Tribrid Orbitrap mass spectrometer (Thermo Scientific) in a data independent acquisition (DIA) mode using the BoxCarDIA method (Mehta et al., 2022; Scandola et al., 2022). Dissolved peptides (1 µg) were injected using an Easy-nLC 1200 system (LC140; Thermo Scientific) and separated on a 50 cm Easy-Spray PepMap C18 Column (ES803A; Thermo Scientific). A spray voltage of 2.2 kV, funnel RF level of 40 and heated capillary at 300ºC was deployed, with all data acquired in profile mode using positive polarity, with peptide match turned off and isotope exclusion selected. All gradients were run at 300 nL/min with the analytical column temperature set to 50ºC. Peptides were eluted using a segmented solvent B gradient of 0.1% (v/v) FA in 80% (v/v) ACN from 4% - 41% B (0 - 107 min). FAIMS using compensation voltages (CVs) of -30, -50, and -70 were used with a static gas flow rate of 3.5 L/min. Within each CV, BoxCar DIA acquisition was performed as previously described (Mehta et al., 2022; Scandola et al., 2022). MS1 analysis was performed by using two multiplexed targeted SIM scans of 10 BoxCar windows each, with detection performed at a resolution of 120,000 at 200 m/z and normalized AGC targets of 100% per BoxCar isolation window. Windows were custom designed as previously described (Mehta et al., 2022; Scandola et al., 2022). An AGC target value for MS2 fragment spectra was set to 2000%. Twenty-eight 38.5 m/z windows were used with an overlap of 1 m/z. Resolution was set to 30,000 using a dynamic maximum injection time and a minimum number of desired points across each peak set to 6.

2.2.5 BoxCarDIA LC–MS/MS Data Analysis

All acquired BoxCar DIA data was analyzed in a library-free DIA approach using Spectronaut v16 (Biognosys AG) using default settings. Key search parameters employed include: a protein, peptide and PSM FDR of 1%, trypsin digestion with 1 missed cleavage, fixed modification including carbamidomethylation of cysteine residues and variable modifications including methionine oxidation. Data was Log2 transformed, globally normalized by median subtraction with significantly changing differentially abundant proteins determined and corrected for multiple comparisons (Bonferroni-corrected p-value ≤ 0.05; q-value ≤ 0.05).

2.2.6 Bioinformatics and Data Visualization

To identify the biological functions of enriched proteins, a gene ontology (GO) analysis of biological processes was performed using the ONTOLOGIZER (http://ontologizer.de; Bauer et al., 2008). A parent–child intersection analysis approach was used (Benjamini–Hochberg FDR correction p-value ≤ 0.05). The foreground used for the study were the significantly changing proteins identified between time-points and between genotypes ($Log_2FC > 0.58$; q-value ≤ 0.05) allowing for comparisons of phase-change differences and genotypic alterations, respectively in GO categories, while the background was all proteins identified in the study (6300 proteins). Identified biological process terms corresponding to elucidated GO terms with ≥ 5 and ≤ 80 proteins were subsequently assembled into a heat map based on FDR-adjusted p-value (r package Superheat). Packages were implemented in r 4.2.2 (R Core Team 2022, https://www.r-project.org/). Contextualization of significantly changing proteins ($Log_2FC > 0.58$; q-value ≤ 0.05) was performed through association network analyses using the Cytoscape STRING-DB plugin StringApp (http://apps.cytoscape.org/apps/stringapp; Szklarczyk et al., 2016) with an overall STRING-DB association score threshold of ≥ 0.7. Cytoscape version 3.9.1 (http://www.cytoscape.org/). Proteins with an association score < 0.7 were removed from visualisation. Metabolic pathways were defined using significantly changing proteins ($Log_2FC > 0.58$; q-value ≤ 0.05) and compiled into a heatmap (r package Superheat) by utilizing the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. The number of significantly changing proteins pertaining to each KEGG pathway identifier were exported from Cytoscape after conducting a GO category-informed STRING-DB network at each timepoint.

2.3 RESULTS

2.3.1 Examination of *rve 4 6 8* phenotypes unveils preliminary connection between osmoregulatory stress responses and RVE8-like proteins.

Given the results of Scandola et al. (2022), which resolved STRING-DB clusters for ABA and S metabolism proteins between WT and *rve 4 6 8* plants and that these two interconnected metabolic processes are related to osmotic stress, it suggests that RVE8-like proteins could be involved in conferring salt and osmotic stress tolerance. If RVE8-like proteins are involved in mitigating drought-like stress responses, then plants with functional *RVE* expression (WT) should fare better than plants lacking in several of the *RVE* genes (*rve 4 6 8*). Correspondingly, I examined WT and *rve 4 6 8* seedlings under three concentrations of mannitol (50 mM, 100 mM, or 200 mM) or NaCl (25 mM, 50 mM, or 100 mM) to see if I could detect a clear phenotype (**Figure 2 - 5**) prior to going further in my study (**Figure 1**). My results showed that the primary root length was longer in WT compared to *rve 4 6 8* (p-value ≤ 0.05; Student's t-test). I have detected that the hypocotyl length was shorter in WT compared to *rve 4 6 8* under 50 mM mannitol (**Figure 2 - 3**). I also found that WT seedling roots were also consistently longer (p-value ≤ 0.05; Student's t-test) than *rve 4 6 8* plants under 50 mM mannitol, beginning at day 5 after being transplanted (**Figure 1**) from CTL plates (**Figure 3**). I did not observe a significant difference between WT and *rve 4 6 8* primary root, hypocotyl length, or final fresh weight measurements under 100 mM mannitol or 200 mM mannitol (**Figure 2 - 3**), suggesting a moderate susceptibility to osmotic stress conditions (**Figure 2 - 3**; Kumar et al., 2019). I also observed that the primary root and hypocotyl length was longer and shorter in WT, respectively under 100 mM NaCl, relative to *rve 4 6 8* (**Figure 4 - 5**). I was not able to observe a discernable phenotypic difference between WT and *rve 4 6 8* plants under 25mM NaCl or 50mM NaCl (**Figure 4 - 5**). Given my phenotyping screen of WT and *rve 4 6 8* plants under osmoregulatory stress, it appears that plants that lack RVE8-like proteins are more susceptible to mannitol stress, over salt stress (**Figure 2 - 5**). Moreover, my data also suggests that WT plants do better than *rve 4 6 8* plants under mannitol and salt stress (**Figure 2 - 5**).

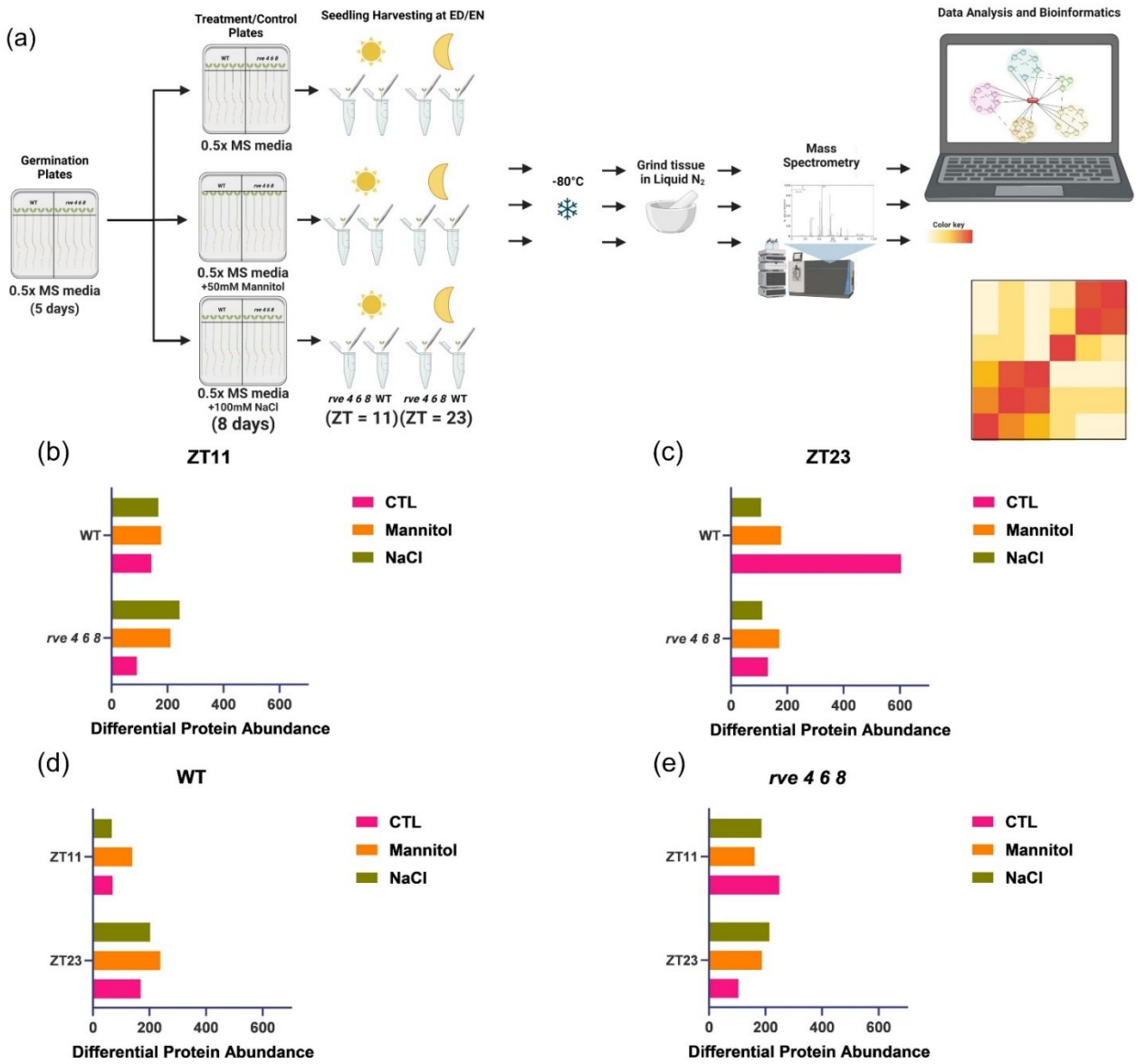

Figure 1: Total proteome changes between time-points and genotypes. Experimental workflow schematic (a) and number of significantly changing proteins ($Log_2FC > 0.58$; q-value ≤ 0.05) across biological replicates (n=4) at ZT11 (b), ZT23 (c), and within WT (d) and *rve 4 6 8* (e) genotypes under CTL, mannitol, and NaCl conditions.

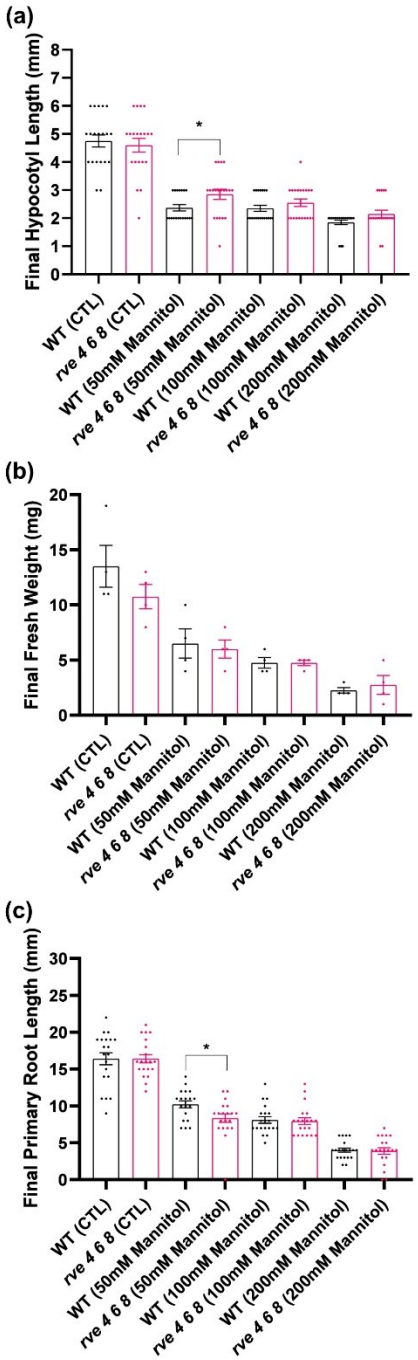

Figure 2: Phenotypic differences between WT and *rve 4 6 8* plants under osmotic stress. Quantitative phenomic analysis of the final hypocotyl (a), plate-wise final fresh weight measurements (b), and final primary root lengths (c) of seedlings at the end of 8 days in diel conditions after being transplanted from CTL germination plates onto CTL, 50mM mannitol, 100 mM mannitol, or 200 mM mannitol experimental plates. An asterisk (*) denotes statistical significance (p-value $\leq$ 0.05; Student's t-test). All measurements were conducted with 20 biological replicates with 5 plants per genotype, per plate (a total of 4 plates were used per treatment). Data are presented are mean ± standard deviation**.**

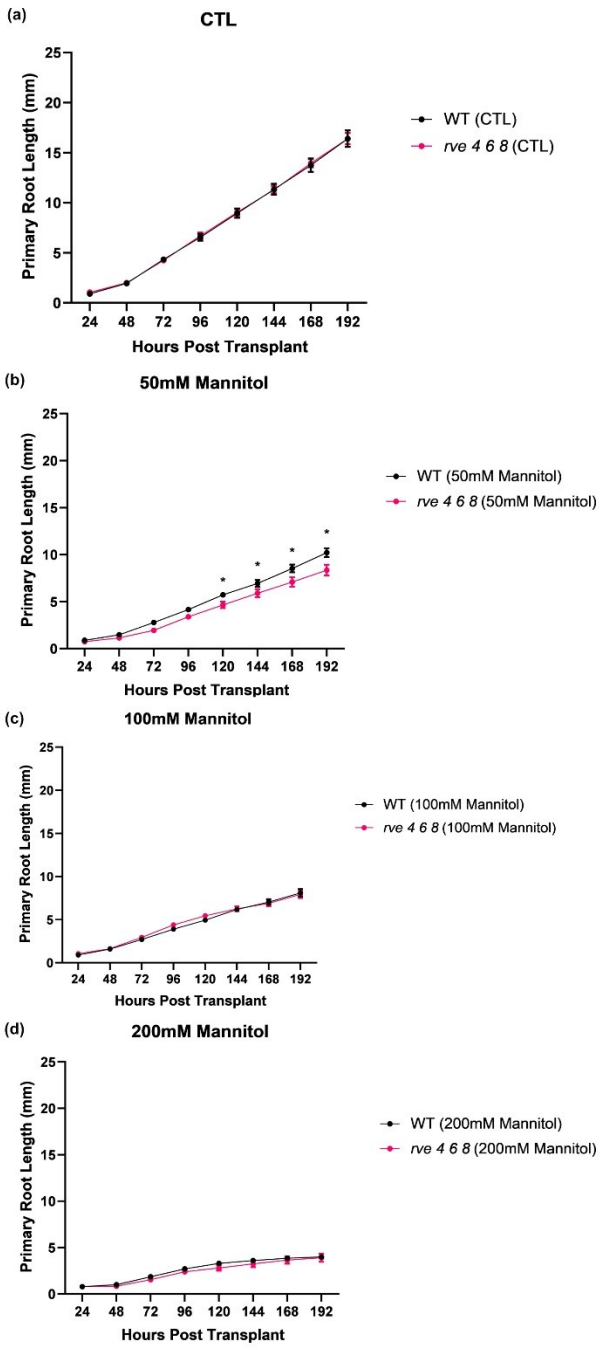

Figure 3: Time-dependent primary root differences between WT and *rve 4 6 8* plants under osmotic stress. Quantitative phenomic analysis of the changing root lengths of seedlings across 8 days in diel conditions after being transplated from CTL germination plates when exposed to CTL (a), 50 mM mannitol (b), 100 mM mannitol (c), or 200 mM mannitol (d) experimental plates. An asterisk (*) denotes statistical significance (p-value ≤ 0.05; Student's t-test). All measurements were conducted with 20 biological replicates. Data are presented are mean ± standard deviation**.**

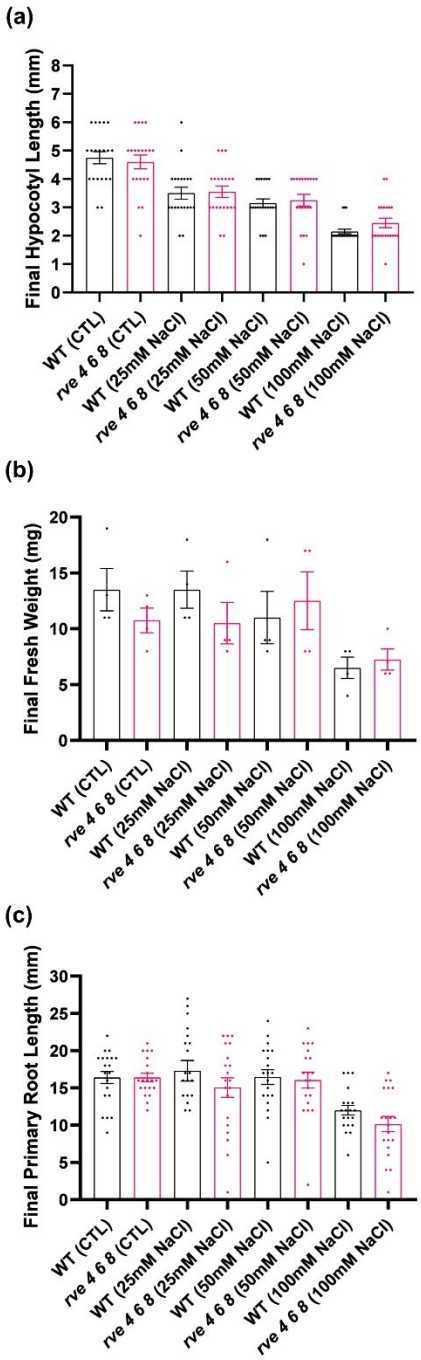

Figure 4: Phenotypic differences between WT and *rve 4 6 8* plants under salt stress. Quantitative phenomic analysis of the final hypocotyl (a), plate-wise final fresh weight measurements (b), and final primary root lengths (c) of seedlings at the end of 8 days in diel conditions after being transplanted from CTL germination plates onto CTL, 25mM NaCl, 50 mM NaCl, or 100 mM NaCl experimental plates. An asterisk (*) denotes statistical significance (p-value ≤ 0.05; Student's t-test). All measurements were conducted with 20 biological replicates with 5 plants per genotype, per plate (a total of 4 plates were used per treatment). Data are presented are mean ± standard deviation**.**

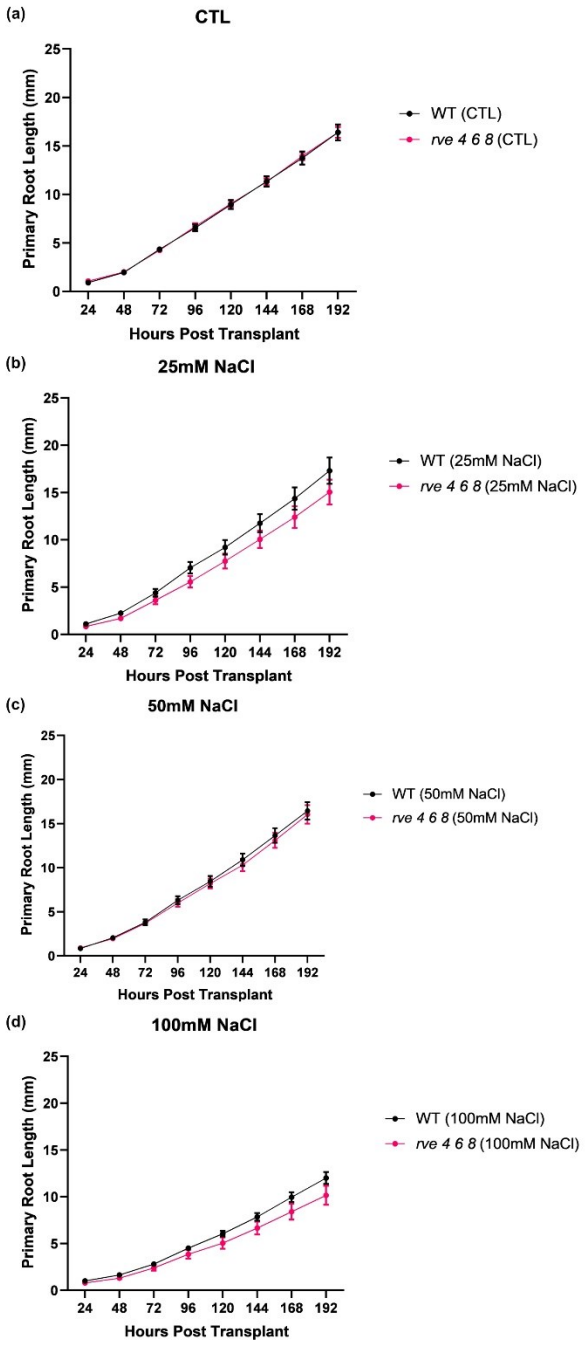

Figure 5: Time-dependent primary root differences between WT and *rve 4 6 8* plants under salt stress. Quantitative phenomic analysis of the changing root lengths of seedlings across 8 days in diel conditions after being transplated from CTL germination plates when exposed to CTL (a), 25 mM NaCl (b), 50 mM NaCl (c), or 100 mM NaCl (d) experimental plates. An asterisk (*) denotes statistical significance (p-value $\leq$ 0.05; Student's t-test). All measurements were conducted with 20 biological replicates. Data are presented are mean ± standard deviation**.**

2.3.2 Quantification of protein abundance changes between time-points and genotypes

Based on my preliminary phenotyping screen (**Figure 2 - 5**), I then decided to look at the changing proteins ($Log_2FC > 0.58$; q-value ≤ 0.05) of WT and *rve 4 6 8* plants under CTL, osmotic (50 mM mannitol), and salt stress (100 mM NaCl) at ZT11 and ZT23 to further examine the role of RVE8-like proteins under these drought-like conditions (**Figure 1**). I quantified a total of 6630 proteins, with 1029, 1303, 1101, and 885 significantly changing proteins ($Log_2FC > 0.58$; q-value ≤ 0.05) at ZT11 and ZT23 and between time-points, respectively (**Figure 1**). At ZT11 in WT plants, a total of 142, 177, and 167 proteins were detected to be significantly changing ($Log_2FC > 0.58$; q-value ≤ 0.05) under CTL, mannitol and NaCl conditions, respectively (**Figure 1**), while a total of 90, 210, and 243 proteins were observed to be significantly changing ($Log_2FC > 0.58$; q-value ≤ 0.05) in *rve 4 6 8* under the same respective conditions (**Figure 1**). At ZT23 in WT plants, a total of 604, 178, and 107 proteins were observed to be significantly changing ($Log_2FC > 0.58$; q-value ≤ 0.05) under CTL, mannitol and NaCl conditions, respectively (**Figure 1**), while a total of 131, 172, and 111 proteins were observed to be significantly changing ($Log_2FC > 0.58$; q-value ≤ 0.05) in *rve 4 6 8* under the analogous respective conditions (**Figure 1**).

In WT plants at ZT11, a total of 70, 139, and 67 proteins were observed to be significantly changing ($Log_2FC > 0.58$; q-value ≤ 0.05) under CTL, mannitol, and NaCl conditions, respectively, while 249, 161, and 186 proteins were observed to be significantly changing ($Log_2FC > 0.58$; q-value ≤ 0.05) in *rve 4 6 8* seedlings at ZT11 under the same respective conditions (**Figure 1**). At ZT23 in WT organisms, a total of 169, 238, and 202 proteins were observed to be significantly changing ($Log_2FC > 0.58$; q-value ≤ 0.05) under CTL, mannitol and NaCl conditions, respectively, however, 104, 104, and 214 proteins were observed to be significantly changing ($Log_2FC > 0.58$; q-value ≤ 0.05) in *rve 4 6 8* plants at ZT23 under identical respective conditions (**Figure 1**). The total proteome data reveals differences in protein abundance across CTL, mannitol, and NaCl conditions between time-points and genotypes (**Figure 1**). Differences between time-points within a genotype relate to potential phase-related differences, while differences between WT and *rve 4 6 8* at a time-point illustrate genotypic differences at that specific time-point (**Figure 1**).

2.3.3 Total proteome between WT and *rve 4 6 8* under CTL conditions further alludes to connection between RVE8-like proteins and drought-like responses

Before exploring the changing proteome between WT and *rve 4 6 8* under mannitol and salt stress, I wanted to examine the cellular processes changing under CTL conditions at ZT11 and ZT23 to see if I could detect similar results to Scandola et al., (2022), with respect to drought-like proteins. My experimental setup differs from Scandola et al., (2022) in several key aspects (**Figure 1**). I grew my seedlings in nutrient-supplemented (0.5x MS) media under 100 µmol/m^2/s of florescent light at 22ºC, while Scandola et al., (2022) grew their plants in the soil under precision narrow-band LED light at a temperature regiment ranging between 21ºC during the day and 19ºC at night. However, given these key experimental differences between my experiments and Scandola et al., (2022), I wanted to see whether I could detect similar protein differences between WT and *rve 4 6 8* in my proteomics experiments. Scandola et al., (2022) and I both looked at the changing proteome ($Log_2FC > 0.58$; q-value ≤ 0.05) between WT and *rve 4 6 8* genotypes at ZT11 and ZT23, thus, it would be interesting to see whether I could replicate key observations identified by Scandola et al., (2022) in my series of experiments.

As a result, I performed a GO enrichment (**Figure 6 - 9**; **Figure 11**) and KEGG identifier (**Figure 10**) analysis for GO terms pertaining to biological processes and KEGG pathways. I found a series of drought-related STRING-DB cluster proteins **(Figure 6 - 10)** that have previously been shown to be enriched by Scandola et al., (2022). These include (**Figure 6 - 8**; **Figure 11**): jasmonic acid biosynthetic process (GO:0009695), response to abscisic acid (GO:0009737), response to temperature stimulus (GO:0009266), and response to oxidative stress (GO:0006979) (**Figure 6 - 9 Figure 11**), which were similarly higher in WT plants versus *rve 4 6 8*. I also found several drought-like STRING-DB clusters in my experiment that were absent in Scandola et al., (2022), such as: response to salt stress (GO:0009651), response to mannitol (GO:0010555), sulfur compound metabolic process (GO:0006790), and reactive oxygen species metabolic process (GO:0072593) (**Figure 6 - 9**; **Figure 11**). In my experiments, there are more drought-related proteins in WT than in *rve 4 6 8*, which further alludes to RVE8-like proteins being involved in drought-stress responses in Arabidopsis.

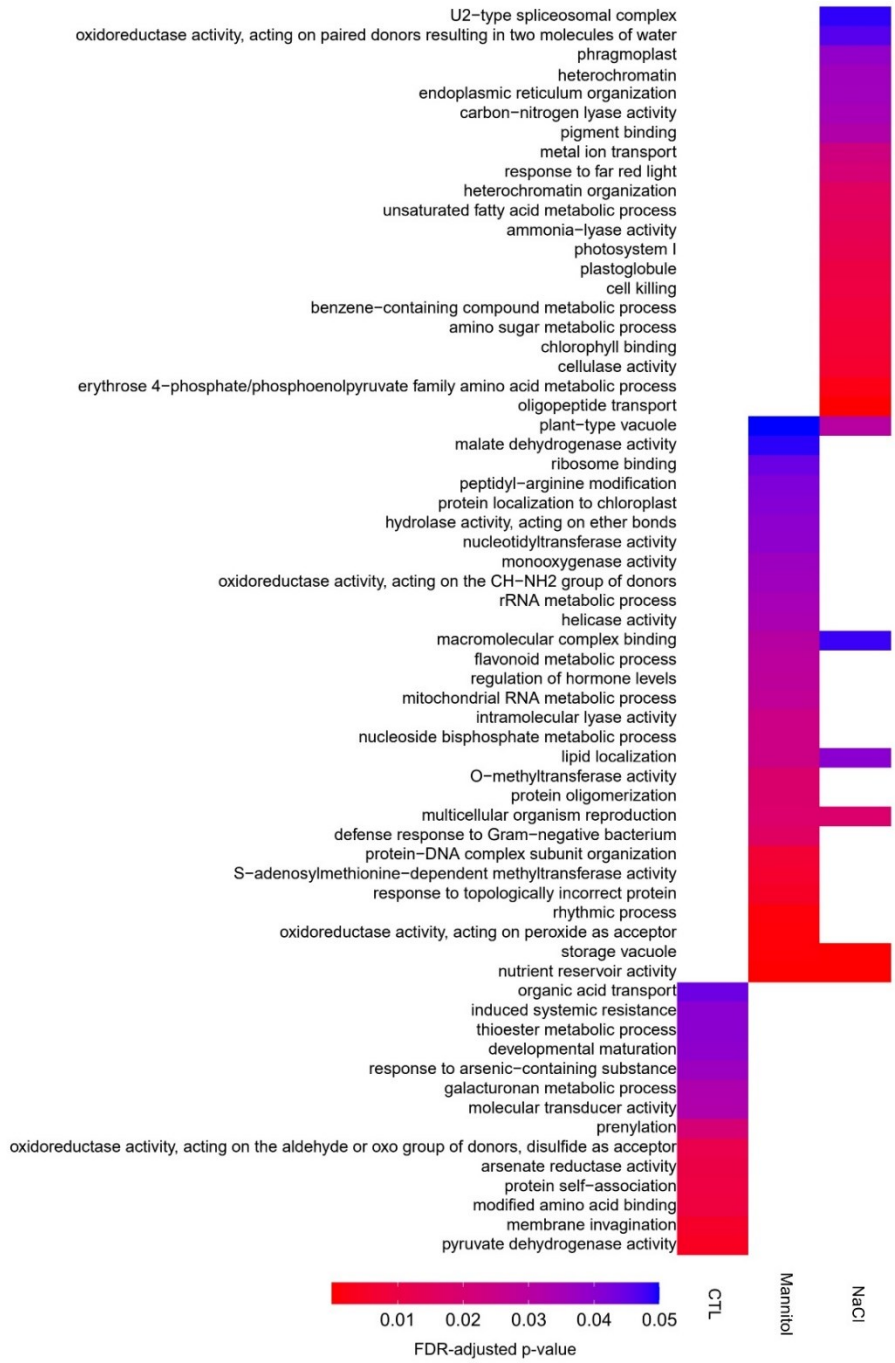

Figure 6: Gene ontology (GO) enrichment analysis of the identified proteome between *rve 4 6 8* and WT at ZT11. A heat map of GO biological processes with the false discovery rate (FDR)-adjusted p-value data (FDR) ≤ 0.05 and ≥ 5 and ≤ 80 proteins. Red-to-blue coloration represents decreasing FDR p-values from 0.05 towards 0. The resulting heat map was generated using the r package superheat (https://rlbarter.github.io/superheat/) from the significant GO terms pertaining to biological processes, which were initially obtained from the ONTOLOGIZER (http://ontologizer.de; Bauer et al., 2008).

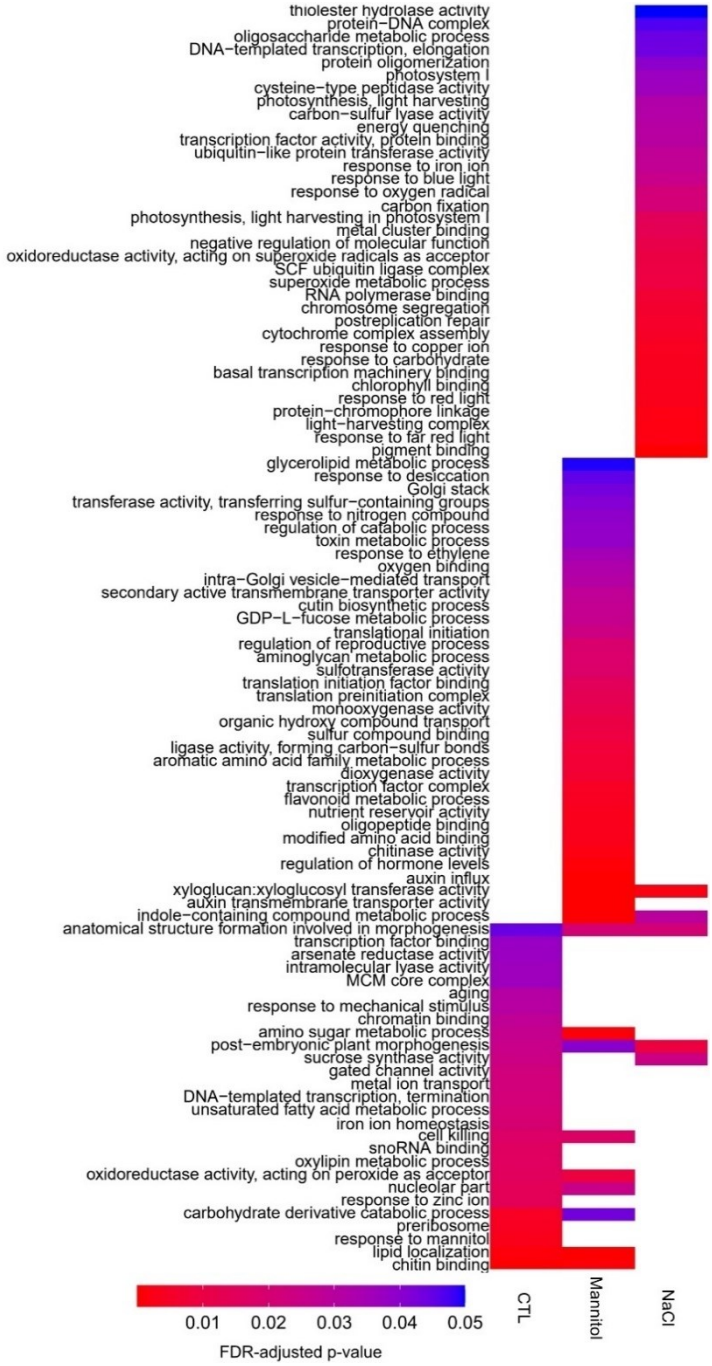

Figure 7: Gene ontology (GO) enrichment analysis of the identified proteome between *rve 4 6 8* and WT at ZT23. A heat map of GO biological processes with the false discovery rate (FDR)-adjusted p-value data (FDR) ≤ 0.05 and ≥ 5 and ≤ 80 proteins. Red-to-blue coloration represents decreasing FDR p-values from 0.05 towards 0. The resulting heat map was generated using the r package superheat (https://rlbarter.github.io/superheat/) from the significant GO terms pertaining to biological processes, which were initially obtained from the ONTOLOGIZER (http://ontologizer.de; Bauer et al., 2008).

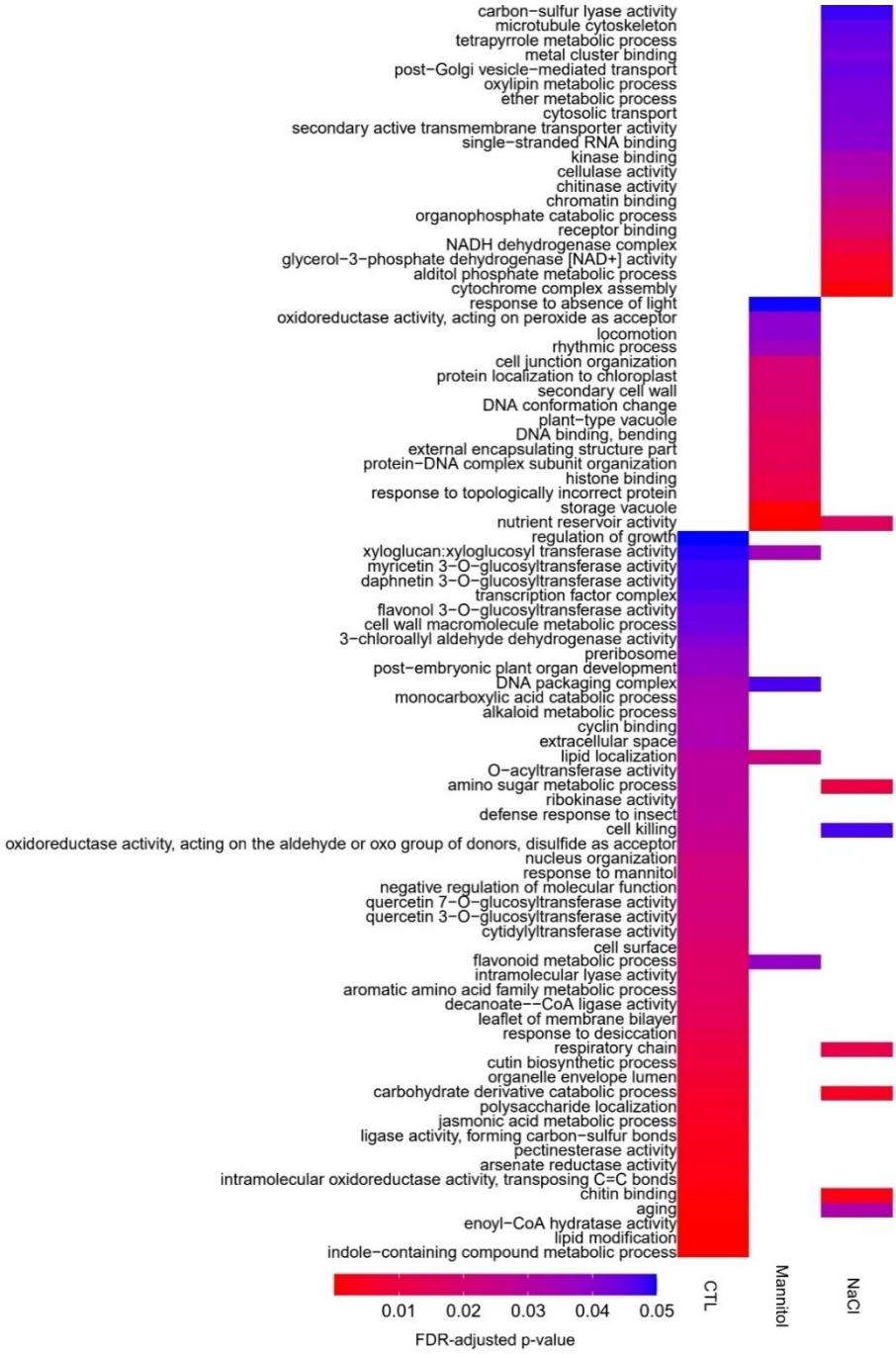

Figure 8: Gene ontology (GO) enrichment analysis of the identified proteome between ZT11 and ZT23, within WT plants. A heat map of GO biological processes with the false discovery rate (FDR)-adjusted p-value data (FDR) ≤ 0.05 and ≥ 5 and ≤ 80 proteins. Red-to-blue coloration represents decreasing FDR p-values from 0.05 towards 0. The resulting heat map was generated using the r package superheat (https://rlbarter.github.io/superheat/) from the significant GO terms pertaining to biological processes, which were initially obtained from the ONTOLOGIZER (http://ontologizer.de; Bauer et al., 2008).

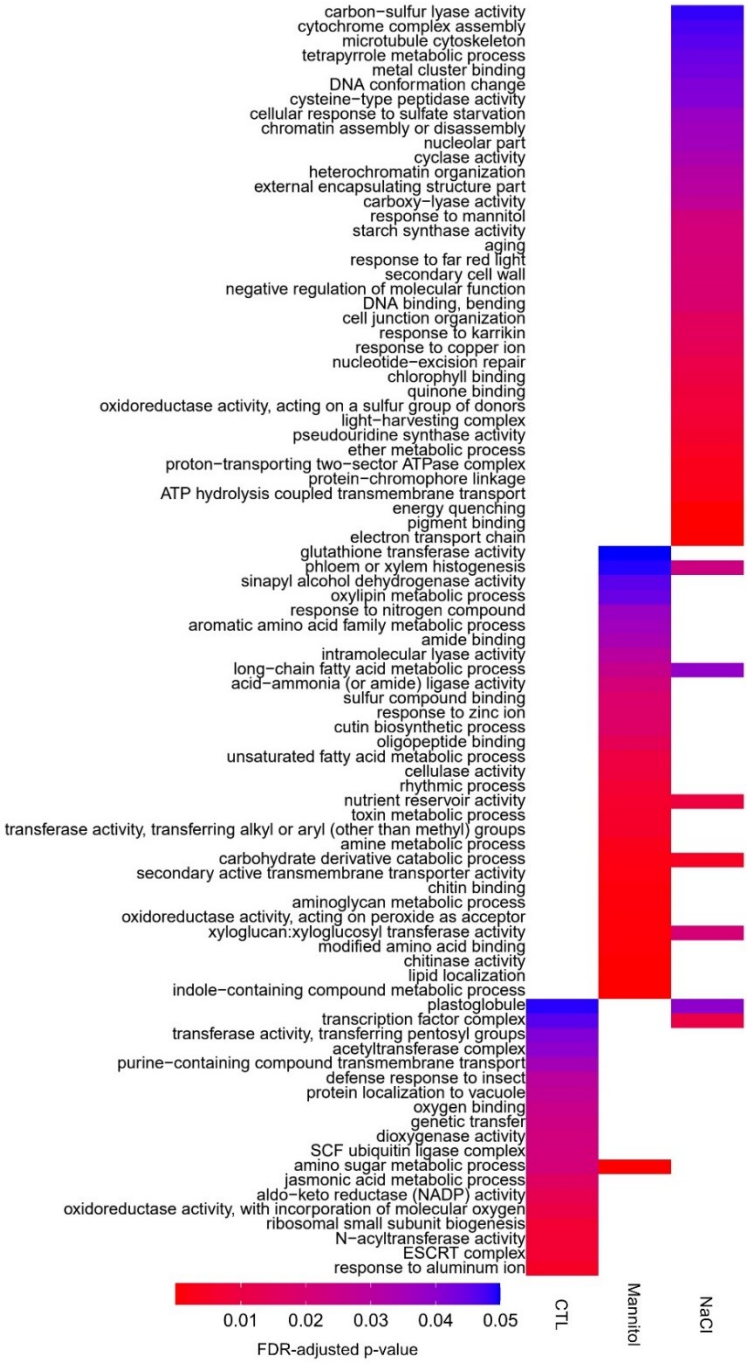

Figure 9: Gene ontology (GO) enrichment analysis of the identified proteome between ZT11 and ZT23, within *rve 4 6 8* plants. A heat map of GO biological processes with the false discovery rate (FDR)-adjusted p-value data (FDR) ≤ 0.05 and ≥ 5 and ≤ 80 proteins. Red-to-blue coloration represents decreasing FDR p-values from 0.05 towards 0. The resulting heat map was generated using the r package superheat (https://rlbarter.github.io/superheat/) from the significant GO terms pertaining to biological processes, which were initially obtained from the ONTOLOGIZER (http://ontologizer.de; Bauer et al., 2008).

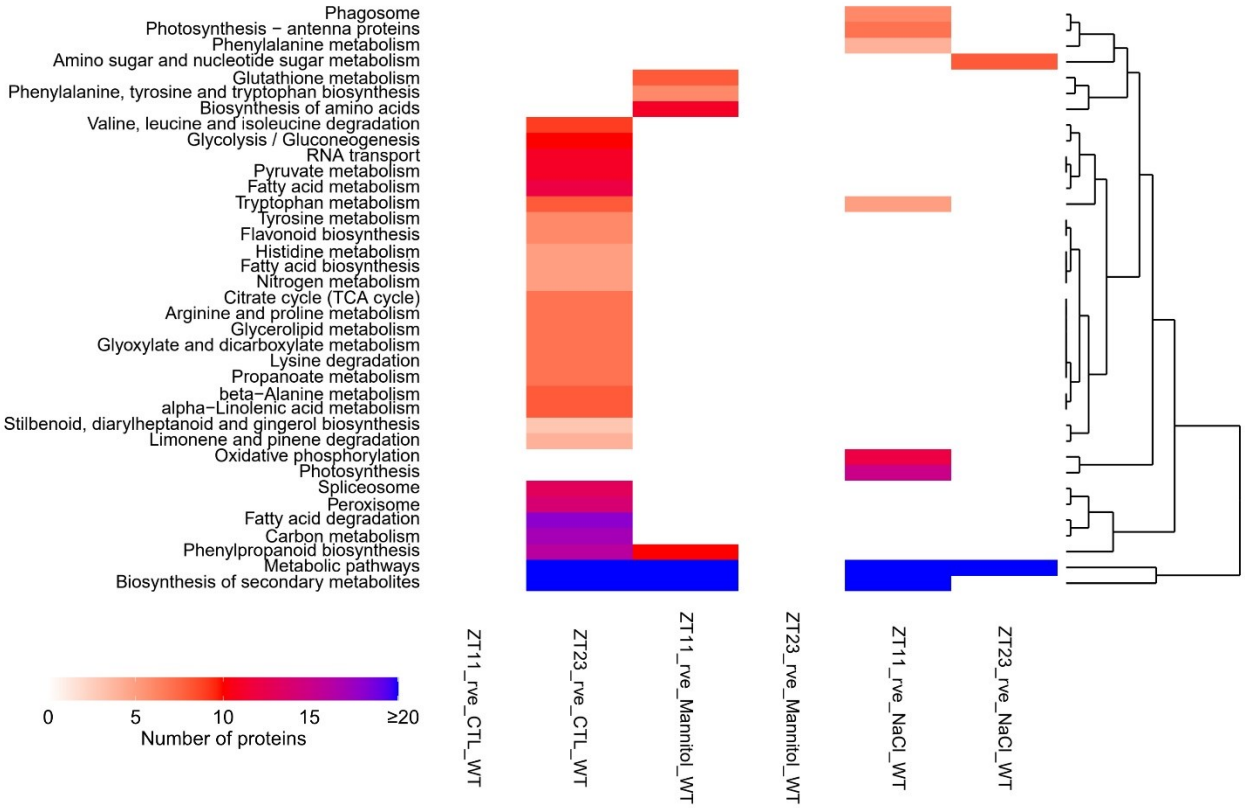

Figure 10: KEGG pathway enrichment analysis of proteomes at ZT11 and ZT23 under control (CTL), mannitol, and NaCl conditions. A heat map summarizing the number of differentially abundant proteins identified per pathway. Red-to-blue coloration represents an increasing number of proteins from 0 to ≥ 20. The resulting heat map was generated using the r package superheat (https://rlbarter.github.io/superheat/).

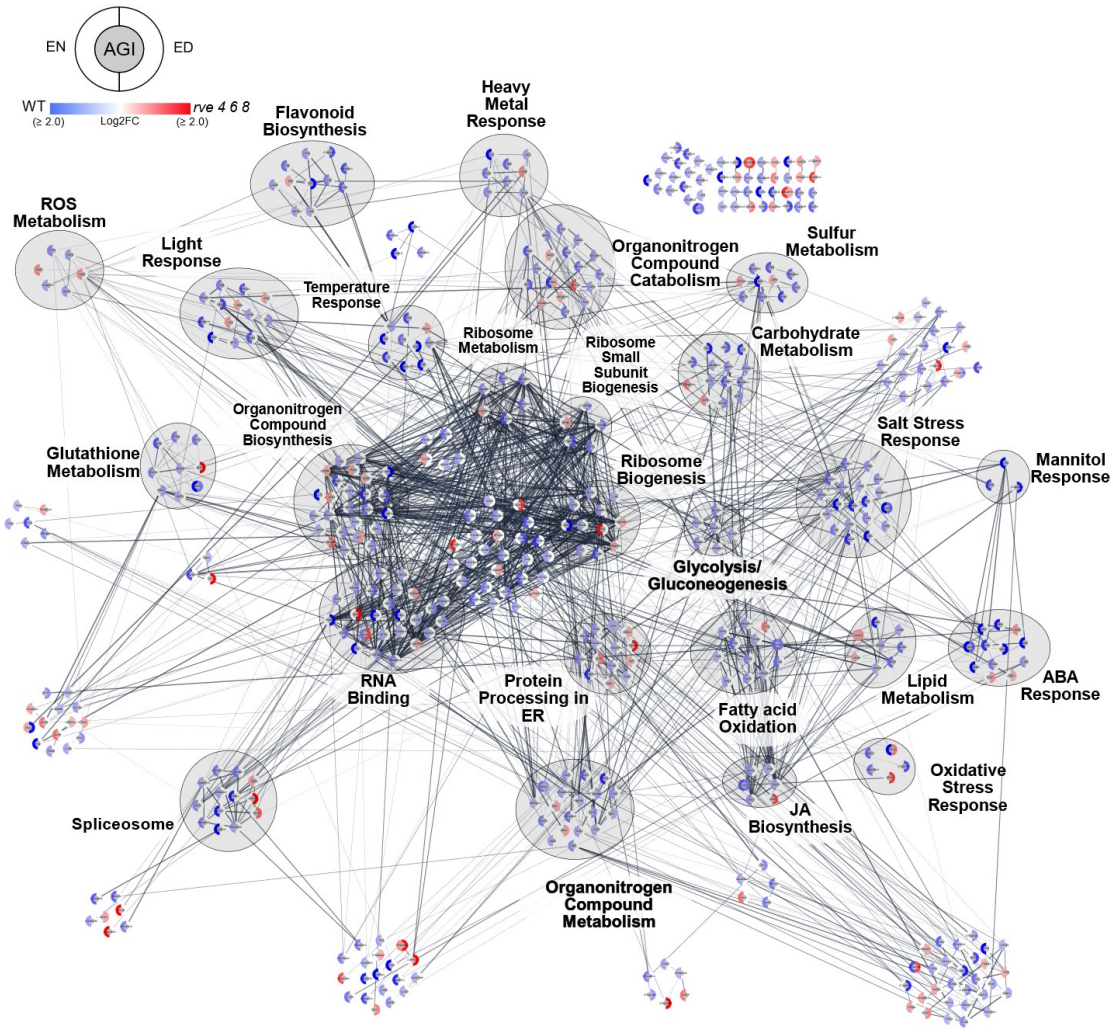

Figure 11: Association network of significant genotypic (WT vs *rve 4 6 8*) total proteome fluctuations between ED (ZT11) and EN (ZT23) under control conditions (CTL). STRING database (STRING-DB) association network illustrates significant Log2FC fluctuations in protein abundance between WT and *rve 4 6 8* at ZT11 versus ZT23 (q-value ≤ 0.05). Networks were generated using Cytoscape (version 3.9.1) and the STRING-DB and enhancedGraphics plugin applications. Only nodes with edges ≥ 0.7 were included. Increasing blue (WT) or red (*rve 4 6 8*) coloration intensity indicates the relative increase in measured Log2FC protein abundance for that corresponding genotype. Highlighted node clusters were manually curated using the STRING-DB provided GO annotations for biological processes.

2.3.4 Proteome changes between WT and *rve 4 6 8* under osmotic stress

After analyzing the genotypic differences in total proteome between WT and *rve 4 6 8* under CTL (**Figure 6 - 11**), I went on to look at the changing proteome ($Log_2FC > 0.58$; q-value ≤ 0.05) under mannitol (**Figure 6 - 10; 12**) and salt stress (**Figure 6 - 10; 13**) to elucidate what plant cell processes were aiding WT plants to do better than *rve 4 6 8* seedlings under 50mM mannitol and 100mM NaCl (**Figure 1 - 5**). Upon compiling a drought-related STRING-DB association network (**Figure 6 - 9; 12**), I found protein clusters related to reactive oxygen species metabolic process (GO:0072593), sulfur compound metabolic process (GO:0006790), response to oxidative stress (GO:0006979), and response to temperature stimulus (GO:0009266) to all be enriched. However, more proteins appear to be differentially abundant in *rve 4 6 8* under mannitol **(Figure 12)**, relative to *rve 4 6 8* under CTL **(Figure 11)**, suggesting that plants which lack RVE8-like proteins increase the abundance of drought-like proteins under osmotic stress (**Figure 12**). Under osmotic stress, I also detect that proteins which are part of glutathione metabolism (ath00480) remain differentially abundant in WT plants (**Figure 10; 12**).

2.3.5 Proteome changes between WT and *rve 4 6 8* under NaCl stress

After analyzing the drought-related STRING-DB clusters under NaCl between WT and *rve 4 6 8*, I was able to uncover drought-related STRING-DB cluster proteins (**Figure 6 - 9; 13**), such as: jasmonic acid biosynthetic process (GO:0009695), response to abscisic acid (GO:0009737), and response to oxidative stress (GO:0006979). Jasmonic acid biosynthesis proteins remain differentially abundant in WT plants, relative to *rve 4 6 8* under salt stress, suggesting that plants that lack RVE8-like proteins have an inability to regulate jasmonic acid-related processes (**Figure 6 - 9; 13**). Response to abscisic acid proteins also remain differentially abundant in WT plants, relative to *rve 4 6 8* under salt stress, illustrating that plants that lack RVE8-like proteins have a hampered ability to regulate drought-like stress responses via ABA signaling. Oxidative stress response proteins are less abundant in WT, relative to *rve 4 6 8*, suggesting that plants that lack RVE8-like proteins have an exaggerated response to oxidative stress. I also detect that proteins which are part of glutathione metabolism become differentially abundant in *rve 4 6 8* plants, suggesting that glutathione metabolism protein pools are largely altered in plants which lack RVE8-like proteins under NaCl stress (**Figure 13**).

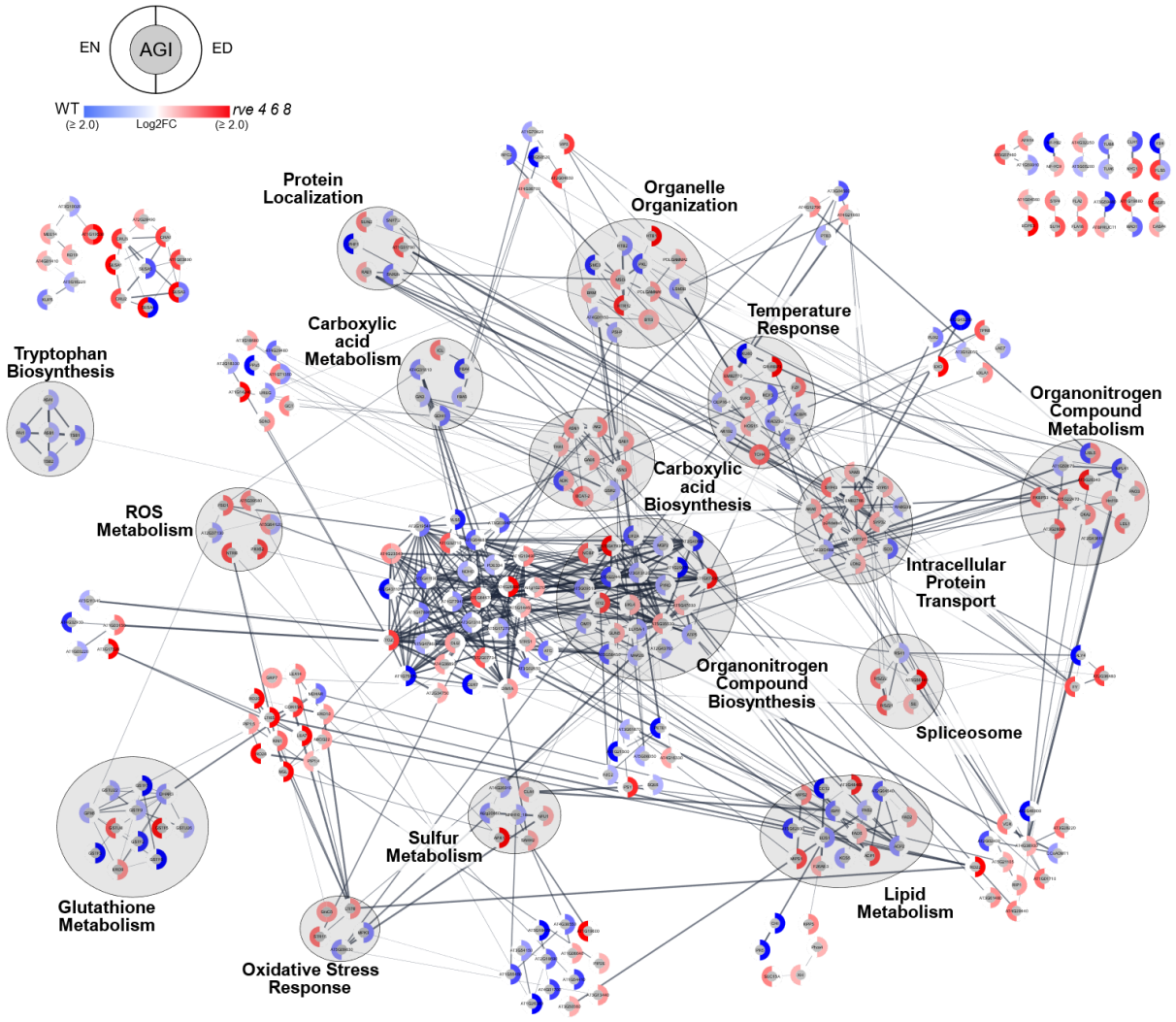

Figure 12: Association network analysis of significant genotypic (WT vs *rve 4 6 8*) total proteome fluctuations between ED (ZT11) and EN (ZT23) under osmotic stress (mannitol). STRING database (STRING-DB) association network illustrates significant Log2FC fluctuations in protein abundance between WT and *rve 4 6 8* at ZT11 versus ZT23 (q-value ≤ 0.05). Networks were generated using Cytoscape (version 3.9.1) and the STRING-DB and enhancedGraphics plugin applications. Only nodes with edges ≥ 0.7 were included. Increasing blue (WT) or red (*rve 4 6 8*) coloration intensity indicates the relative increase in measured Log2FC protein abundance for that corresponding genotype. Highlighted node clusters were manually curated using the STRING-DB provided GO annotations for biological processes.

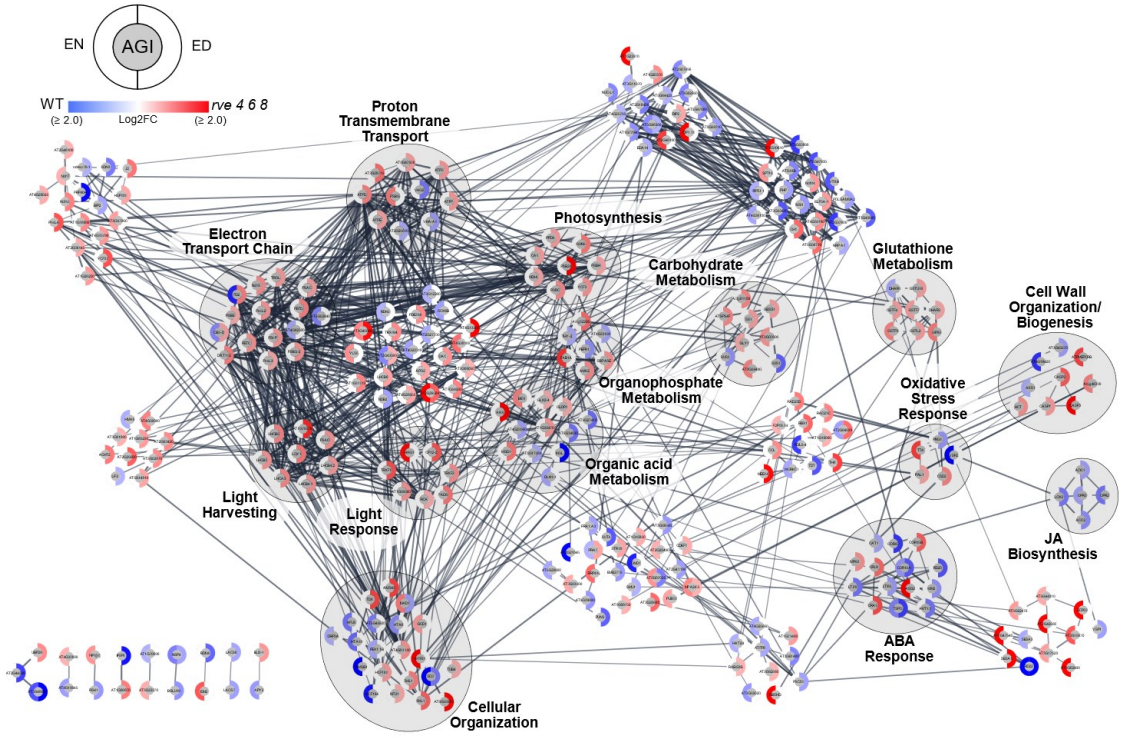

Figure 13: Association network analysis of significant genotypic (WT vs *rve 4 6 8*) total proteome fluctuations between ED (ZT11) and EN (ZT23) under salt stress (NaCl). STRING database (STRING-DB) association network illustrates significant Log2FC fluctuations in protein abundance between WT and *rve 4 6 8* at ZT11 versus ZT23 (q-value ≤ 0.05). Networks were generated using Cytoscape (version 3.9.1) and the STRING-DB and enhancedGraphics plugin applications. Only nodes with edges ≥ 0.7 were included. Increasing blue (WT) or red (*rve 4 6 8*) coloration intensity indicates the relative increase in measured Log2FC protein abundance for that corresponding genotype. Highlighted node clusters were manually curated using the STRING-DB provided GO annotations for biological processes.

2.4 DISCUSSION

2.4.1 Seedling and rosette organ proteomes confer different diel protein-level responses

Scandola et al., (2022) and I both analyzed the changing proteome ($Log_2FC > 0.58$; q-value ≤ 0.05) between WT and *rve 4 6 8* at ZT11 and ZT23 (**Figure 1**), but at different stages of plant development. As mentioned, there were key experimental differences between the experimental setup of Scandola et al., (2022) and me. In particular, I grew my seedlings in nutrient-supplemented (0.5x MS) media under 100 µmol/m^2/s of florescent light at 22ºC, while Scandola et al., (2022) grew their plants in the soil under precision narrow band LED light, using a temperature range between 21ºC to 19ºC. Given these marked differences in experimental design, it seems likely that we should detect similar (identical plant genotypes), but may see different (diverging experimental setups and developmental stages sampled) total proteome data (Scandola et al., 2022; **Figure 1**). In my experiment approach, I detected several drought-related protein groups (STRING-DB clusters) that were previously reported by Scandola et al., (2022), such as: jasmonic acid biosynthetic process (GO:0009695) and response to temperature stimulus (GO:0009266) (**Figure 6 - 8**; **11**) in addition to others. Consistent with Scandola et al 2022, the proteins highlighted within the jasmonic acid biosynthetic process cluster are more abundant in WT in both of our diel proteomes (**Figure 6 - 9**; **11;** Scandola et al., 2022).

In my study, I also found key differences between my total proteome data and the data of Scandola et al., (2022). I was able to uncover enriched carbohydrate metabolic process (GO:0005975) proteins in my experiments (**Figure 6 – 9**; **11**), which were also highlighted by Scandola et al., (2022), however, I (**Figure 6 - 9**; **11**) could not replicate the data which illustrates that RVE8-like proteins are regulators of proteasome activity (Scandola et al., 2022). Given these observations, it seems, that although we have broad consistency with respect to drought-like protein abundances, it appears that my whole seedling analysis (**Figure 6 - 9**; **11**) and the rosette analysis of Scandola et al., (2022) have total WT and *rve 4 6 8* proteomes that differ from one another, suggesting that the clock has organ-specific responses in Arabidopsis. Distinct shoot and root circadian clocks have been elucidated (Bordage et al., 2016), where the root systems are entrained by low light intensities. CCA1 periods are longer with a smaller amplitude in the guard cell compared to mesophyll cells in leaves (Yakir et al., 2011). The comparison also suggests that the drought-response pertaining to RVE8-like proteins is likely not organ-specific, but rather genotypic, as we could detect similar responses with respect to the abundance of osmoregulatory

proteins at the whole seedling (**Figure 6 - 9**; **11**) and rosette (Scandola et al., 2022) organ-level between WT and *rve 4 6 8*.

2.4.2 WT plants perform better than *rve 4 6 8* plants under osmotic and NaCl stress

Upon dissecting the total proteome between WT and *rve 4 6 8* whole seedlings at ZT11 and ZT23, it appears that plants which lack RVE8-like proteins have a hampered response to drought-like stress (**Figure 6 - 9**; **11**). I found a series of previously identified (Scandola et al., 2022) and novel (**Figure 6 - 9**; **11**) drought-related STRING-DB clusters between WT and *rve 4 6 8*. These include (**Figure 6 - 9**; **11**): jasmonic acid biosynthetic process (GO:0009695), response to abscisic acid (GO:0009737), response to temperature stimulus (GO:0009266), response to oxidative stress (GO:0006979), response to salt stress (GO:0009651), response to mannitol (GO:0010555), sulfur compound metabolic process (GO:0006790), and reactive oxygen species metabolic process (GO:0072593) (**Figure 6 - 9**; **11**). I identified several Responses to Mannitol (GO:0010555) proteins to be differentially abundant in WT at ZT23 relative to *rve 4 6 8*, including: SUCROSE SYNTHASE 1 (SUS1; AT5G20830; $Log_2FC = 1.45$) and SUCROSE SYNTHASE 3 (SUS3; AT4G02280; $Log_2FC = 1.51$) (**Figure 6 - 9**; **11**). I also characterized several Response to Salt Stress (GO:0009651) proteins to be differentially abundant in WT at ZT23 relative to RVE8-lacking plants including: ARABIDOPSIS THALIANA SULFOTRANSFERASE 1 (SOT12; AT2G03760; $Log_2FC = 1.01$), ARABIDOPSIS THALIANA CALEOSIN 3 (RD20; AT2G33380; $Log_2FC = 1.76$), and DELTA1-PYRROLINE-5-CARBOXYLATE SYNTHASE 1 (P5CS1; AT2G39800; $Log_2FC = 1.72$) (**Figure 6 - 9**; **11**).

SUS1 and SUS3 are part of the sucrose synthase (SUS) family enzymes in Arabidopsis (Xu et al., 2019). SUS enzymes catalyze the reversible reaction of sucrose and uridine diphosphate into uridine diphosphate-glucose and fructose (Avigad & Milner, 1966). Under mannitol stress comparable to that applied here, *SUS1* and *SUS3* expression was shown to increase by 4X and 37X, respectively, suggesting that RVE8-like proteins could be mediating the osmotic response in Arabidopsis through SUS enzymes to modify metabolism and/or the osmotic environment of the cell (Baud et al., 2004), by increasing the abundance of sugar-containing compounds and thereby, decreasing the osmotic potential (Dejardin et al., 1999). SOT12 proteins sulphonate (where a sulfonate group is added onto a target) salicylic acid in plants in response to stressful conditions, where plants which over-express *SOT12* seem to fare better under stressful conditions and *sot12*

plants are more sensitive to plant stressors (Baek et al., 2010). *SOT12* expression is induced in response to the application of exogenous methyl jasmonate, which suggests that SOT12 activity could be JA-mediated (Lacomme & Roby, 1996). *SOT12* expression is induced in response to different abiotic stressors (200mM sorbitol for 12h, 100mM NaCl for 12h, and 100µM ABA for 3h) (Baek et al., 2010). Further, WT plants have a higher germination rate over *SOT12*-lacking (*sot12*) plants under 8 days of 100mM NaCl or 0.5µM ABA, illustrating that plants with lower SOT12 abundance fare worse under drought-like stress (Baek et al., 2010).

RD20 proteins belong to the caleosin family of proteins which are used by plants to store energy in the form of oil bodies (Partridge & Murphy, 2009). *RD20* expression is induced by ABA during drought-like conditions (Gordon et al., 2008). Under 150 mM NaCl conditions, *rd20* plants have more leaf senescence relative to WT, illustrating that plants deficient in RD20 are more vulnerable to salt stress via ABA-mediated responses (Aubert et al., 2010). Lastly, P5CS1 has been characterized as a key protein in the biosynthesis of proline, a critical metabolite for mitigating NaCl stress. Proline works by counteracting the ionic potential that is experienced by plant cells under NaCl stress, by preferentially increasing K^+ influx and decreasing NaCl intake (de Freitas et al., 2019). Proline also stimulates the biosynthesis of antioxidant enzymes to confer osmotolerance under high salt conditions (Nounjan et al., 2012). Plants with compromised *P5CS1* expression (*p5cs1*) have a 15-30% lower amount of proline within rosette leaves under CTL conditions, as P5CS1 enzymes catalyze the committed (rate-limiting) step of proline biosynthesis (Szekely et al., 2008). Compared to WT plants, *p5cs1* plants exhibited reductions in primary root length under salt stress (150mM and 200mM NaCl) due to a difference in proline profile (Szekely et al., 2008). These examples support my quantitative primary root phenotyping data which found that plants deficient in RVE8-like proteins fare worse when subjected to drought-like stress conditions (**Figure 2 - 5**). Under osmoregulatory stress, WT plants have longer primary roots relative to *rve 4 6 8* plants (**Figure 2 - 5**). Osmotic (Cajero-Sanchez et al., 2019) and salt (West et al., 2004) stress causes plants to have shorter primary roots due to smaller root apical meristem (RAM) cell numbers and shorter cell lengths in the elongation zone (Kiani et al., 2007). Under drought-like conditions, plants preferentially synthesize ABA to reduce growth promotion and increase stomatal closure to mitigate water loss through transpiration (Figueiredo et al., 2008; Ooi et al., 2017). JA also seems to also intervene, as it is characterized to partially regulate the primary root responses by decreasing the number and/or size of cells in the elongation zone (Valenzuela et al.,

2016). It appears that plants which lack RVE8-like proteins fare worse due to a distinct lack of osmoprotectant proteins and protein products (**Figure 6 - 8**; **11**), causing a discernable reduction in plant root growth in response to drought-like stress (**Figure 2 - 5**).

2.4.3 Osmolytes likely confer added tolerance to WT under mannitol relative to RVE8-lacking plants

Under mannitol stress, I detect several tryptophan biosynthetic process (GO:0000162) proteins to be differentially abundant in WT at ZT11, including: PHOSPHORIBOSYLANTHRANILATE ISOMERASE 1 (PAI1; AT1G07780; $Log_2FC = 0.98$), ANTHRANILATE SYNTHASE BETA SUBUNIT 1 (ASB1; AT1G25220; $Log_2FC = 0.60$) A-METHYL TRYPTOPHAN RESISTANT 1 (ASA1; AT5G05730; $Log_2FC = 0.62$) TRYPTOPHAN SYNTHASE BETA-SUBUNIT 1 (TSB1; AT5G54810; $Log_2FC = 0.99$) TRYPTOPHAN SYNTHASE BETA-SUBUNIT 2 (TSB2; AT4G27070; $Log_2FC = 0.84$), suggesting that WT seedlings likely have longer primary root under mannitol, relative to *rve 4 6 8* due to elevated tryptophan biosynthesis-mediated osmotolerance (**Figure 2 – 5; 12; 14**). Melatonin is an osmoprotecting osmolyte, which is synthesized in plants from tryptophan amino acids (Chen et al., 2009; Mannino et al., 2021). White lupine plants treated with exogenous tryptophan (100, 200, or 300 μM) or melatonin (50, 100, or 150 μM) have higher shoot lengths, number of leaves, and fresh weight under drought conditions (Sadak & Ramadan, 2021). SEROTONIN N-ACETYLTRANSFERASE 1 (SNAT1; AT1G32070) is an enzyme that catalyzes one of the rate-limiting steps in melatonin biosynthesis *in vivo* (Lee & Back, 2018). Under increasing titrations of mannitol, progressively higher *SNAT1* relative expression is documented (Wang et al., 2021). WT plants fare better than plants deficient in proper *SNAT1* (*snat1*) expression under osmotic stress (300mM mannitol over 5days), due to reduced endogenous melatonin pools under mannitol stress (Wang et al., 2021). Together, it appears that plants which lack RVE8-like proteins fare worse under mannitol stress due to a distinct lack of tryptophan biosynthesis, partially causing *rve 4 6 8* plants to have shorter roots than WT under osmotic stress (**Figure 2 – 5; 12; 14**).

2.4.4 Differing jasmonic acid biosynthesis activity possibly explains root response differences between RVE8-lacking plants under osmotic and salt stress

Under salt stress, JA has been shown to partially regulate the primary root response by decreasing the number or size of cells in the elongation zone, restricting root elongation (Valenzuela et al., 2016). Prior transcriptomics studies have shown that *AOC1* and *AOC2* expression is induced in response to salt stress (Jiang & Deyholos, 2006). In wheat, the overexpression of *AOC1* (*TaAOC1*) showed enhanced tolerance to salt stress (200 mM for 8 days) (Zhao et al., 2013). AOC1 and AOC2 JA biosynthetic enzymes have been previously shown to be more abundant in WT relative to *rve 4 6 8* in plant rosette tissue (Scandola et al., 2022). I identified several jasmonic acid (JA) biosynthetic proteins to be differentially abundant in WT at ZT23, including: AOC1 ($Log_2FC = 1.09$) and AOC2 ($Log_2FC = 0.83$) (**Figure 11)**. Under salt stress, I also detect a differential abundance of AOC1 ($Log_2FC = 0.68$) and AOC2 ($Log_2FC = 0.82$) proteins in WT at ZT11. Given this, it seems plausible that the root truncation between WT and *rve 4 6 8* under salt is less significant than under mannitol, due to a distinct lack of JA biosynthesis in RVE8-lacking plants under NaCl stress.

2.4.5 Glutathione metabolism partially explains why RVE8-lacking plants fare worse under osmotic stress

Glutathione is a sulfur-containing plant metabolite comprised of glutamine, cysteine, and glycine (Lim et al., 2007). Plants completely devoid of glutathione do not survive past the embryo stage in the life cycle, illustrating how essential glutathione production is to the lives of plants (Cairns et al., 2006). Glutathione reductase (GR) enzymes produce glutathione by reducing glutathione disulfide (Wu et al., 2015). WT plants have a smaller reduction in root length under salt stress (100 mM NaCl for 7 days) relative to GR3-deficient (*gr3*) rice plants (Wu et al., 2015), illustrating that reduced glutathione production can increase plant susceptibility to drought-like stress. Upon comparing the total proteome between WT and *rve 4 6 8* whole seedlings at ZT11 and ZT23, under CTL, mannitol, and NaCl stress, it appears that glutathione metabolism (ath00480) proteins remain enriched (**Figure 6 - 13**), suggesting that glutathione metabolizing proteins may partially explain the differing phenotypes between WT and RVE8-lacking proteins under osmotic and salt stress.

Under CTL conditions, I detect several glutathione transferase (GST) proteins to be differentially abundant in WT at ZT23, including: GLUTATHIONE S-TRANSFERASE PHI (GSTF) 5 (AT1G02940; $Log_2FC = 2.04$), DEHYDROASCORBATE REDUCTASE (DHAR) 1

(AT1G19570; Log_2FC = 0.87), and GLUTATHIONE S-TRANSFERASE TAU (GSTU) 1 (AT2G29490; Log_2FC = 0.98), suggesting that WT plants likely have an elevated GST-mediated osmotolerant response under CTL due to higher GST protein levels (**Figure 11**). GST enzymes participate in detoxification reactions by adding glutathione to various plant toxins such as xenobiotics, tagging the toxic substances for vacuolar isolation (Mauch & Dudler, 1993). Arabidopsis possesses 54 GST proteins that are part of 7 classes, including: phi (GSTF), dehydroascorbate reductase (DHAR), and tau (GSTU) classes (Dixon et al., 2002). GST enzymes are ubiquitous with plant osmotolerance, such that a high degree of redundancy has been elucidated within the supergene family due to a large degree of functional (Gullner et al., 2018) and genetic (Sappl et al., 2009) overlap. In response to 100mM NaCl over 7 days, *GSTF4*, *DHAR2*, *GSTU6*, *GSTU23*, and *GSTU26* expression was induced in tomato crops (Csiszár et al., 2014). Further, the overexpression of rice *GSTU4* (*OsGSTU4*) in *Escherichia coli* has elevated growth % (relative to untransformed colonies) under 300mM NaCl and 300mM mannitol, illustrating that overexpressing *GSTs* can confer osmoprotective properties (Sharma et al., 2014).

In Arabidopsis, GST levels have been shown to increase in response to abiotic stress (Sappl et al., 2004). Under 10 mM of H_2O_2 (a proxy for oxidative stress) for 3 hours, transcript levels of several Arabidopsis *GSTF* and *GSTU* genes are induced, as well as *DHAR2* (AT1G75270), relative to CTL (Sappl et al., 2009). Under mannitol conditions, I detect several GST proteins to be differentially abundant in WT including: GSTF2 (AT4G02520; Log_2FC = 1.75), GSTF3 (AT2G02930; Log_2FC = 1.94), GSTF7 (AT1G02920; Log_2FC = 3.49), GSTF6 (AT1G02930; Log_2FC = 3.47), GSTF9 (AT2G30860; Log_2FC = 0.62), GSTU26 (AT1G17190; Log_2FC = 0.62), and GSTU22 (AT1G78340; Log_2FC = 0.72), suggesting that WT seedlings likely have a less significant shortening of the primary root under mannitol due to elevated GST-mediated osmotolerance relative to their responses under NaCl (**Figure 2 – 5; Figure 12**). However, under NaCl, many GST proteins are differentially abundant in *rve 4 6 8*, including: GSTF4 (AT1G02950; Log_2FC = 0.68), GSTF6 (Log_2FC = 0.89), GSTF7 (Log_2FC = 0.68), DHAR3 (Log_2FC = 0.61), and GSTU18 (AT1G10360; Log_2FC = 0.69) (**Figure 13**). It appears that NaCl conditions cause a greater induction in GST-mediated responses, relative to mannitol, suggesting that *rve 4 6 8* plants fare worse under mannitol versus NaCl, due to a lack of GST protein abundance (**Figure 2 – 13**).

Summary

Together, the data presented within **chapter 2** suggests that RVE8-like proteins are involved in the osmotic and salt stress response (**Figure 1 - 14**). More specifically, it seems that plants with *rve 4 6 8* alleles fare worse under mannitol and salt stress, illustrating that proper *RVE8-like* expression (WT) is needed to confer osmotolerance (**Figure 2 - 5**). I have shown that WT plants have more osmoprotecting proteins, relative to *rve 4 6 8* which might partially explain why RVE8-lacking plants fare worse, because they do not have a sufficient pool of osmoprotectants (**Figure 11**). I also explain why *rve 4 6 8* plants do worse under mannitol stress as opposed to salt stress, as salt stress appears to increase the amount of osmoportection-conferring proteins (**Figure 12-13**). As *rve 4 6 8* plants fare worse under drought-like stress, proper RVE8-like protein levels are needed for crops to be resilient to drought-like conditions.

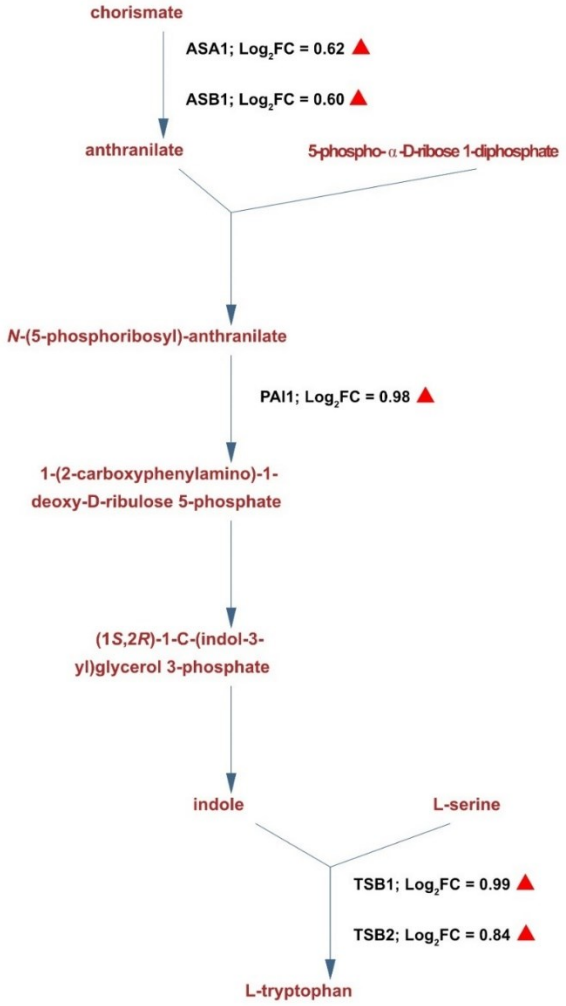

Figure 14: Tryptophan biosynthesis pathway. Tryptophan biosynthetic pathway highlighting the differentially abundant proteins in WT at ZT11 under mannitol stress, with the corresponding Log_2FC.

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Chapter 3:

Exploring the *Arabidopsis* circadian responses to nitrogen, phosphorus, and sulfur nutrient stress responses through phenomics and quantitative metabolomics

3.1 INTRODUCTION

Plants require a diel cycle consisting of light and dark to grow and develop properly (Mehta et al., 2021). To do this, plants rely on a functional circadian clock comprised of a series of transcription factors that function to interpret internal and external cues and control time of day outputs (Creux & Harmer, 2019; Hsu et al., 2013). In *Arabidopsis*, the core clock consists of morning, afternoon, midday, and evening expressed proteins that form a series of highly regulated negative feedback loops (Kamioka et al., 2016). The morning loop consists of LHY, CCA1, PRR7, and PRR9, while the evening loop consists of PRR5, TOC1, and the evening complex (EC) proteins (Creux & Harmer, 2019; Mehta et al., 2021). Approximately 40% of all genes in *Arabidopsis* have been suggested to be under direct circadian regulation (Romanowski et al., 2020), indicating how integral circadian control is to the daily lives of plants.

Plants deficient in these core circadian clock transcription factors have a number of observable phenotypes. Plants with *lhy cca1* alleles flower earlier, are smaller, maintain paler leaves, possess shorter stems, and have smaller rosette leaves (Mizoguchi et al., 2002). Plants with *prr5 prr7* alleles (Nakamichi et al., 2005a), as well as *prr7 prr9,* or *prr5 prr7 prr9* alleles all have longer petioles and hypocotyls under diel conditions (Nakamichi et al., 2005b). Triple knockout *prr5 prr7 prr9* plants have very tall and thick stems, coupled with abnormally dark green leaves (Nakamichi et al., 2005b). Plants deficient in TOC1 proteins flower earlier under SD and LD conditions (Somers et al., 1998) and have elongated hypocotyls, relative to WT (Strayer et al., 2000). Plants with *elf3* (Zagotta et al., 1996), *elf4* (Doyle et al., 2002), or *lux* (Hazen et al., 2005) alleles all flower earlier and have longer hypocotyls than WT plants.

Despite the extensive control of the circadian clock over plant cellular processes, the role of the *Arabidopsis* circadian circuit in managing diel nutrient-related cell processes has not been resolved (see **General Introduction**). Recently however, under phosphorus (P) stress, the expression of *CCA1*, *LHY*, and *PRR9* were all found to be induced, implicating morning loop transcription factors in the regulation of P-dependent homeostasis and P metabolism in *Arabidopsis* (Scheible et al., 2022). Further, previous examination of the diel *Arabidopsis* rosette proteome revealed Nitrate Metabolic Processes (GO:0042126) was enriched in the dark-to-light

transition (ZT23 – ZT1) in WT plants, suggesting that nitrogen (N) metabolism could be regulated in a diel manner (Uhrig et al., 2020). The studies above suggest that nutrient acquisition and metabolism could be under circadian control, while not directly addressing whether diel nutrient-related metabolic processes are regulated by the circadian circuitry.

In chapter 3, I attempt to assess whether the clock is involved in the regulation of N, P, and/or S nutrition by utilizing a variety of well-characterized circadian clock mutants exposed to different nutrient stressors (**Table 1**). To do this, I initially examined the nutrient-dependent photomorphogenic (primary root) and skotomorphogenic (hypocotyl etiolation) responses of *lhy cca1*, *prr7-3 prr9-1*, *prr5-11 prr7-11*, *prr5-11, prr5-1, prr7-11, toc1, elf3*, *elf4,* and *lux* seedlings when subjected to -N (lacking N), -P (lacking P), or -S (lacking S) conditions (**Table 1**). I hypothesize that if the clock is involved in the diel regulation of plant nutrition, that there should be a discernable difference in seedling phenotypes between nutrient fed and starved plants. I then decided to further examine the diel metabolome of *prr5-11 prr7-11*, *prr5-11,* and *prr7-11* plants under CTL, -N, -P, and -S conditions, as *prr5*-related mutants exhibited the most diverse nutrient-dependent phenotypes. After conducting a gas chromatography mass spectrometry (GC-MS) characterization of *prr5-11 prr7-11*, *prr5-11,* and *prr7-11* plants under nutrient stress, I go on to explain plausible reasons as to why PRR5-lacking mutants tend to fare worse under nutrient starvation through the differential pools of metabolites detected (**Table 2**). I specifically use a 12h light: 12h dark photoperiod for my study, and not free-running conditions (e.g., lights always on), as it is representative of what is observed in nature. My study attempts to address which of the circadian clock mutants could become clear targets for agricultural biotechnology, while also systematically elucidating which of the clock components are affected by nutrition-lacking conditions.

3.2 MATERIALS AND METHODS

3.2.1 Nutrient-dependent photomorphogenesis primary root phenomics

WT (wild-type, Columbia ecotype), *lhy cca1* (Blair et al., 2019), *prr7-3 prr9-1* (Farré et al., 2005), *prr5-11 prr7-11* (Yamashino et al., 2008)*, prr5-11* (Yamashino et al., 2008)*, prr5-1* (Eriksson et al., 2003)*, prr7-11* (Yamashino et al., 2008), *toc1* (Más et al., 2003)*, elf3* (Hicks et al., 1996), *elf4* (Khanna et al., 2003)*,* and *lux* (Hazen et al., 2005) seeds (all in the Columbia background) were rinsed firstly in a 70% (v/v) ethanol solution for 2 minutes, followed by a 30%

(v/v) bleach (Clorox® 7.5%) wash for 7 minutes, and lastly with three sequential washes with distilled water. The seeds were then immediately imbibed on 0.5x MS CTL (Caisson Labs MS Media with macronutrients and micronutrients; MSP01) media, 1% sucrose, and 7 g/L of agar (regular plates) at pH 5.8 (with KOH). For photomorphogenesis root assays, seeds were stratified for 3 days at 4°C in the dark and then exposed to a 12h light and 12h dark photoperiod of 100µmol/m^2/s of florescent light for 5 days at 22°C, before being transferred onto experimental plates. Seedlings were transferred to CTL, -N (MS media with macronutrients, and micronutrients, without ammonium nitrate; MSP05), -P (MS media with macronutrients and micronutrients without phosphate; MSP19), or -S (Caisson Labs MS media with macronutrients and micronutrients without sulfur; MSP44) experimental plates for a subsequent 8 days under 12h light and 12h dark photoperiod of 100µmol/m^2/s of florescent light at 22°C. All plants were grown vertically with their roots covered to limit root light exposure (Gao et al., 2021). Primary root measurements were taken over an 8-day time-course, with representative photographs obtained at the end of the 8 days.

3.2.2 Nutrient-dependent skotomorphogenesis phenomics

WT, *lhy cca1*, *prr7-3 prr9-1*, *prr5-11 prr7-11*, *prr5-11*, *prr5-1*, *prr7-11, toc1, elf3*, *elf4,* and *lux* seeds were sterilized as previously described. The seeds were then immediately imbibed on CTL, -N, -P, or -S plates, with 1% sucrose, and 7 g/L of agar at pH 5.8. All seeds were stratified for 3 days at 4°C in the dark, and then exposed to a 6h pulse of florescent light (100µmol/m^2/s at 22°C), followed by 4 days of darkness with a temperature of 22°C as previously described (Woloszynska et al., 2017). Hypocotyl etiolation measurements and representative photographs were taken at the end of four days in darkness.

3.2.3 Plant growth and harvesting for metabolomic analysis

WT, *prr5-11 prr7-11*, *prr5-11*, and *prr7-11* seeds were sterilized, stratified, germinated (CTL) as described above, then transferred onto, and grown on experimental plates (CTL, -N, -P, or -S) for 8 days, as previously described. Whole seedlings were then harvested at ZT 0, 4, 8, and 12, after which, the plant matter was immediately snap frozen in liquid N_2. Samples were stored at -80°C until they were ready to be grounded in liquid N_2 to be used for metabolomics data analysis.

3.2.4 Time-course Nutrient-Dependent Metabolomics by utilizing Gas Chromatography Mass Spectrometry (GC-MS)

Metabolite extraction and preparation was performed with modifications as previously described (Hill & Roessner, 2013; Liu et al., 2016; Scandola et al., 2022). Approximately ~50 mg of pulverized tissue was prepared and homogenized in 700 µl of iced-cold methanol, after which, the samples were vortexed twice for 30 seconds. Samples were incubated for 15 min at 70ºC and 850 xg in a table-top shaking incubator (Eppendorf). Tubes were then centrifuged for 20 min at 12000 xg, whereby the supernatant was transferred into new tubes. Samples were extracted twice, with the second extraction containing the internal standard (0.4 $mg.mL^{-1}$ ribitol). The internal standard consisted of 25 µL of ribitol at 0.4 $mg.mL^{-1}$ in water. Both the first and second extracts contained 500µl of supernatant. Extraction supernatants were pooled into one tube (1mL), where 200µL of extracted metabolites were thoroughly dried. Dried metabolite samples were derivatized with 100 µL of 25 $mg.mL^{-1}$ methoxylamine hydrochloride (AC210490050; Fisher Scientific) in pyridine for 60 min at 30ºC and 850 xg in the table-top shaking incubator (Eppendorf), followed by an additional incubation with 50 µL of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, AA4392806; Fisher Scientific) at 55°C for 3hrs and 850 xg in the table-top shaking incubator (Eppendorf). Derivatized samples were injected in splitless mode and analyzed using a 7890A gas chromatograph coupled to a 5975C quadrupole mass detector (Agilent Technologies, Palo Alto, CA, USA). In the same manner 1 µl of retention time standard mixture Supelco C7–C40 saturated alkanes standard (1,000 $µg.mL^{-1}$ of each component in hexane) diluted 100-fold (10 $µg.mL^{-1}$ final concentration) was injected and analyzed. Alkanes were dissolved in pyridine to a final concentration of 0.22 $mg.mL^{-1}$. Chromatic separation was done with a DB-5MS capillary column (30 m × 0.25 mm × 0.25 µm; 5183-4647, Agilent, USA). Inlet temperature was set at 280ºC. Initial GC Oven temperature was set to 80ºC and held for 2 min after injection, after which, GC oven temperature was raised to 300ºC at 7ºC min^{-1}, and held at 300ºC for 10 min. Injection and ion source temperatures were adjusted to 300ºC and 200ºC, respectively with a solvent delay of 5 min. The carrier gas (Helium) flow rate was set to 1 $mL.min^{-1}$. Sample detector was operated in EI mode at 70 eV and in full scan mode (m/z 33–600).

***3.2.5** GC-MS Data Analysis, Bioinformatics, and Data Visualization*

Compounds were identified by a combination of mass spectra and retention time index matching to the mass spectra of the National Institute of Standards and Technology library (NIST20, https://www.nist.gov/) and the Golm Metabolome Database (GMD, http://gmd.mpimp-golm.mpg.de/). Metabolite quantification was performed by using MassHunter (Agilent, USA). Peaks were integrated, and after blank subtraction, samples were subsequently normalized by dividing blank-subtracted sample peak values with the peak area values of the internal standard ribitol and the sample fresh weight (ng per mg of sample fresh weight). Identified metabolite pools pertaining to *prr5-11 prr7-11*, *prr5-11*, and *prr7-11* genotypes were each normalized to WT and Log2 transformed (relative Log2 fold change). The corresponding metabolite amounts were assembled into heat maps based on relative Log2 fold change (FC) value (r package Superheat) for each condition (control, nitrogen deficient, phosphorus deficient, and sulfur deficient) at ZT 0, 4 ,8, and 12. Euclidean distance clustering was executed to highlight metabolite profiles that trend with a similar Log_2 fold change (FC) (r package Superheat). Packages were implemented in r 4.2.2 (R Core Team 2022, https://www.r-project.org/).

3.3 RESULTS

3.3.1 Phenotyping data implicates morning loop proteins in the regulation of N, P, and S nutrient stress responses

In order to systematically address whether the circadian clock was involved in regulating plant N, P, or S nutrition, I subjected the seedlings of circadian clock deficient plant lines to nutrient stress to observe the effects of nutrition starvation on primary root elongation and hypocotyl etiolation (Table 1). I saw that plants with *lhy cca1* alleles have shorter and longer hypocotyls under -P and -S conditions, respectively (**Figure 2**; p-value ≤ 0.05; Student's t-test; n ≥ 30). However, I was unable to report any significant differences between *lhy cca1* hypocotyls under CTL verses -N conditions (**Figure 2**), nor between *lhy cca1* primary roots under -N, -P, or -S (**Figure 1**), suggesting that LHY and CCA1 proteins likely do not have an obvious primary root phenotype, nor a hypocotyl response under N, P, or S stress (n ≥ 30). Plants with *prr7-3 prr9-1* alleles have shorter hypocotyls under -N conditions, implicating the morning loop in the -N response in *Arabidopsis* (**Figure 4**; p-value ≤ 0.05; Student's t-test; n ≥ 30). I did not detect significant differences between *prr7-3 prr9-1* hypocotyls under -P nor -S conditions, suggesting that PRR7 and PRR9 proteins likely do not have an obvious hypocotyl response under P or S stress

(**Figure 4**; n ≥ 30). I did not find a significant difference in the primary root of *prr7-3 prr9-1* seedlings under -N, -P, or -S conditions (**Figure 3**; n ≥ 30). I found plants that with *prr5-11 prr7-11* alleles had shorter primary roots under N, P, and S starvation (**Figure 5**; p-value ≤ 0.05; Student's t-test; n ≥ 30). I also found that plants with *prr5-11 prr7-11* alleles had longer hypocotyls under P and S stress (**Figure 6**; p-value ≤ 0.05; Student's t-test; n ≥ 30).

3.3.2 The evening loop appears to preferentially regulate P metabolic processes

Here, I found that *toc1* plants have shorter hypocotyls under -P conditions (**Figure 8**; p-value ≤ 0.05; Student's t-test; n ≥ 30), implicating the evening loop in the P hypocotyl response. I was unable to observe any other clear phenotypes between *toc1* plants under N or S deficient conditions (**Figure 7 - 8**; n ≥ 30). I was also able to report that *elf4* seedlings have longer primary roots when subjected to P stress (**Figure 11**; p-value ≤ 0.05; Student's t-test; n ≥ 30). I was unable to detect any discernable phenotypes between *elf4* seedlings under N or S stress (**Figure 11 - 12**; n ≥ 30). I was also unable to detect any significant phenotypic differences between *elf3* (**Figure 9 - 10**; n ≥ 30) and *lux* (**Figure 13 - 14**; n ≥ 30) seedlings under -N, -P, nor -S. Given the evaluation of *lhy cca1, prr7-3 prr9-1*, *prr5-11 prr7-11*, *toc1, elf3, elf4*, and *lux* seedlings for nutrient dependent responses, I believe that the morning and evening loop is involved in the N, P, and S responses, but to differing degrees (**Figure 21**). Of the circadian clock deficient genotypes examined, I found the most extensive phenotype response to be between nutrient stress and PRR5 and PRR7 through the *prr5-11 prr7-11* mutant (**Figure 5 - 6**). Thus, to tease apart the roles of PRR5 and PRR7 under nutrient deficiency, I then subjected *prr5-11*, *prr7-11*, and *prr5-1* seedlings to nutrient stress conditions (**Figure 21**; Yamashino et al., 2008).

3.3.3 Phenotyping screen implicates PRR5 proteins in the S metabolic response

Seedlings with *prr5-11* alleles have shorter primary roots when subjected to -S conditions (**Figure 15**; p-value ≤ 0.05; Student's t-test; n ≥ 30). Plants with *prr7-11* alleles show longer and shorter hypocotyl lengths when subjected to P and S stress, respectively (**Figure 18**; p-value ≤ 0.05; Student's t-test; n ≥ 30). Plants with *prr5-11 prr7-11* alleles showed a greater number of phenotypic responses to nutrient stress (**Figure 5** – **6**). This suggests that plants that lack both PRR5 and PRR7 have disparate phenotypes compared to plants that lack either PRR5 or PRR7 alone (**Figure 21**). I also analyzed one additional plant line, *prr5-1*, which showed a similar short

primary root phenotype to *prr5-11* when subjected to -S conditions and longer hypocotyls under N, P, and S stress (**Figure 19-20**; p-value ≤ 0.05; Student's t-test; n ≥ 30). Based on this screen and the immediate connections between N, P and S to plant growth and development as macronutrients, I next examined the time-of-day metabolome of WT, *prr5-11 prr7-11*, *prr5-11*, and *prr7-11* plants under N, P, or S limiting conditions at ZT 0, 4, 8, and 12 to elucidate the roles of PRR5 and PRR7 proteins in regulating nutrient-related processes in *Arabidopsis* (**Figure 22**). I chose ZT 0, 4, 8, and 12 for my time-course experimentation as *PRR5* and *PRR7* expression peaks between ZT 6 and ZT 12 under 12L:12D diel conditions, making ZT 0 to 12 the best time-points to elucidate the roles of PRR5 and PRR7 proteins in nutrient-related processes (Nakamichi et al., 2010).

Table 1: Arabidopsis mutants of interest in characterizing the circadian response to nutrient stress.

AGIs	Mutant Line	ARBC Stock ID	Citation
AT1G01060 AT2G46830	*lhy cca1*	N/A	Mizoguchi et al., (2002)
AT5G24470 AT5G02810	*prr5-11 prr7-11*	CS2107711	Nakamichi et al., (2005a)
AT5G02810 AT2G46790	*prr7-3 prr9-1*	N/A	Farré et al., (2005)
AT5G02810 AT2G46790 AT5G24470	*prr5-11 prr7-11 prr9-10*	N/A	Nakamichi et al., (2005b)
AT5G24470	*prr5-11*	CS2107708	Yamamoto et al., (2003)
AT5G24470	*prr5-1*	CS9384	Eriksson et al., (2003)
AT5G02810	*prr7-11*	CS2107709	Yamamoto et al., (2003)
AT5G02810	*prr9-1*	CS9385	Eriksson et al., (2003)
AT5G61380	*toc1*	CS2107710	Más et al., (2003)
AT2G25930	*elf3*	N/A	Hicks et al., (1996)
AT2G40080	*elf4*	CS68093	Khanna et al., (2003)
AT3G46640	*lux*	N/A	Hazen et al., (2005)

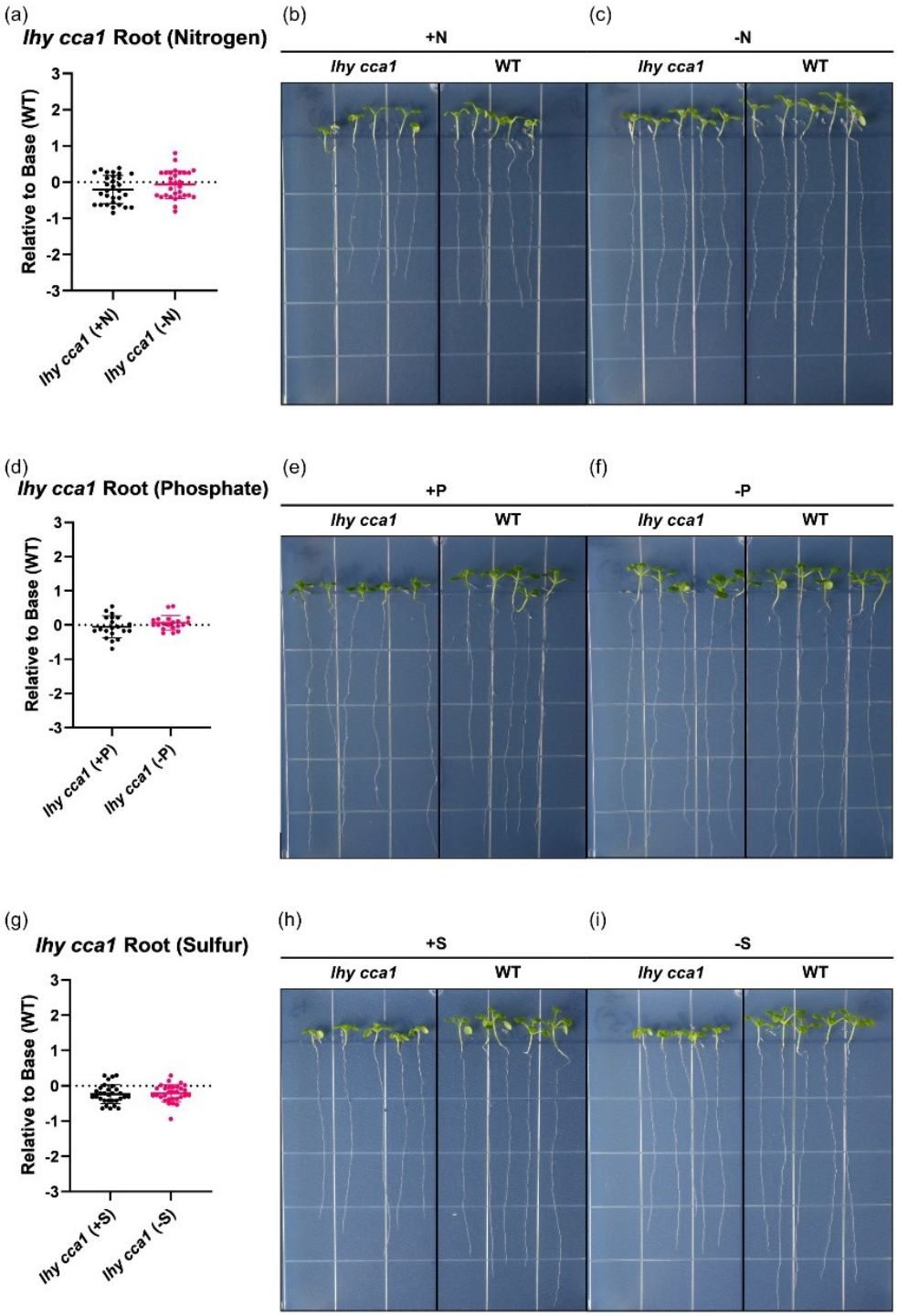

Figure 1: Root length changes in *lhy cca1* plants under nitrogen, phosphorus, and sulfur stress. Quantitative analysis of seedling primary root length after 8 days of growth under control (CTL; a-b, d-e, g-h), -N (a, c), -P (d, f), or -S (g, i) conditions. An asterisk (*) denotes statistical significance (p-value ≤ 0.05; Student's t-test). Data are presented as mean ± standard deviation; n ≥ 30.

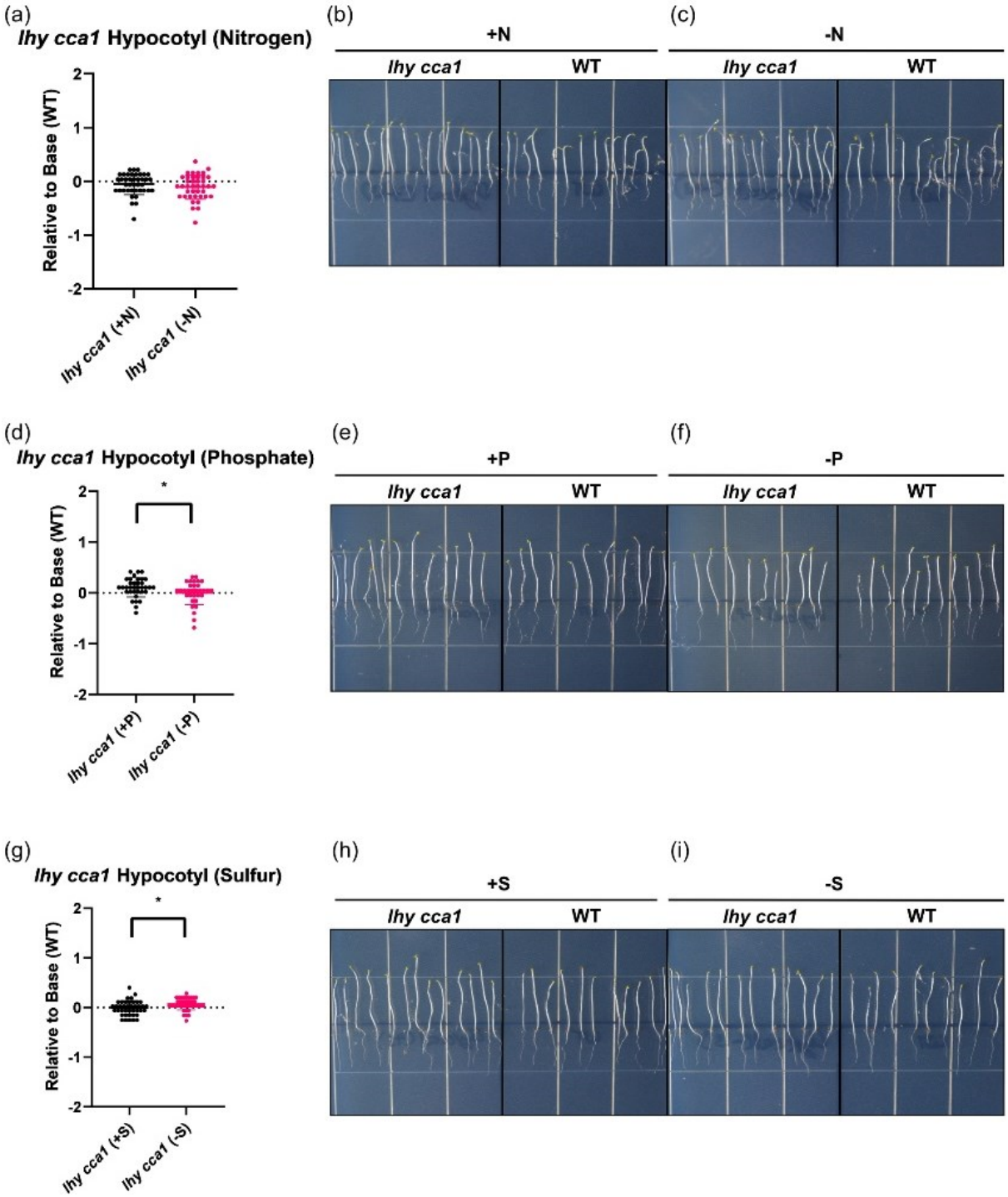

Figure 2: Hypocotyl length changes in *lhy cca1* plants under nitrogen, phosphorus, and sulfur stress. Quantitative analysis of seedling hypocotyl etiolation lengths at the end of 4 days in the dark (post a 6-hour pulse of white light) under control (CTL; a-b, d-e, g-h), -N (a, c), -P (d, f), or -S (g, i) media. An asterisk (*) denotes statistical significance (p-value ≤ 0.05; Student's t-test). Data are presented as mean ± standard deviation; n ≥ 30.

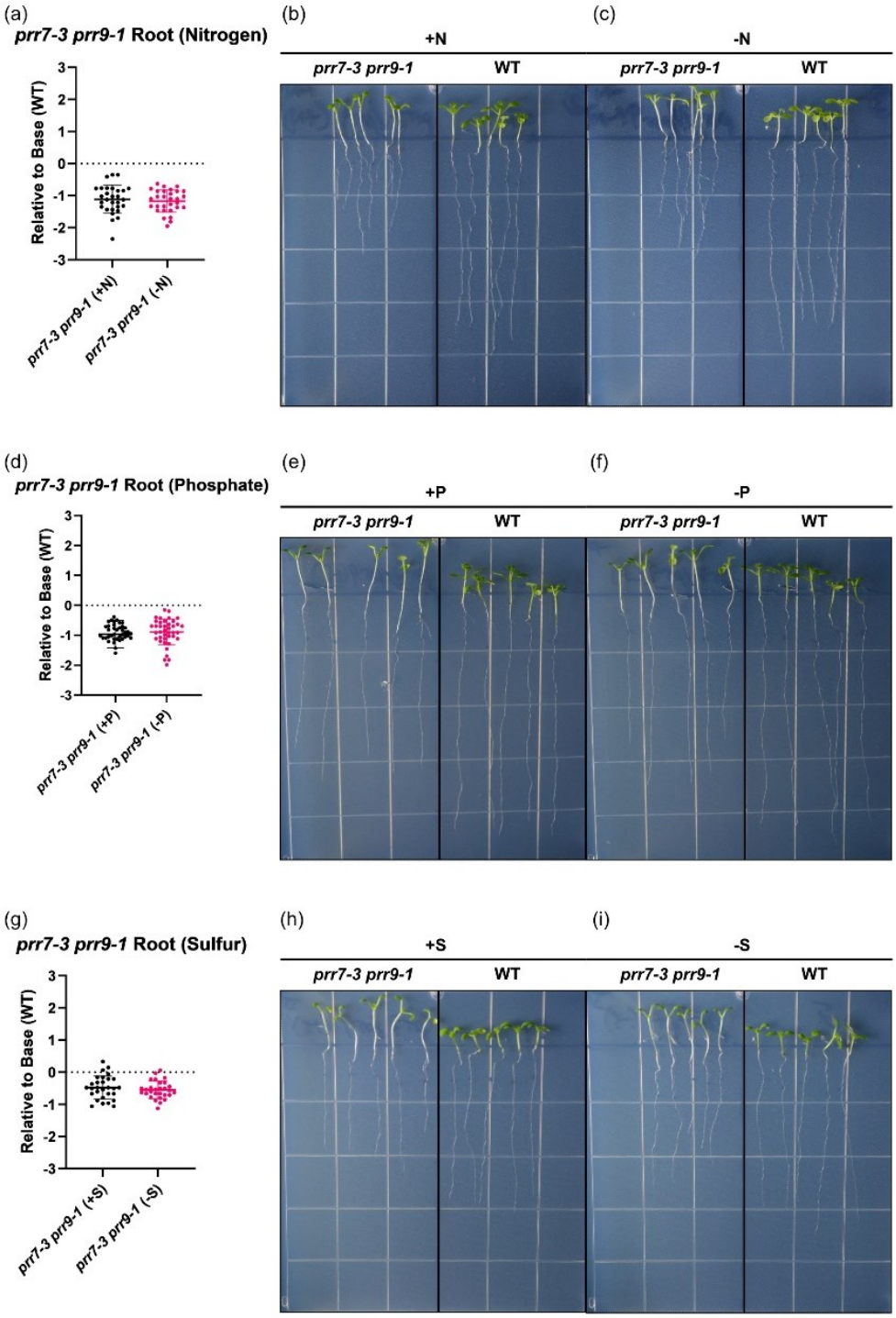

Figure 3: Root length changes in *prr7-3 prr9-1* plants under nitrogen, phosphorus, and sulfur stress. Quantitative analysis of seedling primary root length after 8 days of growth under control (CTL; a-b, d-e, g-h), -N (a, c), -P (d, f), or -S (g, i) conditions. An asterisk (*) denotes statistical significance (p-value ≤ 0.05; Student's t-test). Data are presented as mean ± standard deviation; n ≥ 30.

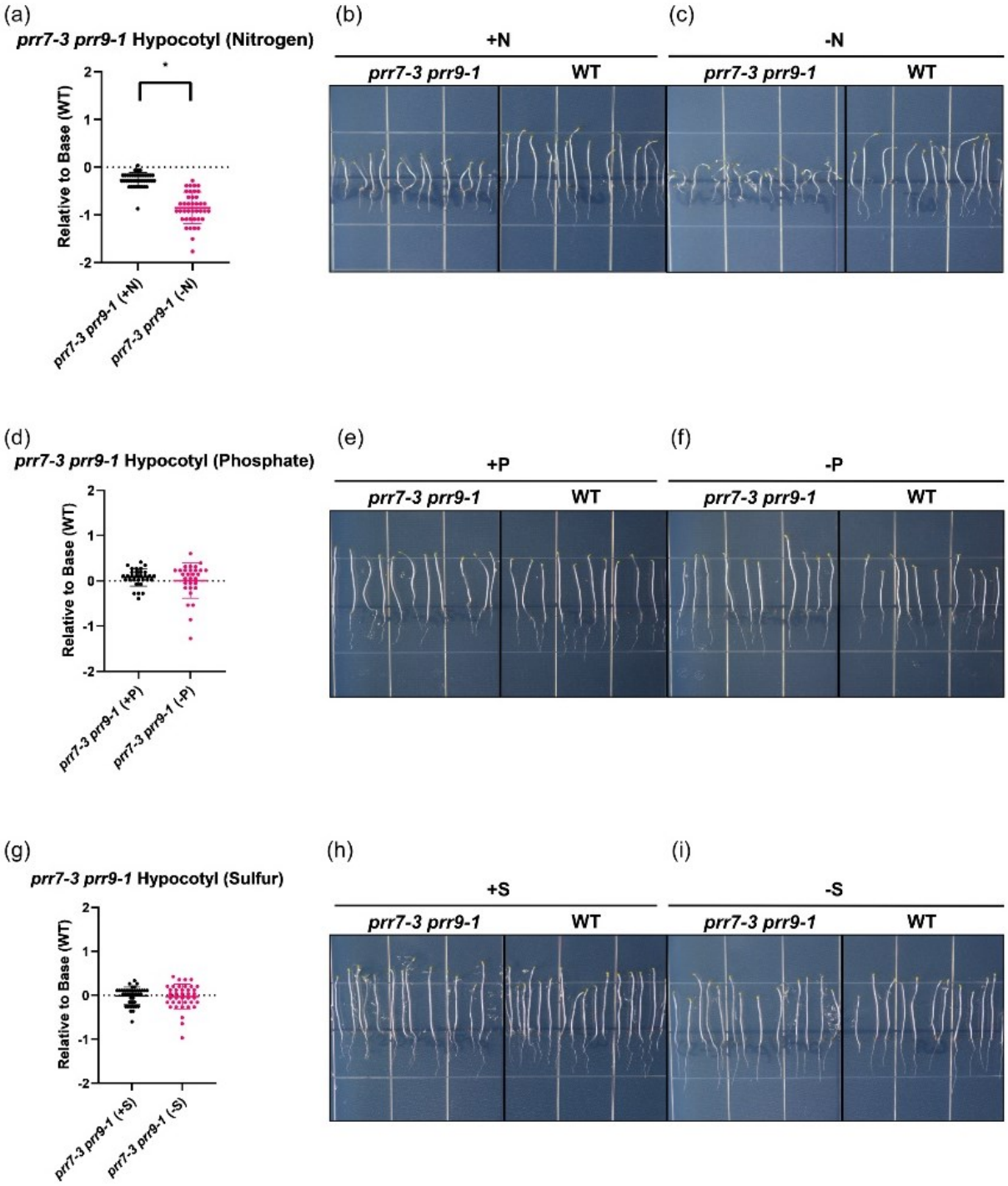

Figure 4: Hypocotyl length changes in *prr7-3 prr9-1* plants under nitrogen, phosphorus, and sulfur stress. Quantitative analysis of seedling hypocotyl etiolation lengths at the end of 4 days in the dark (post a 6-hour pulse of white light) under control (CTL; a-b, d-e, g-h), -N (a, c), -P (d, f), or -S (g, i) media. An asterisk (*) denotes statistical significance (p-value ≤ 0.05; Student's t-test). Data are presented as mean ± standard deviation; n ≥ 30.

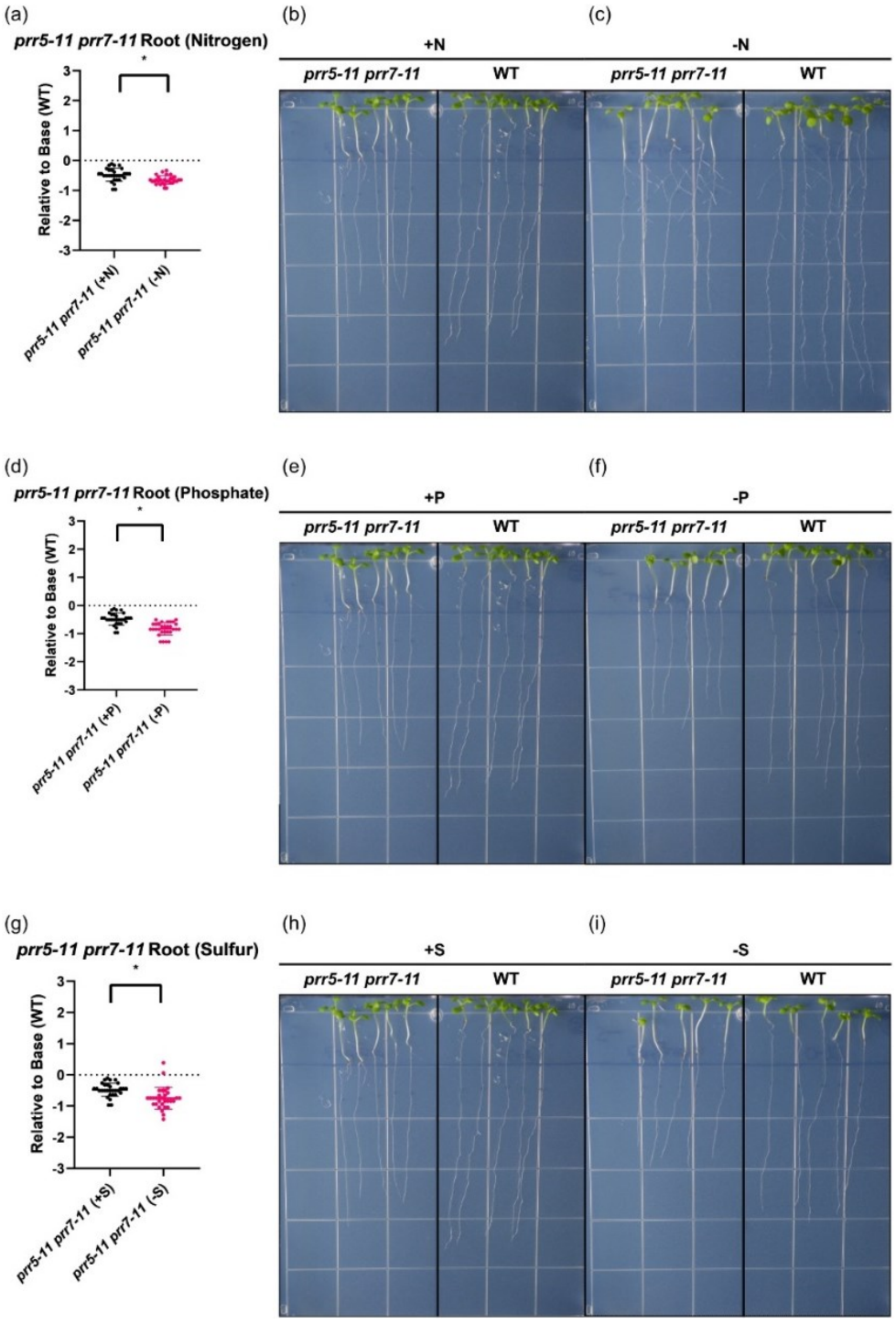

Figure 5: Root length changes in *prr5-11 prr7-11* plants under nitrogen, phosphorus, and sulfur stress. Quantitative analysis of seedling primary root length after 8 days of growth under control (CTL; a-b, d-e, g-h), -N (a, c), -P (d, f), or -S (g, i) conditions. An asterisk (*) denotes statistical significance (p-value ≤ 0.05; Student's t-test). Data are presented as mean ± standard deviation; $n \geq 30$.

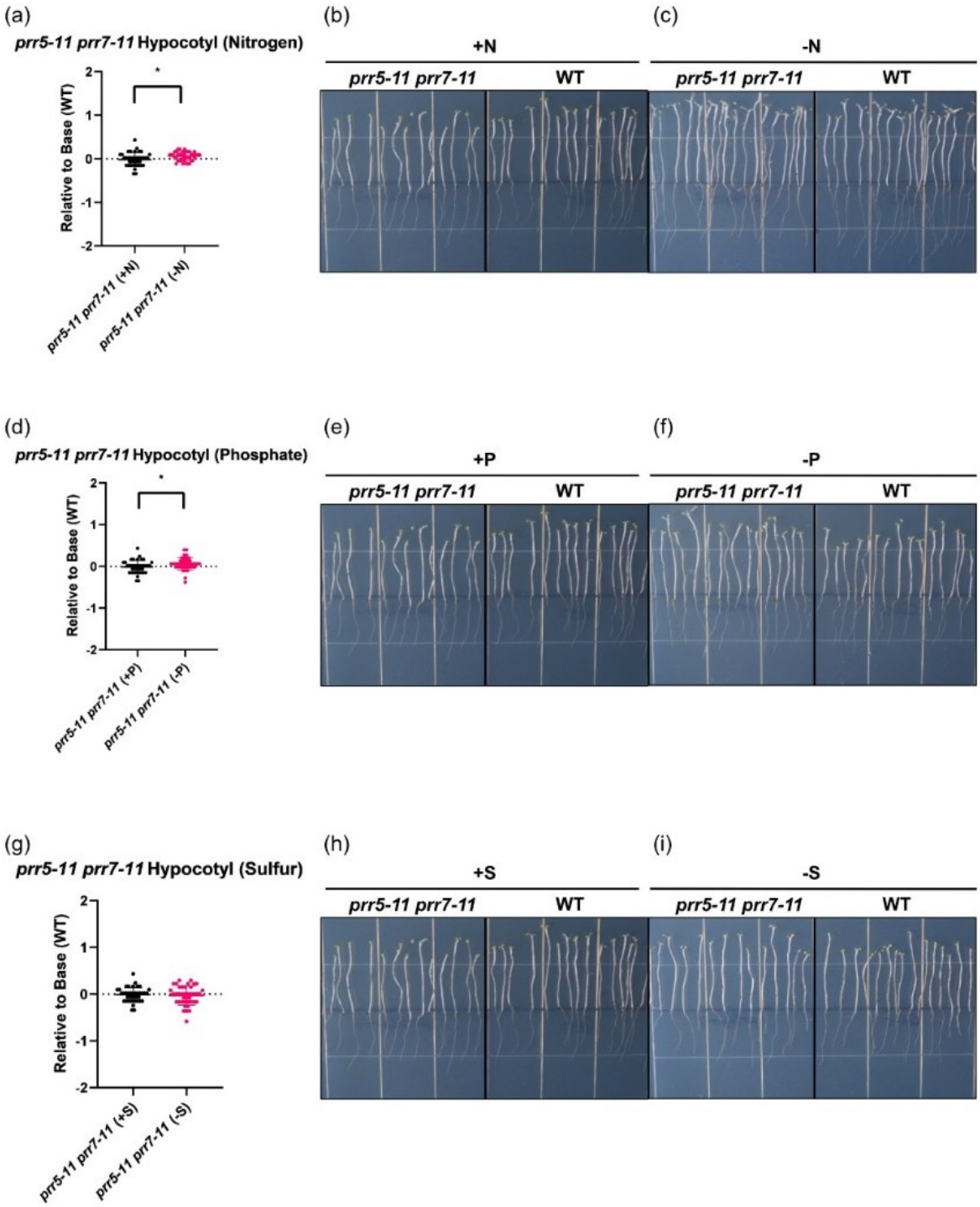

Figure 6: Hypocotyl length changes in *prr5-11 prr7-11* plants under nitrogen, phosphorus, and sulfur stress. Quantitative analysis of seedling hypocotyl etiolation lengths at the end of 4 days in the dark (post a 6-hour pulse of white light) under control (CTL; a-b, d-e, g-h), -N (a, c), -P (d, f), or -S (g, i) media. An asterisk (*) denotes statistical significance (p-value ≤ 0.05; Student's t-test). Data are presented as mean ± standard deviation; n ≥ 30.

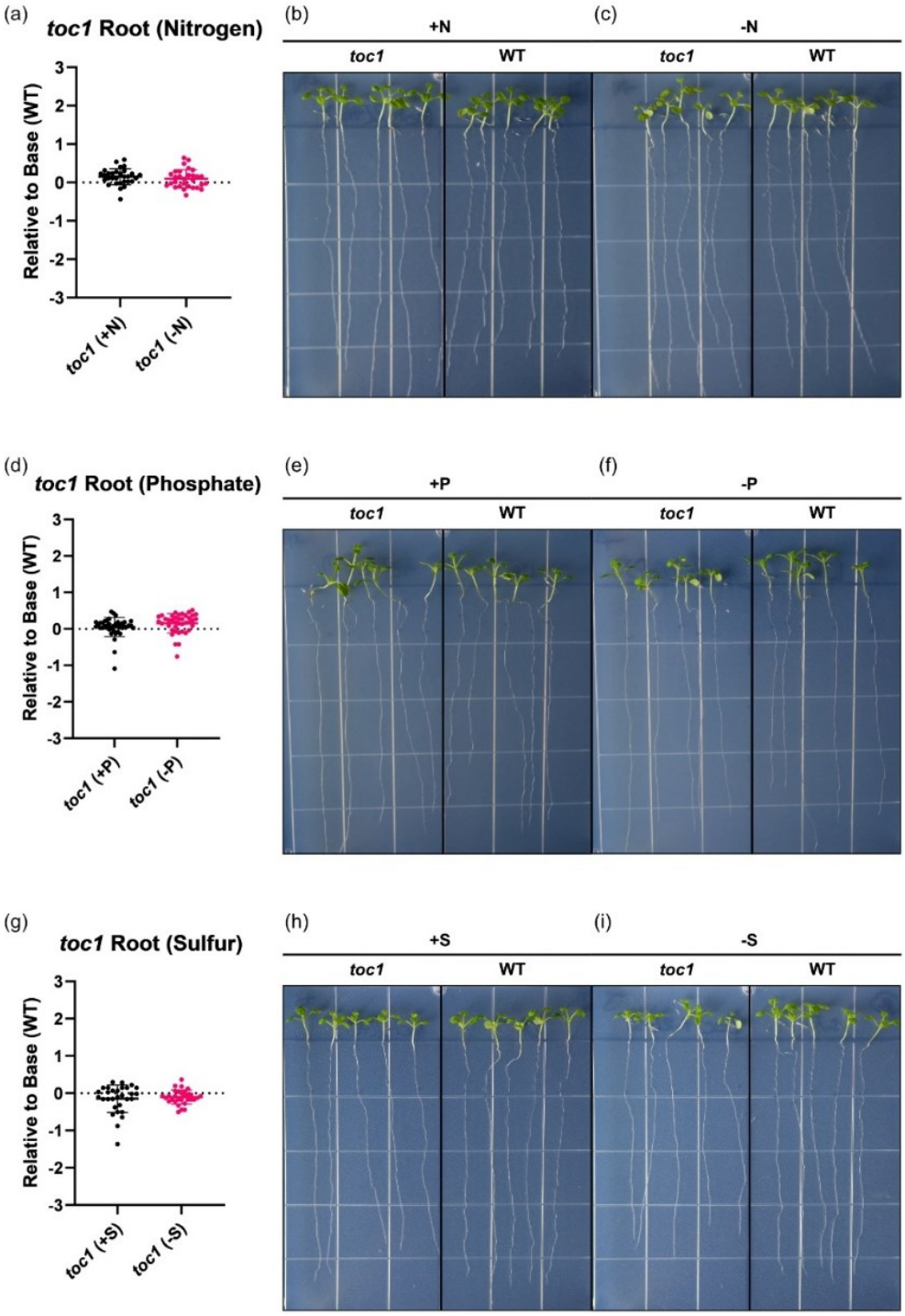

Figure 7: Root length changes in *toc1* plants under nitrogen, phosphorus, and sulfur stress. Quantitative analysis of seedling primary root length after 8 days of growth under control (CTL; a-b, d-e, g-h), -N (a, c), -P (d, f), or -S (g, i) conditions. An asterisk (*) denotes statistical significance (p-value ≤ 0.05; Student's t-test). Data are presented as mean ± standard deviation; n ≥ 30.

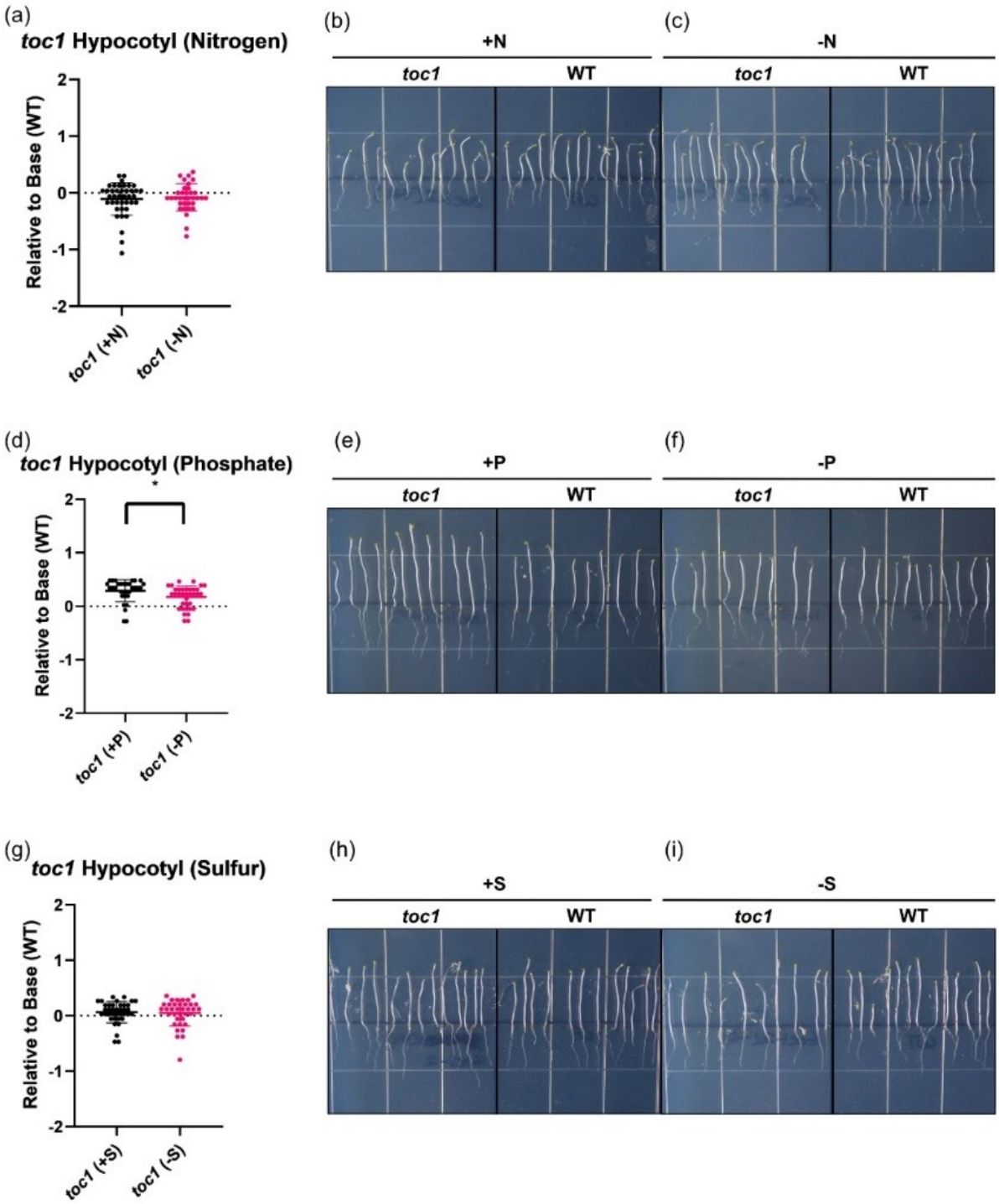

Figure 8: Hypocotyl length changes in *toc1* plants under nitrogen, phosphorus, and sulfur stress. Quantitative analysis of seedling hypocotyl etiolation lengths at the end of 4 days in the dark (post a 6-hour pulse of white light) under control (CTL; a-b, d-e, g-h), -N (a, c), -P (d, f), or -S (g, i) media. An asterisk (*) denotes statistical significance (p-value ≤ 0.05; Student's t-test). Data are presented as mean ± standard deviation; n ≥ 30.

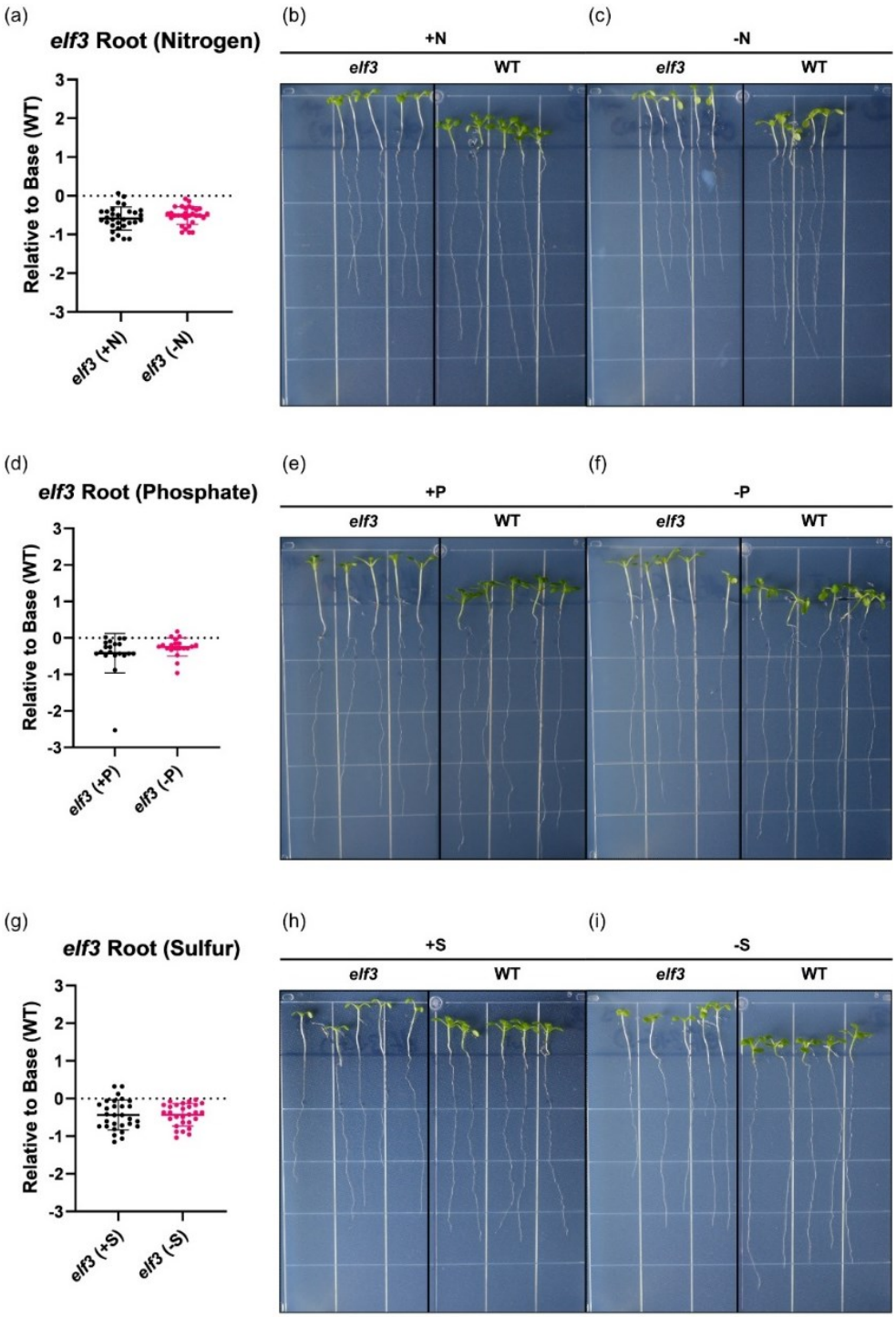

Figure 9: Root length changes in *elf3* plants under nitrogen, phosphorus, and sulfur stress. Quantitative analysis of seedling primary root length after 8 days of growth under control (CTL; a-b, d-e, g-h), -N (a, c), -P (d, f), or -S (g, i) conditions. An asterisk (*) denotes statistical significance (p-value ≤ 0.05; Student's t-test). Data are presented as mean ± standard deviation; n ≥ 30.

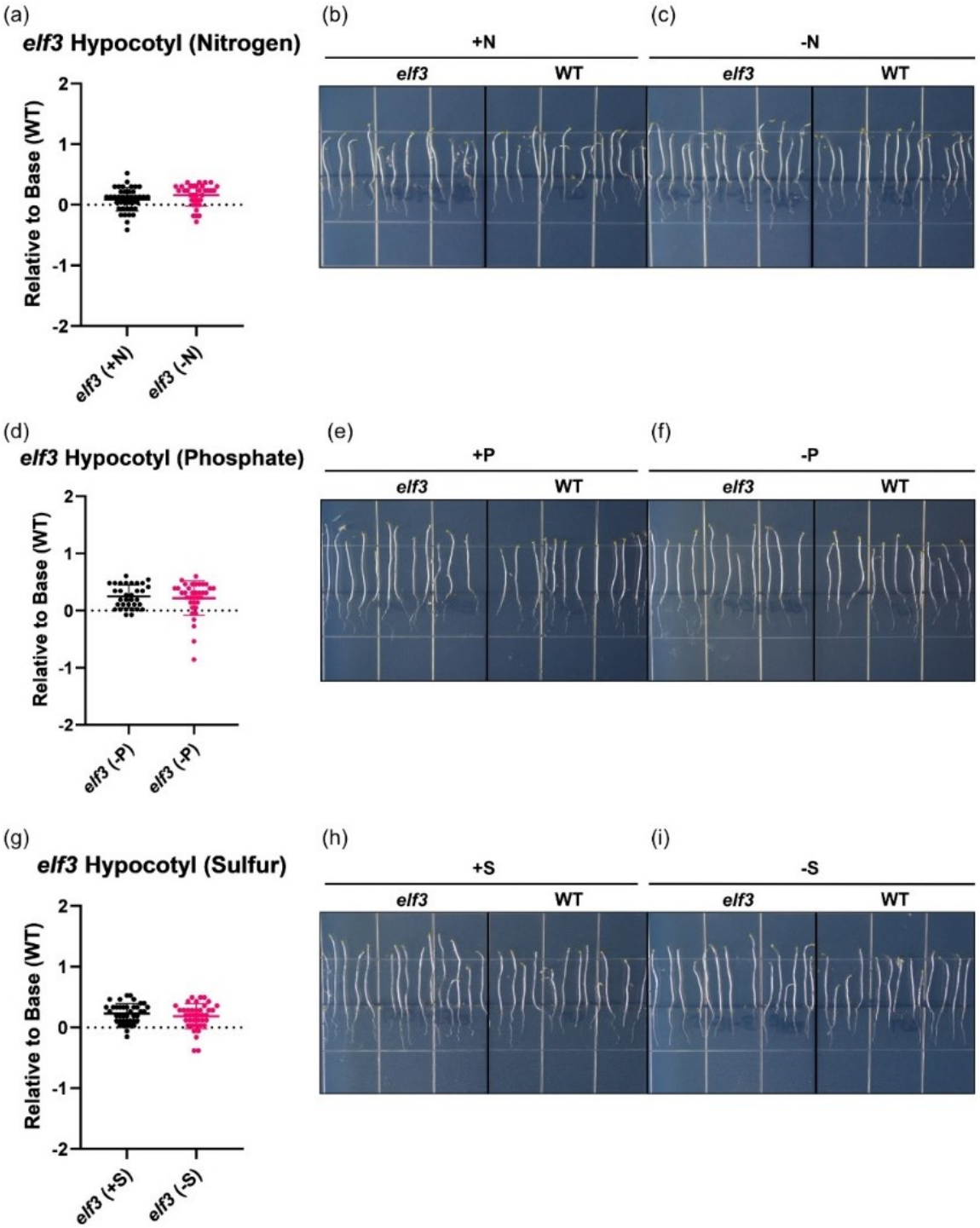

Figure 10: Hypocotyl length changes in *elf3* plants under nitrogen, phosphorus, and sulfur stress. Quantitative analysis of seedling hypocotyl etiolation lengths at the end of 4 days in the dark (post a 6-hour pulse of white light) under control (CTL; a-b, d-e, g-h), -N (a, c), -P (d, f), or -S (g, i) media. An asterisk (*) denotes statistical significance (p-value ≤ 0.05; Student's t-test). Data are presented as mean ± standard deviation; n ≥ 30.

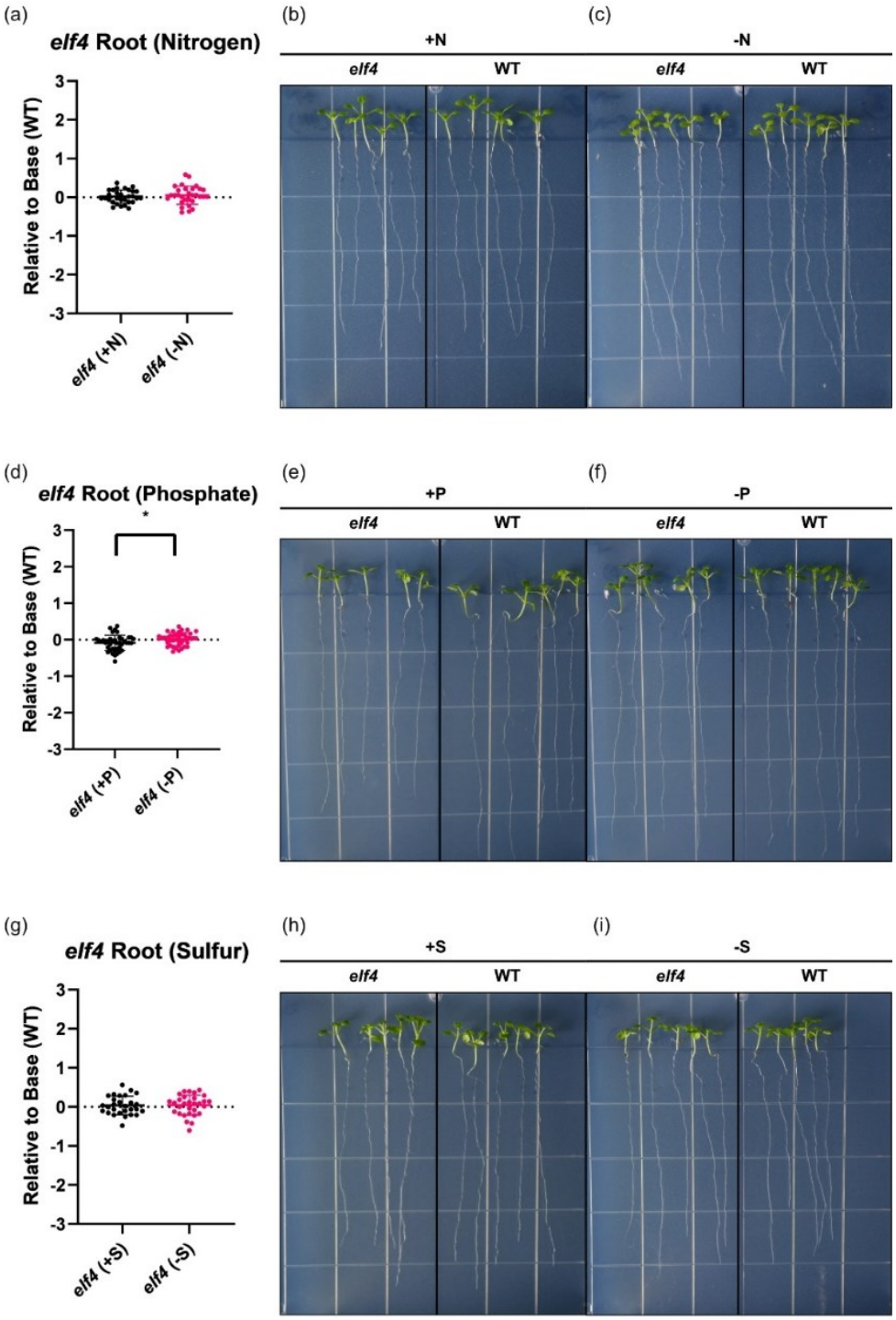

Figure 11: Root length changes in *elf4* plants under nitrogen, phosphorus, and sulfur stress. Quantitative analysis of seedling primary root length after 8 days of growth under control (CTL; a-b, d-e, g-h), -N (a, c), -P (d, f), or -S (g, i) conditions. An asterisk (*) denotes statistical significance (p-value ≤ 0.05; Student's t-test). Data are presented as mean ± standard deviation; n ≥ 30.

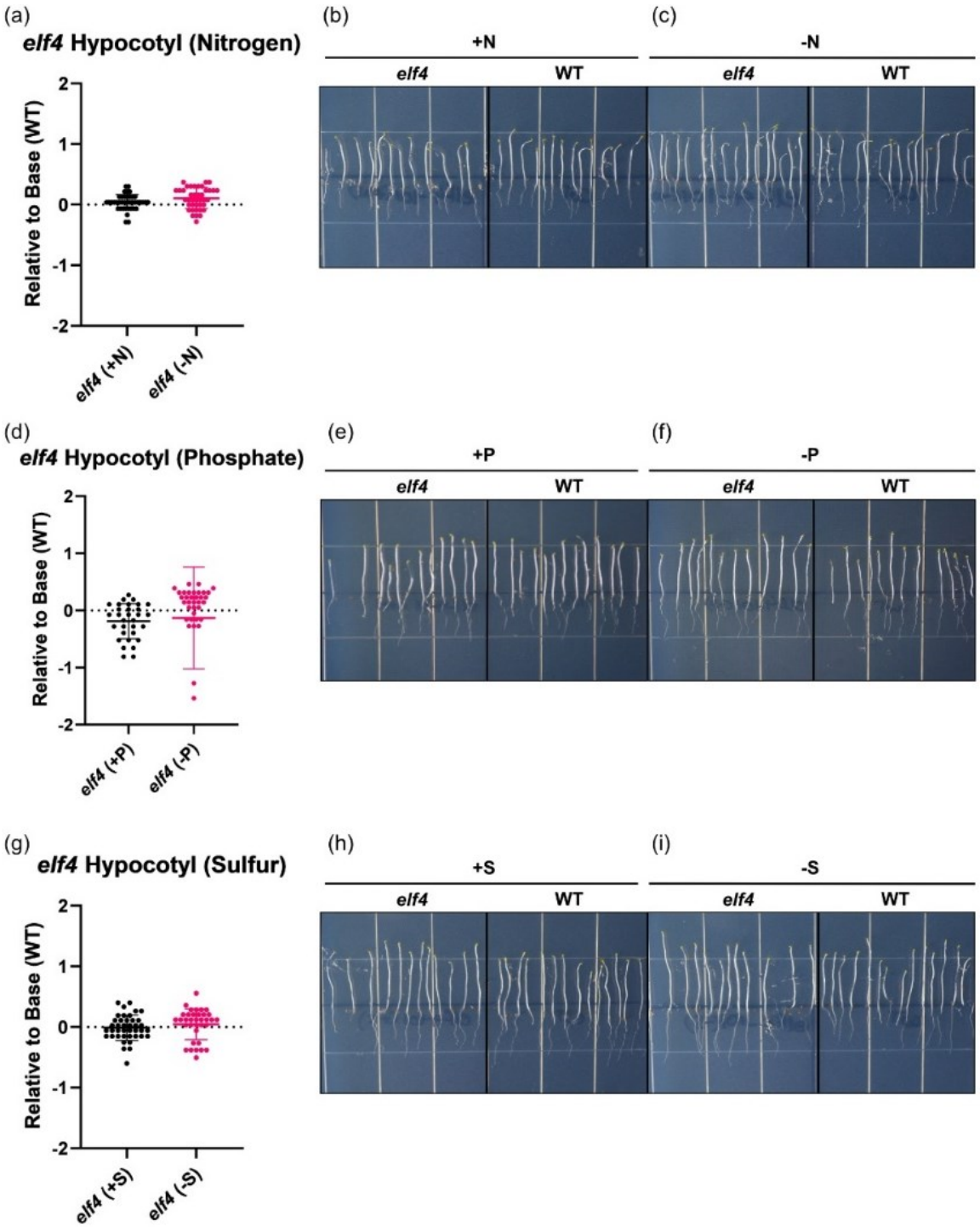

Figure 12: Hypocotyl length changes in *elf4* plants under nitrogen, phosphorus, and sulfur stress. Quantitative analysis of seedling hypocotyl etiolation lengths at the end of 4 days in the dark (post a 6-hour pulse of white light) under control (CTL; a-b, d-e, g-h), -N (a, c), -P (d, f), or -S (g, i) media. An asterisk (*) denotes statistical significance (p-value ≤ 0.05; Student's t-test). Data are presented as mean ± standard deviation; n ≥ 30.

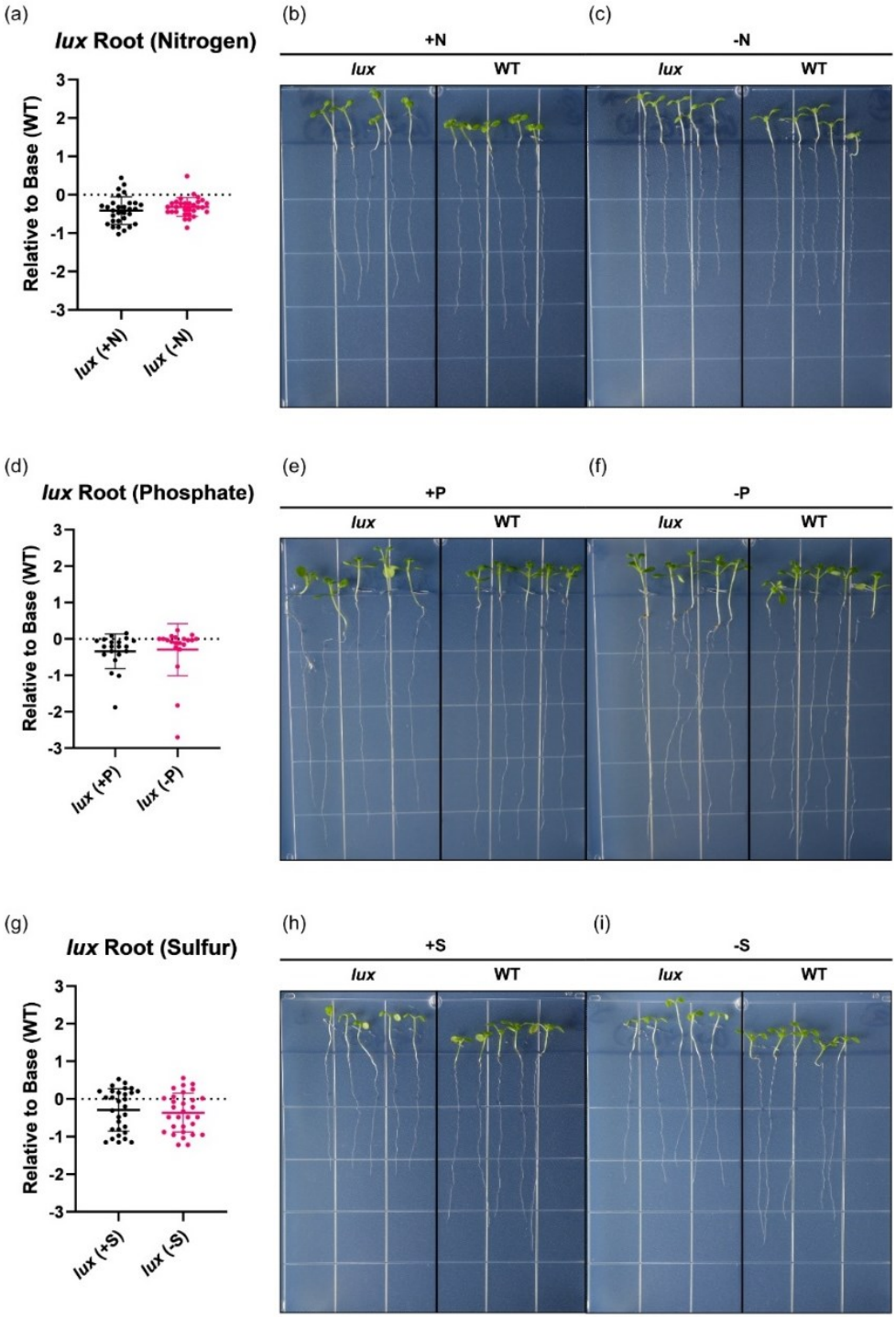

Figure 13: Root length changes in *lux* plants under nitrogen, phosphorus, and sulfur stress. Quantitative analysis of seedling primary root length after 8 days of growth under control (CTL; a-b, d-e, g-h), -N (a, c), -P (d, f), or -S (g, i) conditions. An asterisk (*) denotes statistical significance (p-value ≤ 0.05; Student's t-test). Data are presented as mean ± standard deviation; n ≥ 30.

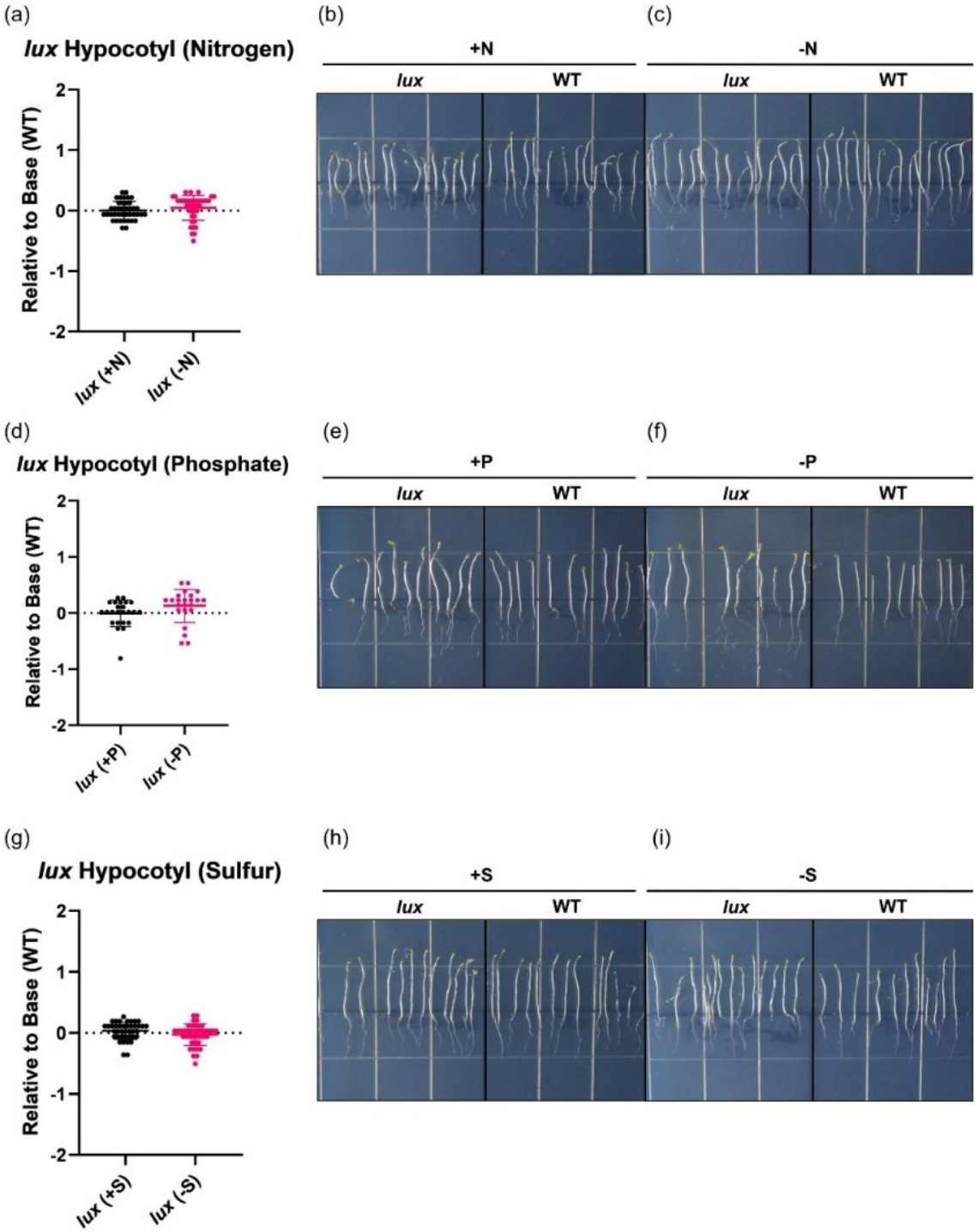

Figure 14: Hypocotyl length changes in *lux* plants under nitrogen, phosphorus, and sulfur stress. Quantitative analysis of seedling hypocotyl etiolation lengths at the end of 4 days in the dark (post a 6-hour pulse of white light) under control (CTL; a-b, d-e, g-h), -N (a, c), -P (d, f), or -S (g, i) media. An asterisk (*) denotes statistical significance (p-value ≤ 0.05; Student's t-test). Data are presented as mean ± standard deviation; n ≥ 30.

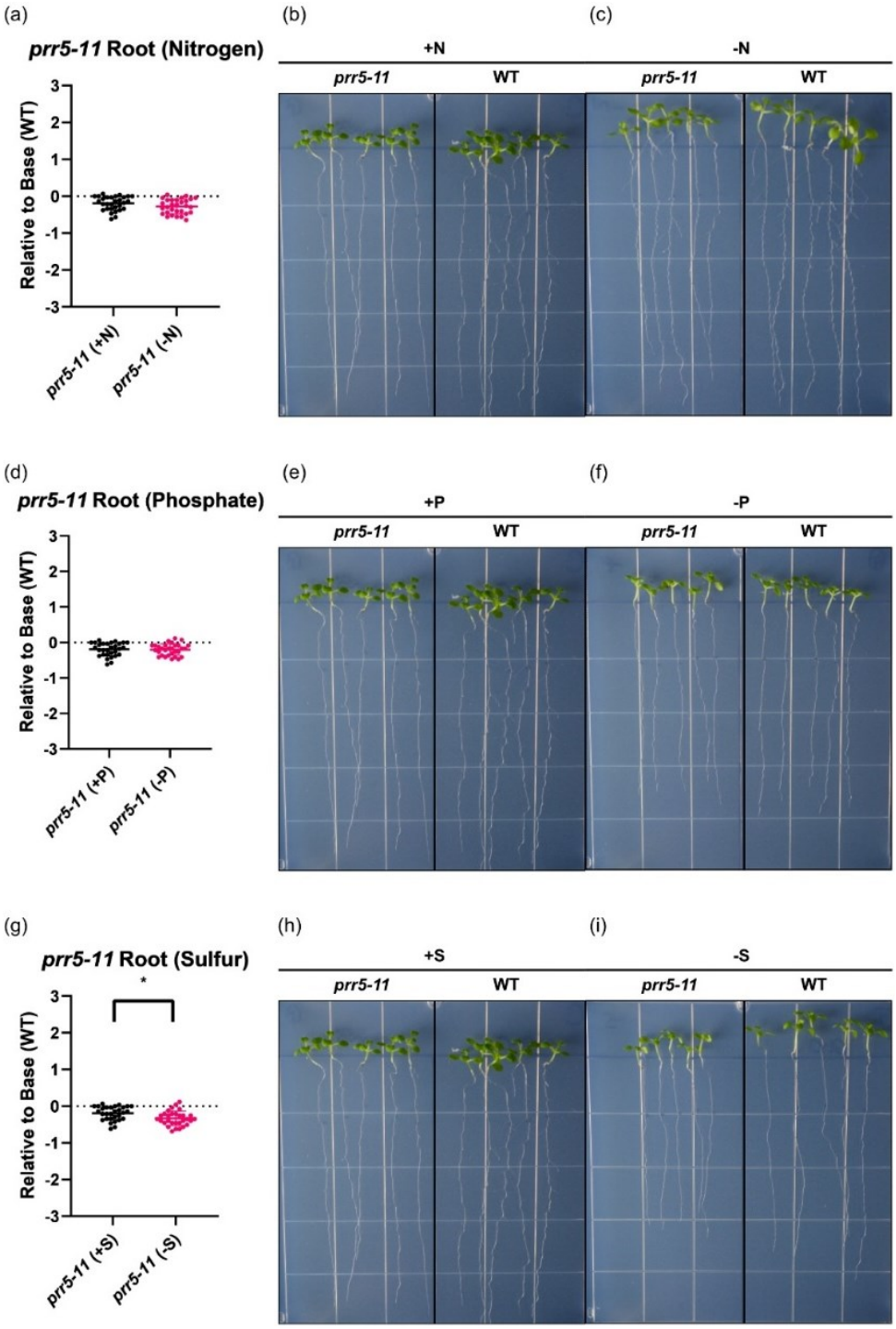

Figure 15: Root length changes in *prr5-11* plants under nitrogen, phosphorus, and sulfur stress. Quantitative analysis of seedling primary root length after 8 days of growth under control (CTL; a-b, d-e, g-h), -N (a, c), -P (d, f), or -S (g, i) conditions. An asterisk (*) denotes statistical significance (p-value ≤ 0.05; Student's t-test). Data are presented as mean ± standard deviation; n ≥ 30.

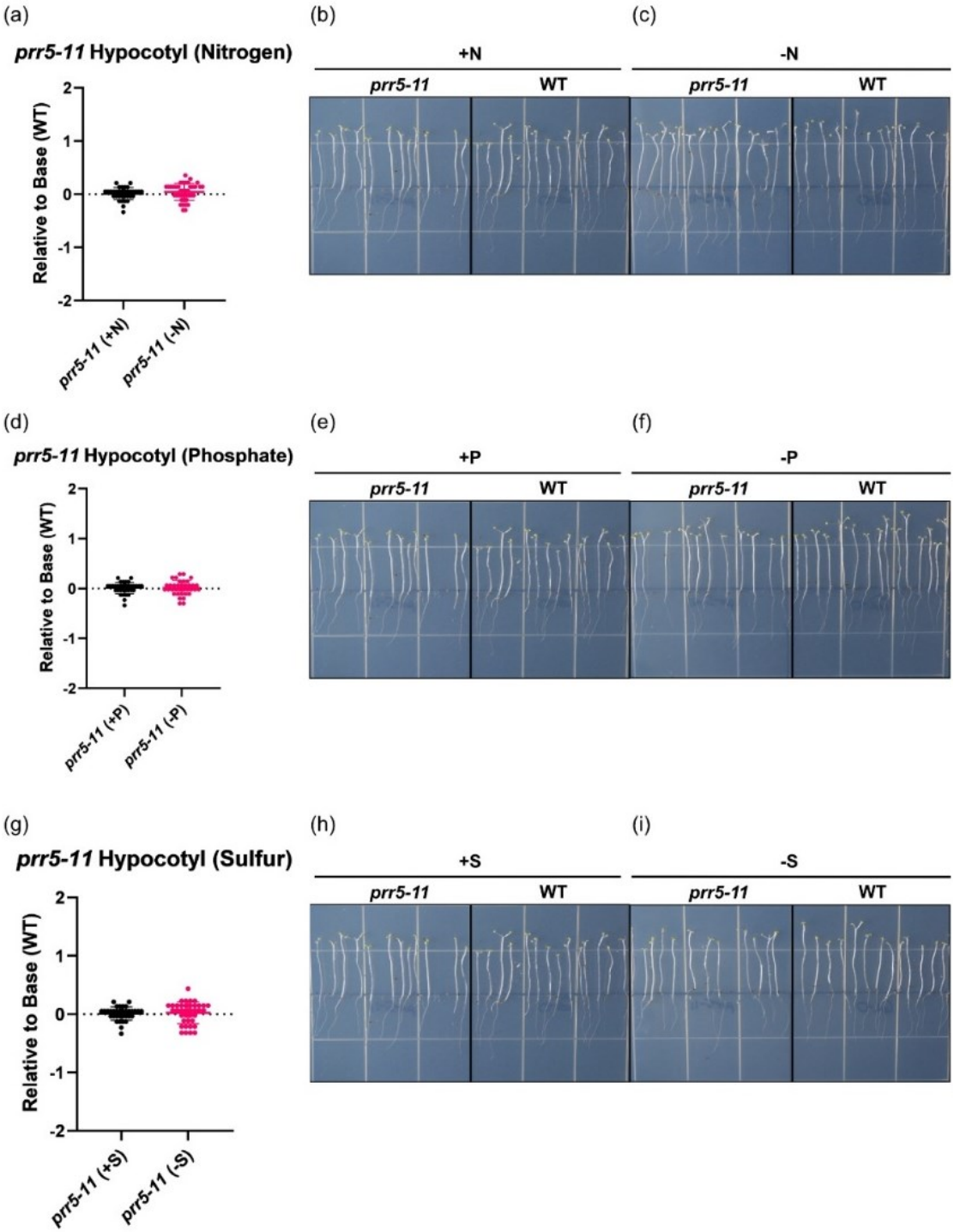

Figure 16: Hypocotyl length changes in *prr5-11* plants under nitrogen, phosphorus, and sulfur stress. Quantitative analysis of seedling hypocotyl etiolation lengths at the end of 4 days in the dark (post a 6-hour pulse of white light) under control (CTL; a-b, d-e, g-h), -N (a, c), -P (d, f), or -S (g, i) media. An asterisk (*) denotes statistical significance (p-value ≤ 0.05; Student's t-test). Data are presented as mean ± standard deviation; n ≥ 30.

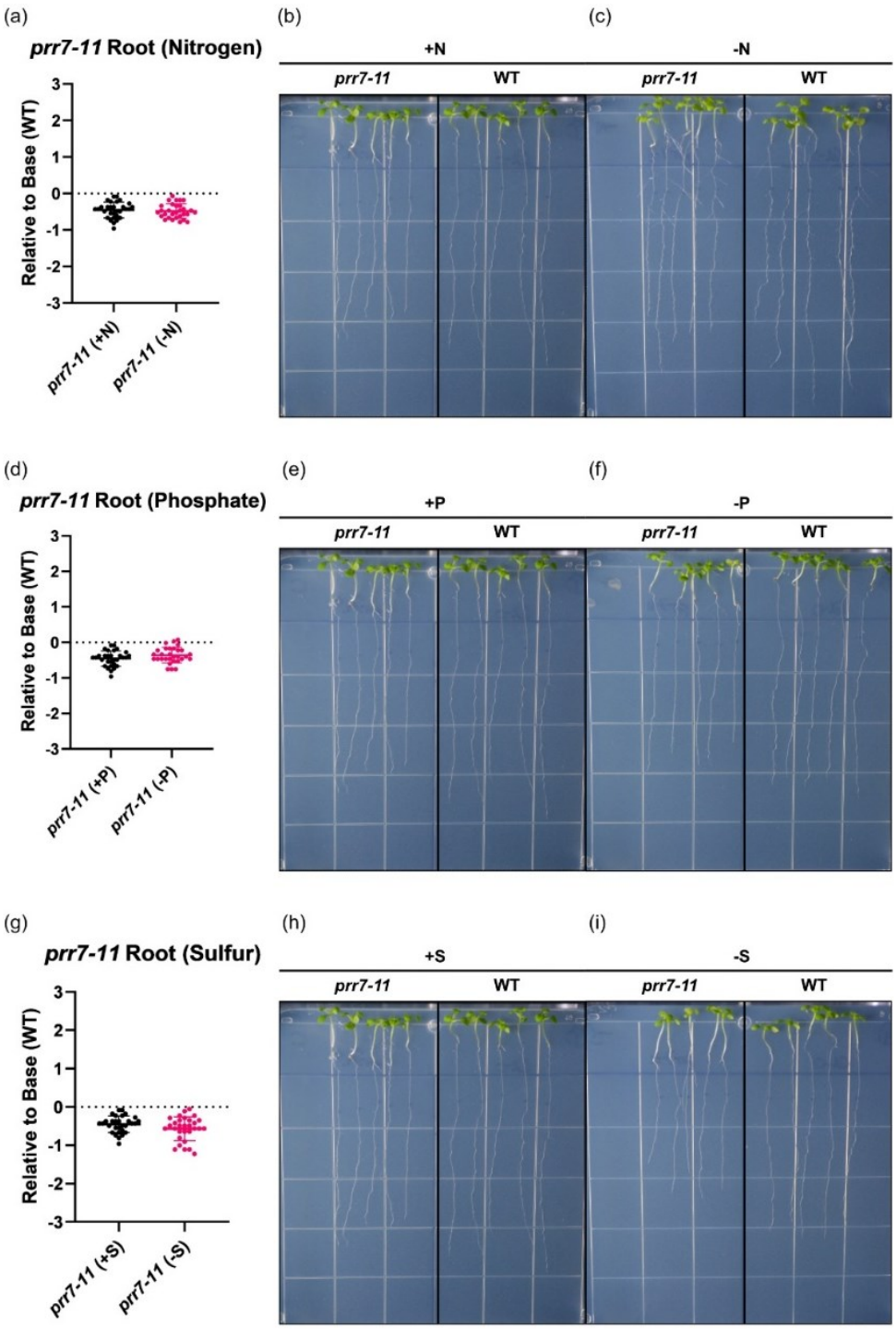

Figure 17: Root length changes in *prr7-11* plants under nitrogen, phosphorus, and sulfur stress. Quantitative analysis of seedling primary root length after 8 days of growth under control (CTL; a-b, d-e, g-h), -N (a, c), -P (d, f), or -S (g, i) conditions. An asterisk (*) denotes statistical significance (p-value ≤ 0.05; Student's t-test). Data are presented as mean ± standard deviation; n ≥ 30.

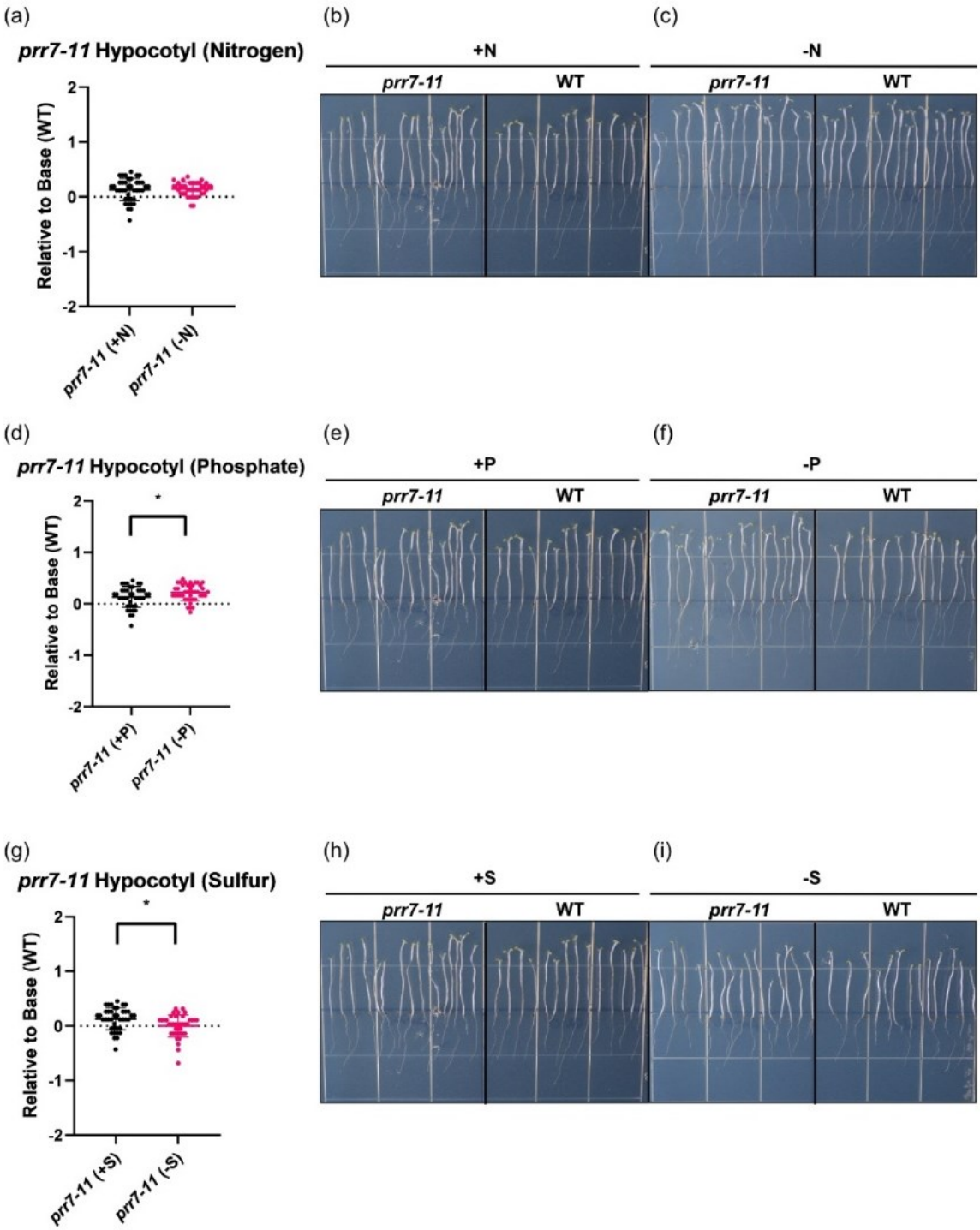

Figure 18: Hypocotyl length changes in *prr7-11* plants under nitrogen, phosphorus, and sulfur stress. Quantitative analysis of seedling hypocotyl etiolation lengths at the end of 4 days in the dark (post a 6-hour pulse of white light) under control (CTL; a-b, d-e, g-h), -N (a, c), -P (d, f), or -S (g, i) media. An asterisk (*) denotes statistical significance (p-value ≤ 0.05; Student's t-test). Data are presented as mean ± standard deviation; n ≥ 30.

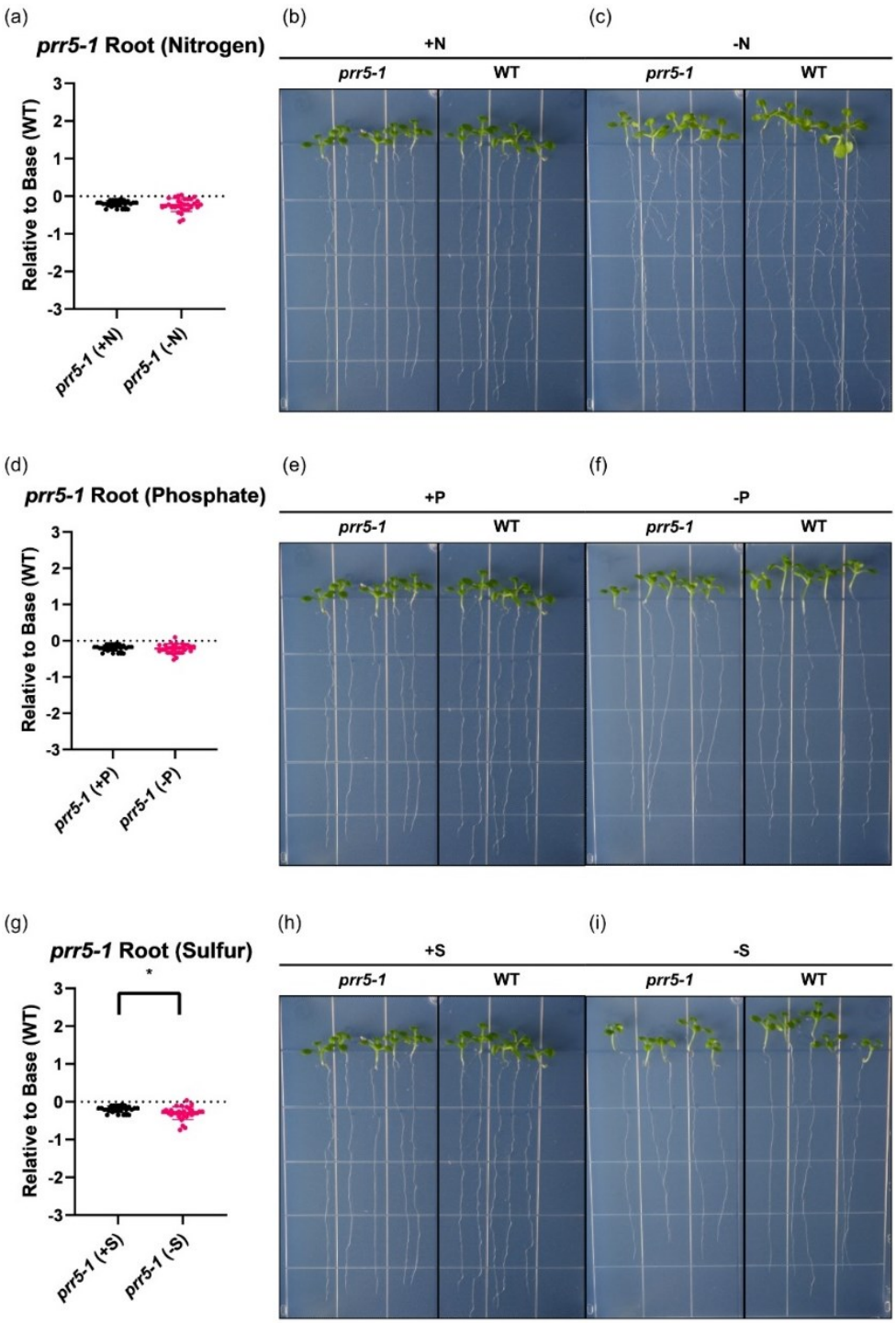

Figure 19: Root length changes in *prr5-1* plants under nitrogen, phosphorus, and sulfur stress. Quantitative analysis of seedling primary root length after 8 days of growth under control (CTL; a-b, d-e, g-h), -N (a, c), -P (d, f), or -S (g, i) conditions. An asterisk (*) denotes statistical significance (p-value ≤ 0.05; Student's t-test). Data are presented as mean ± standard deviation; n ≥ 30.

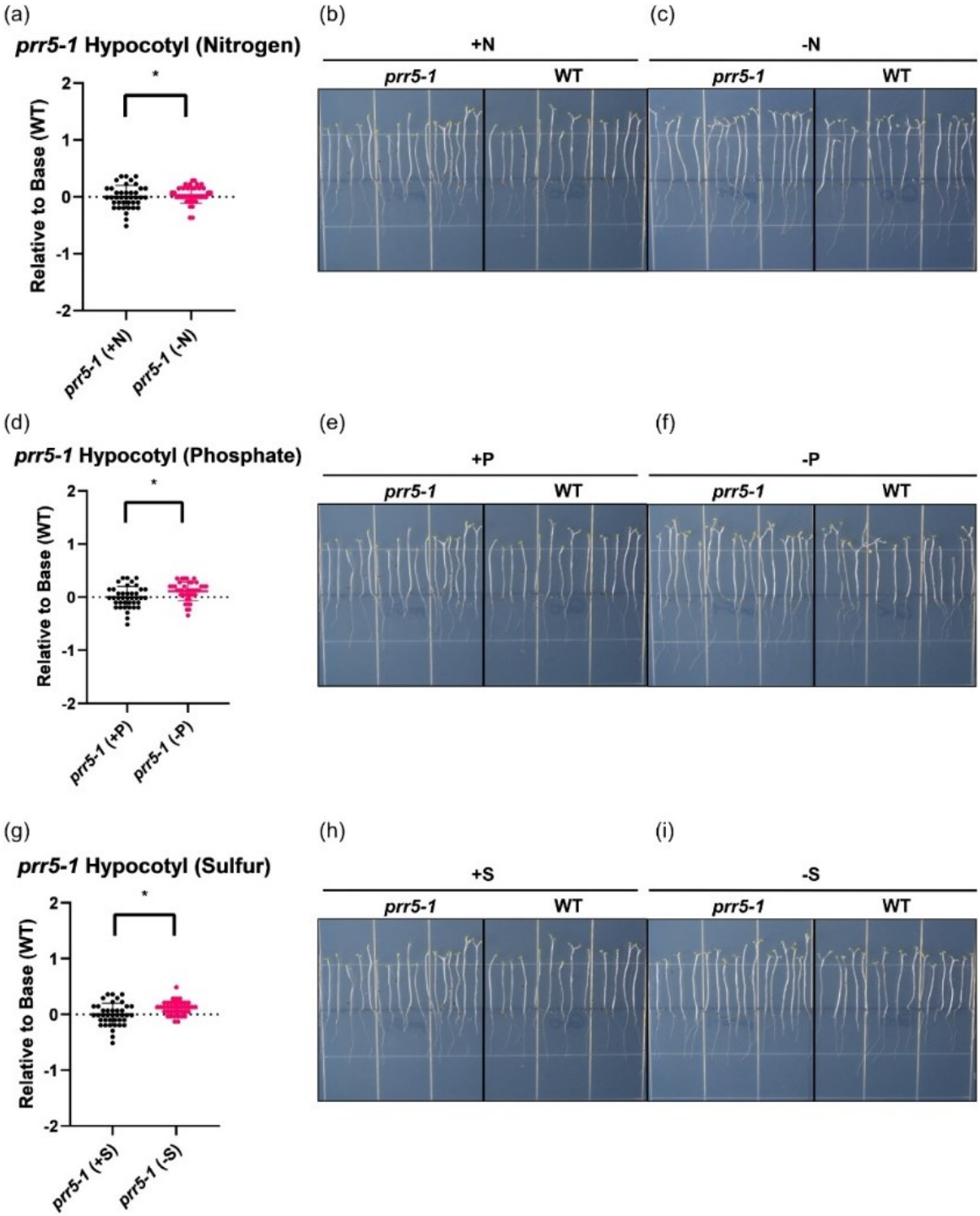

Figure 20: Hypocotyl length changes in *prr5-1* plants under nitrogen, phosphorus, and sulfur stress. Quantitative analysis of seedling hypocotyl etiolation lengths at the end of 4 days in the dark (post a 6-hour pulse of white light) under control (CTL; a-b, d-e, g-h), -N (a, c), -P (d, f), or -S (g, i) media. An asterisk (*) denotes statistical significance (p-value ≤ 0.05; Student's t-test). Data are presented as mean ± standard deviation; n ≥ 30.

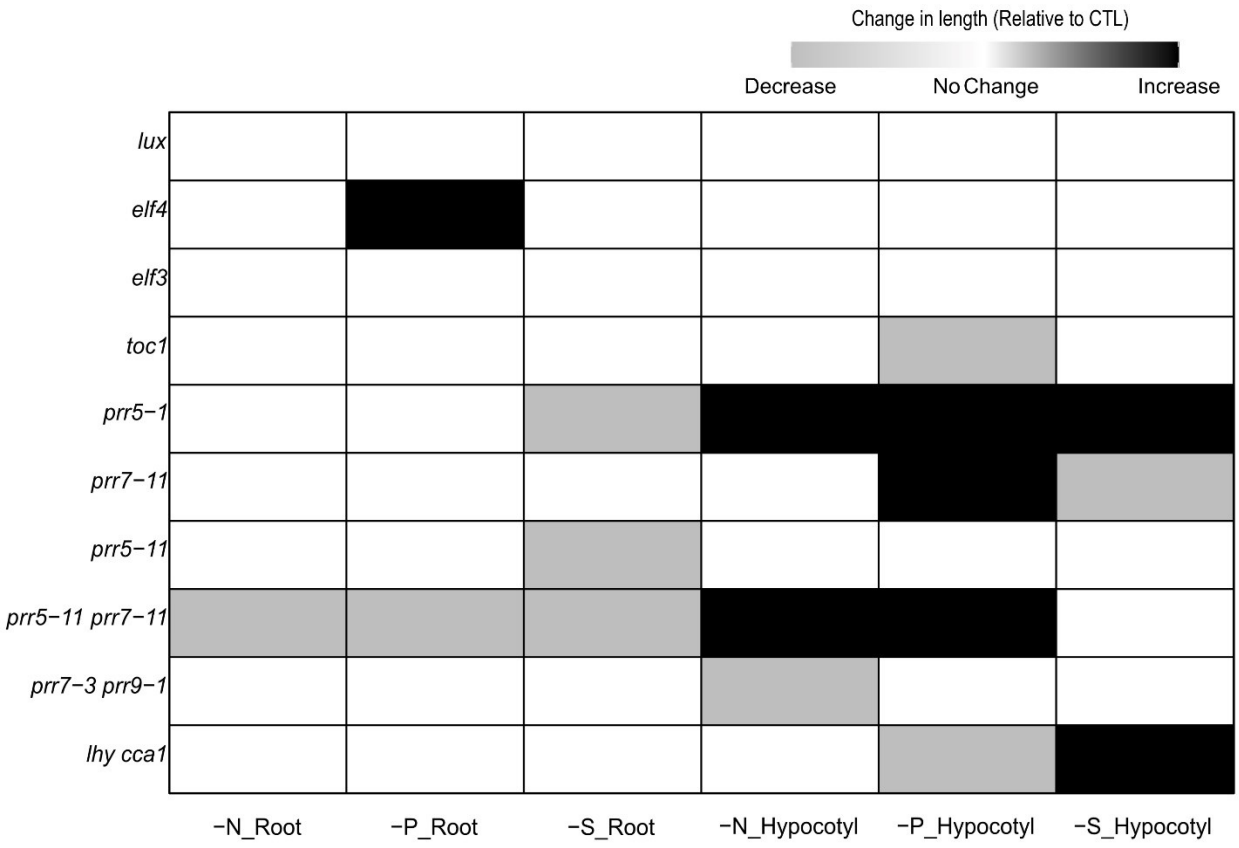

Figure 21: Heatmap summarizing primary root and hypocotyl responses of clock-deficient plants under N, P, or S stress. Coloration represents either a decrease (grey) or increase (black) in root and/or hypocotyl length, respectively, while white coloration indicates no statistically significant change in length. The resulting heat map was generated using the r package superheat (https://rlbarter.github.io/superheat/).

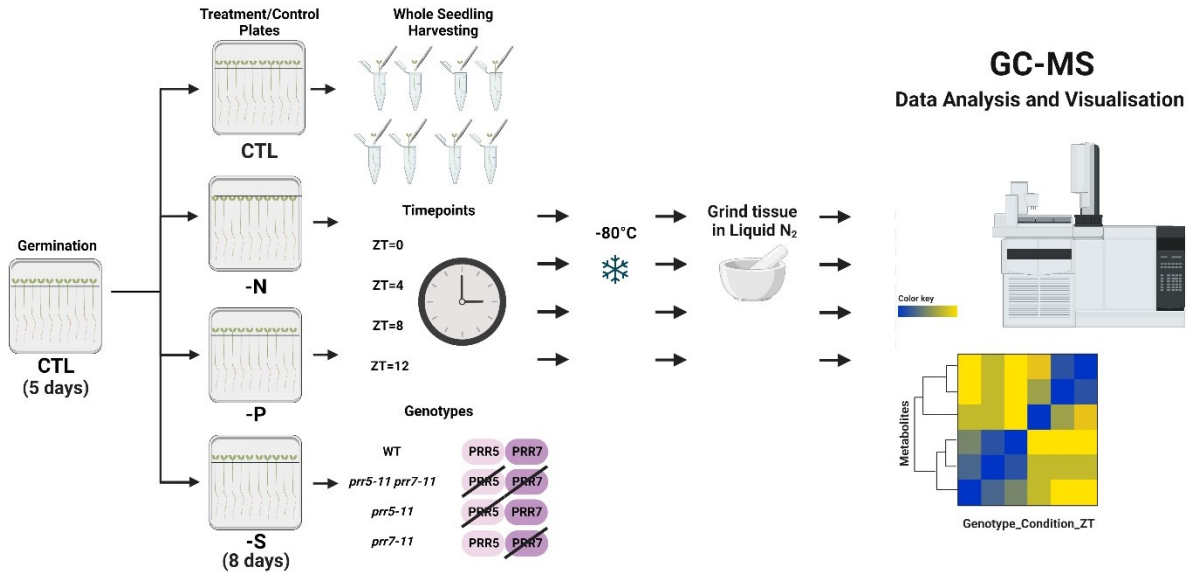

Figure 22: Time-of-day metabolomics workflow schematic. Depicted is the workflow for WT, *prr5-11 prr7-11*, *prr5-11*, and *prr7-11* seedlings under control (CTL), -N, -P, or -S conditions at ZT 0, 4, 8, and 12.

Table 2: Classification of metabolites identified through GC-MS analysis.

Amino Acid	Fatty Acid	Organic Acid	Organic Alcohol	Sugar	Other
Alanine	Benzoic Acid	Aspartic acid	Erythritol	Fructose	Neophytadiene
Asparagine	Oleic Acid	Citric acid	Glycerol	Glucose	Putrescine
Glutamine	Palmitic Acid	Fumaric acid	Myo-inositol	Sucrose	Uracil
Glycine	Stearic acid	Glutamic acid	Stigmasterol		Urea
Lysine		Glyceric acid			
Oxoproline		Glycolic acid			
Phenylalanine		Malic acid			
Serine		Myristic acid			
Threonine		Nicotinic acid			
Valine		Shikimic acid			
		Sinapinic acid			
		Succinic acid			

3.3.4 Diel metabolome analysis implicates PRR5 and PRR7 in N, P, and S nutrient stress responses

Given the results of my phenotyping screen (**Figure 21**), I wanted to further explore the roles of PRR5 and/or PRR7 in N, P, and S nutrient-related cell processes (**Figure 22**). As a result, I have analyzed the changing metabolome of WT, *prr5-11 prr7-11, prr5-11*, and *prr7-11* under control (CTL; **Figure 23**), -N (**Figure 24**), -P (**Figure 25**), or -S (**Figure 26**) conditions at ZT 0, 4, 8, and 12 (**Figure 21**; **Table 2**). The corresponding metabolite changes for each knockout line observed throughout the time-course were assembled into Euclidean distance clustered heat maps based on their Log2 fold change relative to WT metabolite levels for each condition at ZT 0, 4 ,8, and 12. First, to assess genotypic differences in diel metabolite changes, I analyzed each knockout plant line under CTL conditions. By analyzing the changing metabolite pools under CTL conditions, I can explore the genotypic effect of each knockout line early in the day, before adding the effect of nutrient stress. By conducting these series of analyses first, I am able to show that any modification in metabolite pools observed under nutrient stress are due to nutrition stress and not

an unforeseen confounding effect. Under CTL conditions, it appears that the largest pools of amino acids, fatty acids, organic acids, organic alcohols, and sugars, occur in the morning (namely at/between ZT0 and ZT4), while discernable reductions occur later in the day (specifically at/between ZT8 and ZT12) (**Figure 23**; **Table 2**).

Urea which are rich sources of N that can be used to synthesize amino acids *de novo* (Witte, 2011), pools at ZT0 are discernably lower in *prr5-11 prr7-11* and *prr5-11* (**Figure 23**). Citric acid and fumaric acid, which are important tricarboxylic acid (TCA) cycle intermediates used to synthesize ATP (Nakamichi et al., 2009), amounts are all notably elevated in *prr5-11 prr7-11* and *prr5-11* in the morning (**Figure 23**). The pool of aspartic acid, an organic acid which can be used to synthesize amino acids such as threonine (Alfosea-Simón et al., 2021), is the highest at ZT0 and decreases at ZT4 (**Figure 23**). Amounts of asparagine, oxoproline, glutamine, lysine and phenylalanine amino acids all drop in *prr5-11 prr7-11* and *prr5-11* at ZT12 (**Figure 23**). Levels of oleic acid, which is used in the biosynthesis of JA (Gao et al., 2010), metabolites remain elevated at ZT0 in *prr5-11 prr7-11* and *prr5-11*, before dropping at ZT4 and remaining low throughout the day (**Figure 23**). The levels of glucose and sucrose appears to drop around ZT4 in both *prr5-11 prr7-11* and *prr5-11*, before increasing at ZT8. Levels of glucose decrease in *prr5-11 prr7-11* and *prr5-11* at ZT0 and continues to decrease until ZT8, before increasing slightly at ZT12. Under CTL conditions, the metabolite profile seems to be quite similar between *prr5-11 prr7-11* and *prr5-11*, where pools are generally at the highest at ZT0 and lowest at ZT12 (**Figure 23**; **Table 2**).

3.3.5 Time-course metabolomics illustrates that *prr5-11 prr7-11* and *prr5-11* plants have different organic and fatty acid profiles, but similar amino acid pools under N and P stress

After examining metabolite profiles under CTL conditions, I then went on to explore how metabolite levels change under N, P, or S stress to elucidate which select metabolite perturbations might explain the root phenotype response detected under nutrient-deprived conditions (**Figure 21 - Figure 26**). Levels of glycine remain elevated in *prr5-11 prr7-11* and *prr5*-11 at all timepoints, but levels increase marginally around ZT8 to ZT12 in *prr5-11 prr7-11* under N stress (**Figure 24**). Under N stress, glutamine levels rise at ZT8 in *prr5-11 prr7-11* and *prr5-11*, where levels continue to remain elevated at ZT12 for both (**Figure 24**). Asparagine levels rise at ZT4 in *prr5-11 prr7-11* and *prr5-11*, before reaching the highest amounts at ZT8, and dropping at ZT12 in both (**Figure 24**). Levels of phenylalanine and asparagine are lowest at ZT0 for *prr5-11 prr7-11* and *prr5-11*

and reach maximal levels at ZT4 to ZT8 (**Figure 24**). Alanine levels peak at ZT0, before dropping at ZT4 and remaining low in *prr5-11 prr7-11* and *prr5-11* (**Figure 24**).

Levels of palmitic acid, which is important for lipid biosynthesis and metabolism (Carta et al., 2017) have maximum levels at ZT0, before decreasing and remaining relatively low throughout the day in *prr5-11 prr7-11*, *prr5-11*, and *prr7-11* (**Figure 24**). Fumaric acid amounts remain low throughout the day in *prr5-11 prr7-11*, however, levels rise slightly in *prr5-11 prr7-11* around ZT8 (**Figure 24**). Levels of citric acid and shikimic acid, which is important for aromatic amino acid biosynthesis (Leistner, 1999), differ greatly between *prr5-11 prr7-11*, *prr5-11*, and *prr7-11* (**Figure 24**). Levels of citric acid and shikimic acid are elevated at ZT0 in *prr5-11 prr7-11* and continue to rise slightly at ZT8, while levels remain consistently low in *prr5-11* and rise abruptly at ZT8 in *prr7-11* (**Figure 24**). Levels of glucose and fructose are lowest at ZT0 in *prr5-11 prr7-11*, rise at ZT4, and peak at ZT8 before dropping once more at ZT12. Levels of sucrose are the highest in *prr5-11 prr7-11* at ZT0 and drop throughout the day (**Figure 24**).

I then went on to analyze the perturbations in metabolite profiles under P stress to see if I could detect selected metabolite changes which would explain the primary root phenotypes observed in *prr5-11 prr7-11* (**Figure 21; Figure 25**). Levels of glycine rise in *prr5-11 prr7-11*, *prr5-11*, and *prr7-11* around ZT8 to ZT12 under P stress (**Figure 25**). Under P stress, glutamine levels rise at ZT4 in *prr5-11 prr7-11* and reach maximum amounts at ZT8 (**Figure 25**). Asparagine levels rise at ZT4 in *prr5-11 prr7-11* and *prr5-11*, before reaching the maximal amounts at ZT8, and dropping at ZT12 in both (**Figure 25**). Levels of phenylalanine are lowest at ZT0 for *prr5-11 prr7-11* and *prr5-11* and reach the highest levels at ZT8 in both genotypes, before dropping at ZT12 (**Figure 25**). Alanine levels rise at ZT0, in *prr5-11 prr7-11* and *prr5-11*, before reaching the highest levels at ZT4, and dropping at ZT12 in both (**Figure 25**). Fumaric acid pools start to rise at ZT0 in *prr5-11 prr7-11*, before reaching maximum levels at ZT8, and dropping at ZT12 (**Figure 25**). In *prr5-11*, levels of fumaric acid rise around ZT4, where levels reach maximum amounts at ZT12 (**Figure 25**). Levels of glucose are lowest at ZT0 in *prr5-11 prr7-11* and *prr5-11*, where levels rise at ZT4 and peak at ZT8, before dropping once more at ZT12 (**Figure 25**). Levels of fructose are lowest at ZT0 in *prr5-11 prr7-11* and *prr5-11*, but levels rise at ZT4, and peak at ZT8, before dropping once more at ZT12 (**Figure 25**). Levels of sucrose remain low throughout the day in *prr5-11 prr7-11* (**Figure 25**).

3.3.6 Time-of-day metabolomics suggests that *prr5-11 prr7-11* and *prr5-11* plants have similar metabolite profiles under S stress

Lastly, I analyzed the metabolite profile under S stress, to see if I could detect similar metabolite trends between *prr5-11 prr7-11* and *prr5-11* seedlings, which might partially explain why PRR5-deficient plants seem to have a similar phenotype response under S-lacking conditions (**Figure 21; Figure 26**). Levels of alanine rise to reach maximum amounts in both *prr5-11 prr7-11* and *prr5-11* at ZT8, before dropping at ZT12 (**Figure 26**). Under S stress, valine and threonine levels drop at ZT4 in *prr5-11 prr7-11* and *prr5-11*, before reaching maximum amounts at ZT8 (**Figure 26**). Glycine levels remain elevated at all timepoints in both *prr5-11 prr7-11* and *prr5-11* (**Figure 26**). Levels of serine and phenylalanine rise very sharply at ZT8 in *prr5-11 prr7-11* and *prr5-11*, before dropping at ZT12 (**Figure 26**). Glutamine levels drop at ZT0 in *prr5-11 prr7-11* and *prr5-11*, before rising to reach maximal levels at ZT8 and dropping at ZT12 in both (**Figure 26**).

Levels of palmitic acid start to drop at ZT0 before rising to maximum levels at ZT8 in *prr5-11 prr7-11* and *prr5-11* and decrease at ZT12 (**Figure 26**). Levels of oleic acid increase at ZT4 in *prr5-11 prr7-11* and *prr5-11*, before dropping at ZT12 in both (**Figure 26**). Aspartic acid levels decrease slightly from ZT0 to ZT8 in *prr5-11 prr7-11* and *prr5-11* before rising slightly at ZT12 (**Figure 26**). Levels of sinapinic acid, an organic acid that regulates seed germination (Bi et al., 2017), decrease in both *prr5-11 prr7-11* and *prr5-11* at ZT0, before rising at ZT8 and dropping again at ZT12 (**Figure 26**). Citric acid levels drop at ZT0 in *prr5-11 prr7-11* and *prr5-11* before rising to reach the highest levels at ZT8 in both (**Figure 26**). Levels of palmitic acid begin to drop at ZT0 in *prr5-11 prr7-11* and *prr5-11* and rise sharply at ZT8, before dropping once more at ZT12 (**Figure 26**). Levels of glucose are lowest at ZT4 in *prr5-11 prr7-11* and *prr5-11*, rise to peak amounts at ZT8, before dropping once more at ZT12 (**Figure 26**). Levels of sucrose seem to rise steadily from ZT0 in *prr5-11 prr7-11*, where maximum levels are reached at ZT8, before dropping once more at ZT12 (**Figure 26**). Levels of fructose also seem to rise in *prr5-11 prr7-11* and *prr5-11* at ZT4, to peak at ZT8, before dropping once more at ZT12 (**Figure 26**).

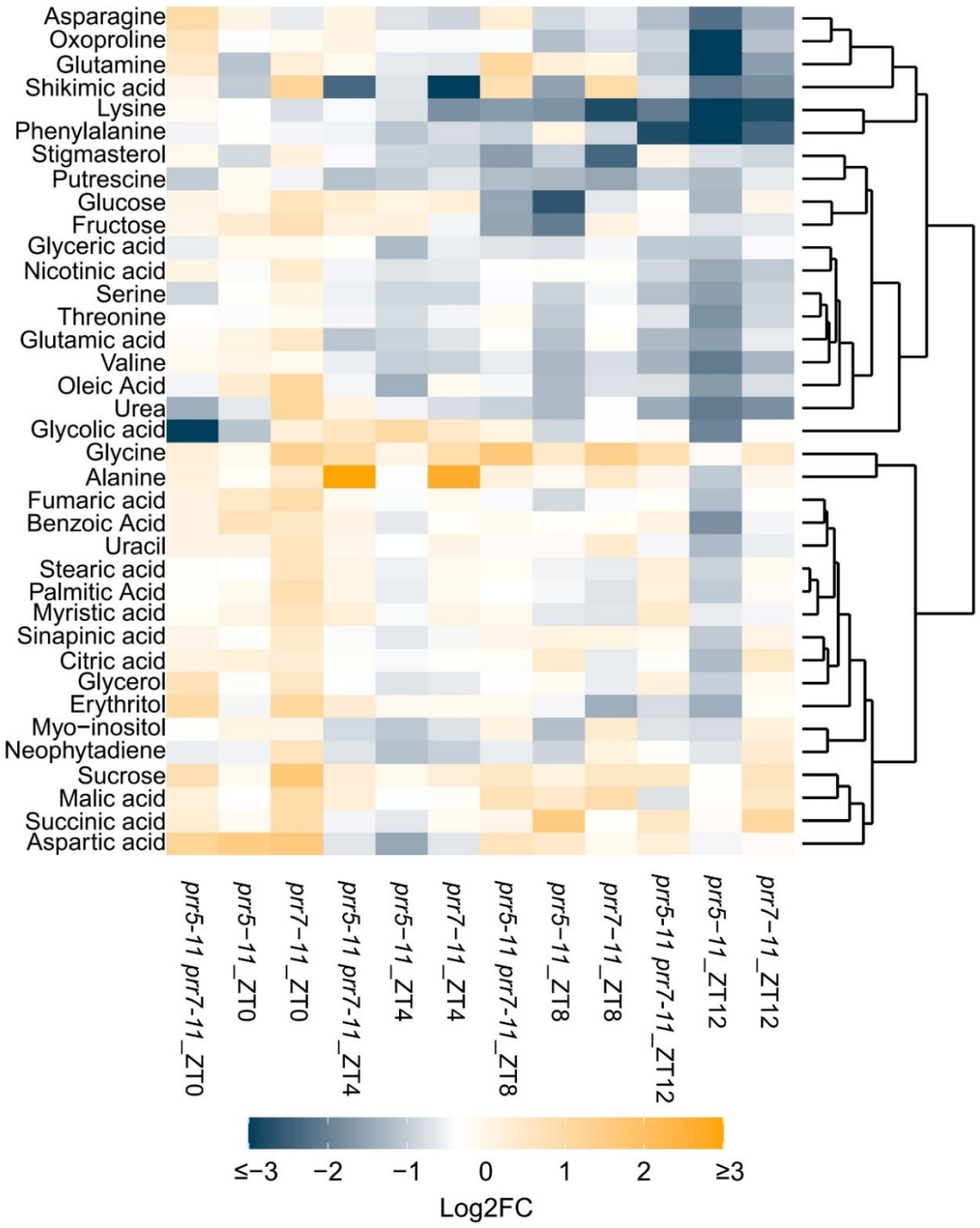

Figure 23: Relative Log2FC in diel metabolite pools in *prr5-11 prr7-11*, *prr5-11*, and *prr7-11* seedlings, relative to WT at ZT0, 4, 8, and 12 under control (CTL) conditions. A heat map of diel metabolite changes within *prr5-11 prr7-11*, *prr5-11*, and *prr7-11* whole seedlings, relative to WT. Scale represents Log2 fold-change (FC). Blue-to-white-to yellow coloration represents increasing Log2FC values. Log2FC values from ≤ -3 towards 0 and values from 0 ≥ 3 are shown. The resulting heat map was generated using the r package superheat (https://rlbarter.github.io/superheat/).

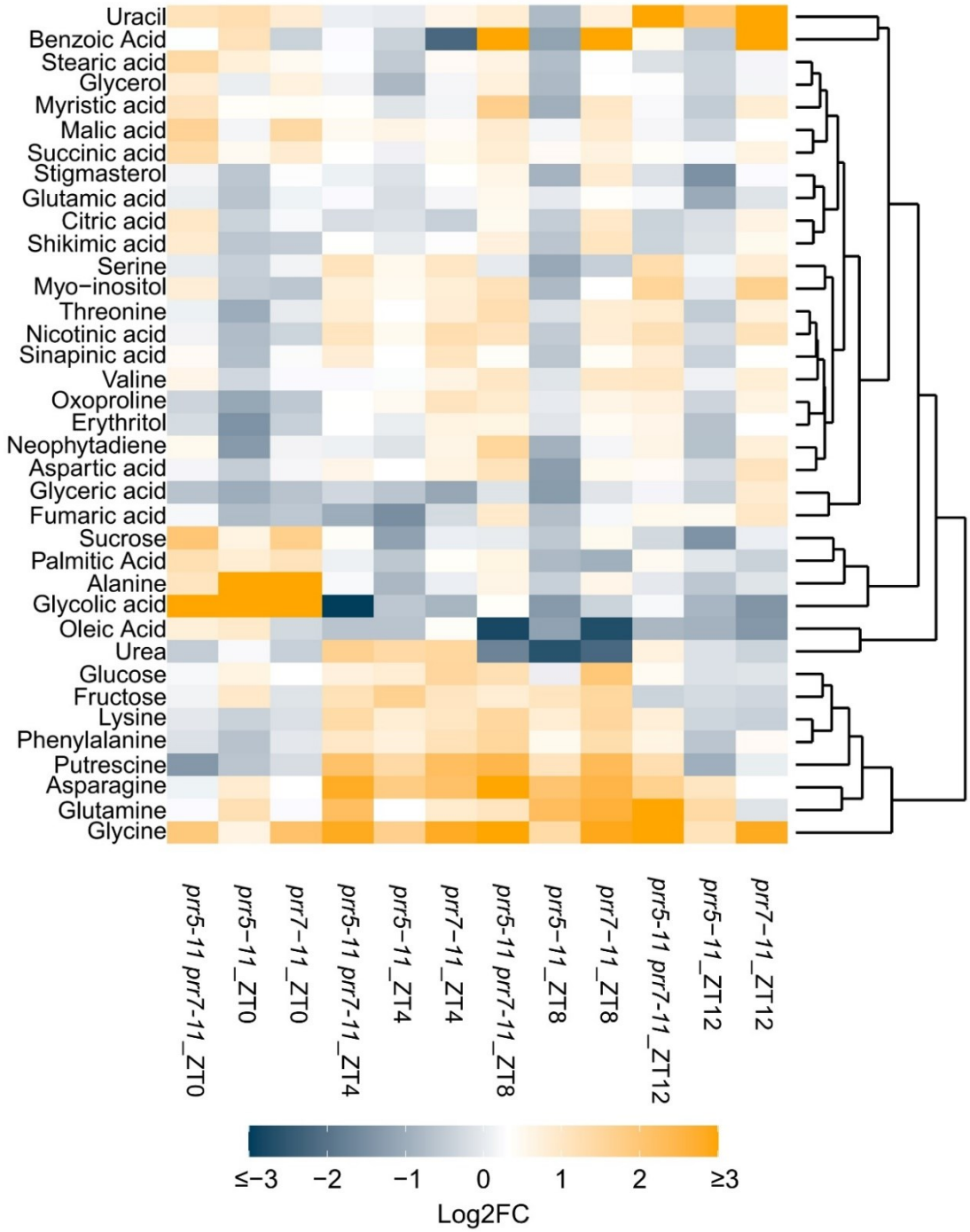

Figure 24: Relative Log2FC in diel metabolite pools in *prr5-11 prr7-11*, *prr5-11*, and *prr7-11* seedlings, relative to WT at ZT0, 4, 8, and 12 under N-deficient (-N) conditions. A heat map of diel metabolite changes within *prr5-11 prr7-11*, *prr5-11*, and *prr7-11* whole seedlings, relative to WT. Scale represents Log2 fold-change (FC). Blue-to-white-to yellow coloration represents increasing Log2FC values. Log2FC values from ≤ -3 towards 0 and values from 0 ≥ 3 are shown. The resulting heat map was generated using the r package superheat (https://rlbarter.github.io/superheat/).

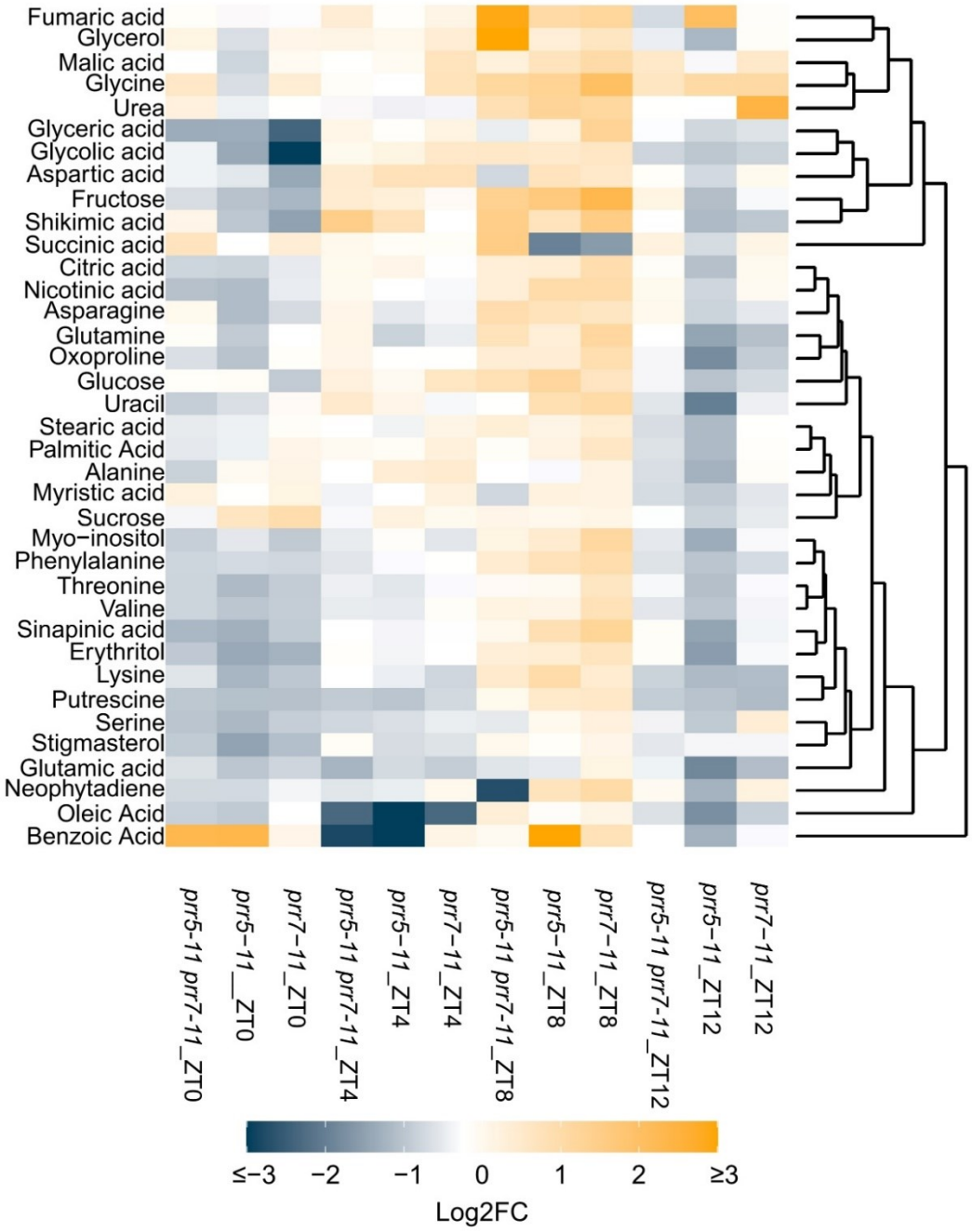

Figure 25: Relative Log2FC in diel metabolite pools in *prr5-11 prr7-11*, *prr5-11*, and *prr7-11* seedlings, relative to WT at ZT0, 4, 8, and 12 under P-deficient (-P) conditions. A heat map of diel metabolite changes within *prr5-11 prr7-11*, *prr5-11*, and *prr7-11* whole seedlings, relative to WT. Scale represents Log2 fold-change (FC). Blue-to-white-to yellow coloration represents increasing Log2FC values. Log2FC values from ≤ -3 towards 0 and values from 0 ≥ 3 are shown. The resulting heat map was generated using the r package superheat (https://rlbarter.github.io/superheat/).

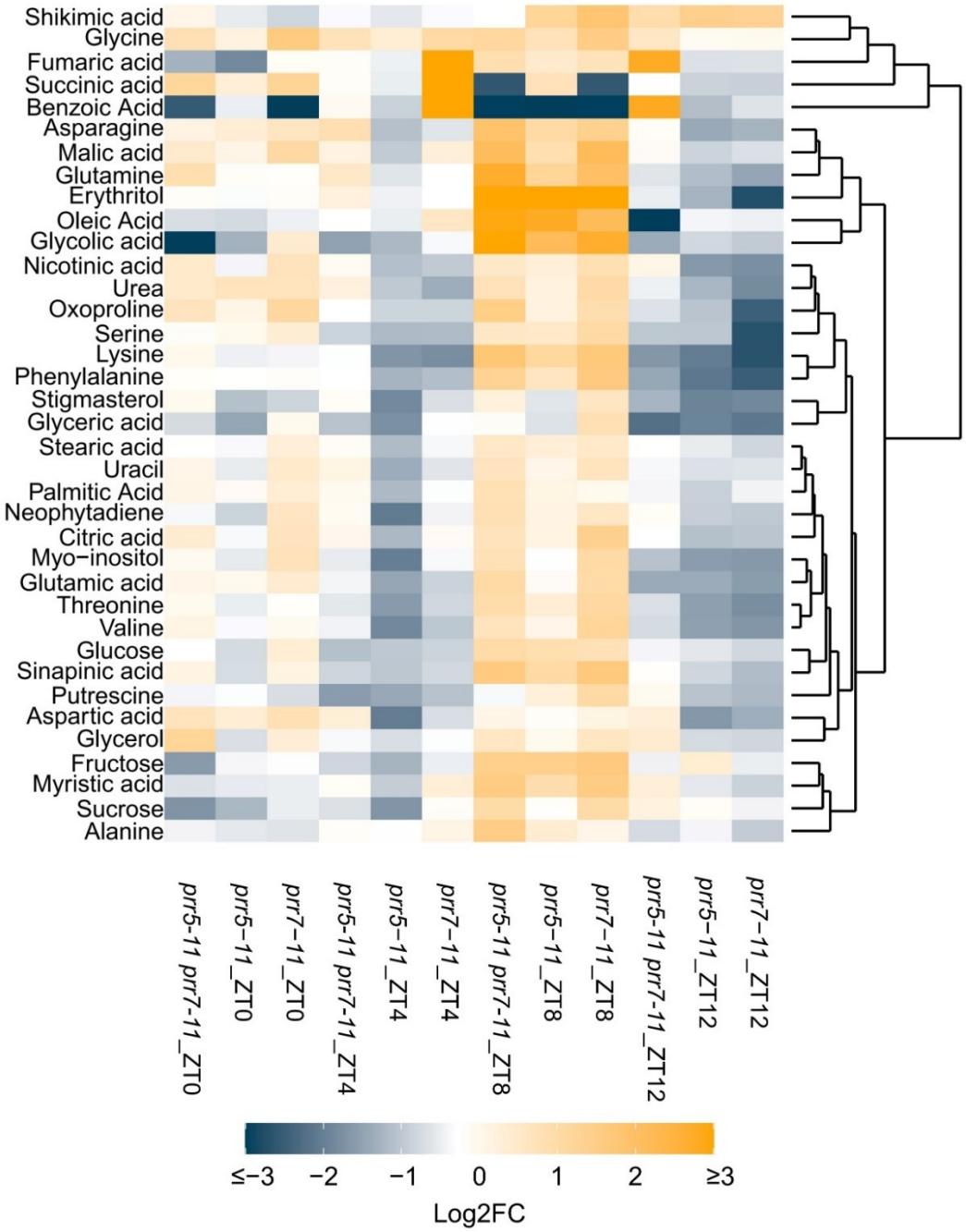

Figure 26: Relative Log2FC in diel metabolite pools in *prr5-11 prr7-11*, *prr5-11*, and *prr7-11* seedlings, relative to WT at ZT0, 4, 8, and 12 under S-deficient (-S) conditions. A heat map of diel metabolite changes within *prr5-11 prr7-11*, *prr5-11*, and *prr7-11* whole seedlings, relative to WT. Scale represents Log2 fold-change (FC). Blue-to-white-to yellow coloration represents increasing Log2FC values. Log2FC values from ≤ -3 towards 0 and values from 0 ≥ 3 are shown. The resulting heat map was generated using the r package superheat (https://rlbarter.github.io/superheat/).

3.4 DISCUSSION

3.4.1 Time-of-day quantitative metabolomics implicates PRR proteins in the regulation organic acid metabolic processes

Metabolomic screens of PRR-family mutants have implicated PRR5, PRR7, PRR9, and TOC1 in regulating plant metabolism (Cervela-Cardona et al., 2021; Flis et al., 2019; Fukushima et al., 2009). Plants with *prr5 prr7 prr9* alleles have enhanced levels of TCA cycle intermediates, including, citrate, fumarate, and succinate from ZT 7 to ZT 19 in diel conditions (Fukushima et al., 2009). Further, levels of proline was shown to be elevated at all time-points in *prr5 prr7 prr9*, relative to WT (Fukushima et al., 2009). Moreover, elevated pools of glycine, glutamate, and shikimate was also reported to be elevated throughout the day in *prr5 prr7 prr9*, relative to WT (Fukushima et al., 2009). Plants lacking in proper *PRR7* and *PRR9* expression have higher amounts of malate, fumarate, citrate, isocitrate, and aconitate between ZT6 and ZT16, along with elevated pools of shikimate (Flis et al., 2019). Plants deficient in TOC1 have increased pools of fumarate at ZT11 and ZT23, consistent with the metabolite pools observed in *prr5 prr7 prr9* (Fukushima et al., 2009), implicating PRR-family proteins in the regulation of TCA cycle intermediates (Cervela-Cardona et al., 2021). In my study, I find that plants deficient in PRR5 and/or PRR7 proteins have high levels of organic acid metabolites in the morning under CTL conditions (**Figure 23**). In particular, I find elevated amounts of fumaric acid (FA) and citric acid (CA) at ZT0 in *prr5-11 prr7-11* ($Log_2FC = 0.06$ and 0.10, respectively) (**Figure 23**). My data illustrates that pools of TCA cycle intermediates are elevated in plants deficient in PRR5, while prior works show elevated levels of the same metabolites in *prr7 prr9* and *toc1* plants (Cervela-Cardona et al., 2021; Flis et al., 2019). Taken together, my work adds to the observations of prior works by showing that plants lacking in only PRR5, have elevated levels of TCA intermediates, which shows that there is specificity with respect to the regulation of TCA intermediates.

My results differ from the more extreme phenotypes of *prr5 prr7 prr9* plants, where pools of FA were observed to be elevated at all timepoints, suggesting that plants deficient in PRR5, PRR7, and PRR9 are unable to regulate TCA pools throughout the day (**Figure 23**; Fukushima et al., 2009). Plants with *prr5 prr7 prr9* alleles are arrhythmic and do not show a typical circadian peak and trough expression pattern throughout the day (Nakamichi et al., 2005b). Carbon metabolism is under tight circadian control, such that plants with clock knockout alleles have starch profiles that differ from WT throughout the day under diel conditions (Flis et al., 2019).

TCA cycle intermediates are used in ATP biosynthesis and carbon assimilation (Nakamichi et al., 2009). Thus, taken together, it seems that arrhythmic nature of *prr5 prr7 prr9* plants prevents these genotypes from regulating carbon stores in a circadian manner.

Plants with *prr7 prr9* alleles have a longer period than WT (Nakamichi et al., 2005b), while *prr5-11 prr7-11* plants have a faster period than WT (Nakamichi et al., 2005b). Flis et al., (2019) show that *prr7 prr9* have elevated pools of FA throughout the day, while I show that FA pools are elevated at ZT0 in *prr5-11 prr7-11*, but levels remain comparable to WT levels later in the day (**Figure 23**). It appears that plants with a faster clock seem to assimilate TCA intermediates faster than plants with a slower clock. Cumulatively, it seems that the clock is intimately tied to TCA intermediate pools and C metabolism, such that plants with a faster clock accumulate TCA pools earlier, while plants with a later clock do not (**Figure 23**; Flis et al., 2019; Fukushima et al., 2009).

3.4.2 Plants with *prr5-11 prr7-11* alleles increase select pools of amino acids and organic acids, partially explaining the primary root response under N stress

Plants with *prr5-11 prr7-11* alleles have shorter primary roots under N-stress, which is not detected in *prr5-11* or *prr7-11* plants (**Figure 21**). Thus, I wanted to elucidate what the impact of N stress is on *prr5-11 prr7-11* plants at the metabolome level (**Figure 24**). Upon subjecting *prr5-11 prr7-11* seedlings to N-stress, glycine pools increase throughout the day from ZT0 (Log_2FC = 1.93) to ZT12 (Log_2FC =3.75) (**Figure 24**). My data suggests that *prr5-11 prr7-11* positively upregulates glycine levels under N stress to mediate the effects of N starvation (**Figure 24**), while also stunting its own primary root growth (**Figure 21**). Interestingly, approximately 18–29% of the total N that is taken up by plants consists of glycine (Ma et al., 2016). Glycine is an organic amino acid that increases carbon assimilation, resulting in higher levels of sucrose and glucose in plants (Liu et al., 2016). The application of exogenous glycine increases the abundance of antioxidant compounds, such as anthocyanin (Yang et al., 2017). N-mediated anthocyanin production aids Arabidopsis plants under nitrogen stress, where plants which cannot accumulate anthocyanin showed a lower germination rate under N stress (Liang & He, 2018). ARABIDOPSIS THALIANA PRODUCTION OF ANTHOCYANIN PIGMENT 1 (PAP1; AT1G56650) is an enzyme that regulates anthocyanin production in plants (Liang & He, 2018). Plants void of proper PAP1 (*pap1*) have a much lower germination rate than WT under N stress (Liang & He, 2018).

Glycine inhibits root growth and causes an increase in carbohydrate concentration in plant roots (Domínguez-May et al., 2013), which is consistent with our observed decrease in root length (**Figure 21**).

Subjecting *prr5-11 prr7-11* to N stress directly causes asparagine pools to increase throughout the day from ZT0 ($Log_2FC = 0.11$) to ZT8 ($Log_2FC = 3.23$) (**Figure 24**). Asparagine is a highly abundant amino acid in plant xylem tissue, being the amino acid with the largest number of N per carbon unit, making asparagine an effective store of nitrogen that moves N throughout the plant (Gaufichon et al., 2010). Asparagine is synthesized *de novo* in plants via a two-step process, where glutamine synthetase (GS) enzymes incorporate free-flowing ammonium into glutamine which can then be synthesized into asparagine via asparagine synthetase (Oddy et al., 2020). Asparagine has protective characteristics, such that plants treated with exogenous asparagine have increased chlorophyll pools (Kaya et al., 2013). Consistent with our results, plants treated with exogenous asparagine also have shorter roots, but perform better, having higher nitrogen use efficiency in N-free conditions, as similar to glycine, asparagine can be utilized as an organic N source (Qu et al., 2019; Han et al., 2022). Thus, it appears that *prr5-11 prr7-11* positively upregulates asparagine levels under N stress to improve its N usage in low N conditions (**Figure 24**). However, while this may confer homeostatic properties, it is also a way in which *prr5-11 prr7-11* seedlings are stunting their primary root growth (**Figure 21**).

Upon subjecting *prr5-11 prr7-11* plants to N stress, benzoic acid (BA) levels increase before the end of the day from ZT4 ($Log_2FC = 0.25$) to ZT8 ($Log_2FC = 3.72$) (**Figure 24**). BA is an organic acid that functions as a precursor to SA (Widhalm & Dudareva, 2015). SA limits root growth under nitrogen-limiting conditions (Conesa et al., 2020), aiding in the plant's response to low N conditions by increasing the concentration of osmoprotecting compounds, such as anthocyanin (Heidari, 2020; Liang & He, 2018). Plants accumulate higher amounts of SA under N-starvation, compared to N-fed conditions (Singh & Chaturvedi, 2012). Plants treated with exogenous SA also have higher N use efficiency, compared to control conditions (Singh & Chaturvedi, 2012). More directly, plants treated with exogenous BA present with shorter primary roots due to shorter and smaller numbers of apical meristematic cells in Arabidopsis root organs (Zhang et al., 2018). Cumulatively, it appears that *prr5-11 prr7-11* positively upregulates BA levels under N-stress, suggesting a likely increase in SA production (**Figure 24**), which could also partially explain its primary root phenotype (**Figure 21**).

3.4.3 Plants with *prr5-11 prr7-11* alleles increase organic alcohols and TCA intermediates under P stress

Plants with *prr5-11 prr7-11* alleles also have shorter primary roots under P-stress (**Figure 21**), to explain why this occurs, I characterized the changing metabolome of *prr5-11 prr7-11* plants under P-stress (**Figure 25**). Upon subjecting *prr5-11 prr7-11* to P-stress, glycerol pools increase early in the day from ZT0 (Log_2FC = 0.18) to ZT8 (Log_2FC = 3.46) (**Figure 25**). Glycerol represents an important precursor to the central primary metabolite glycerol-3-phosphate (Venugopal et al., 2009). The application of exogenous glycerol results in a buildup of glycerol-3-phosphate in plant cells, as opposed to bioavailable phosphate (Aubert et al., 1994). The buildup of glycerol-3-phosphate then prevents metabolic flux through the pentose-phosphate pathway due to a lack of endogenous phosphate, leading to stalled plant growth and development (Andriotis & Smith, 2019). Plants under phosphate stress also have increased levels of glycerol-3-phosphate (Ramaiah et al., 2011). Further, the addition glycerol increases pools of SA in plants (Kachroo et al., 2005), which is also increased in response to P-stress (Gulabani et al., 2021). In particular, PHOSPHATE TRANSPORTER 4;1 (PHT4;1; AT2G29650) acts upstream of SA-biosynthesizing gene *SALICYLIC ACID INDUCTION DEFICIENT 2* (*SID2*; AT1G74710), to regulate endogenous pools of SA. Collectively, the upregulation of glycerol in *prr5-11 prr7-11* under P-stress suggests that a combined increase in two downstream root truncating metabolites glycerol-3-phosphate and SA may be responsible for the observed root phenotype.

Upon subjecting *prr5-11 prr7-11* plants to P-stress, FA levels increase throughout the day from ZT0 (Log_2FC = -0.17) to ZT8 (Log_2FC = 2.90) (**Figure 25**). FA is synthesized from succinic acid via succinic dehydrogenase, which is also supported by my dataset, as succinic acid levels also rise from ZT0 (Log_2FC = 0.68) to ZT8 (Log_2FC = 1.56) (**Figure 25**; Jardim-Messeder et al., 2015). TCA cycle intermediates accumulate in plants due to the incomplete oxidation of carbohydrates yielding lower pools of adenosine triphosphate (ATP) (Igamberdiev & Eprintsev, 2016). P-stress reduces the concentration of bioavailable phosphate in plant cells, which can lower the production of ATP (Carstensen et al., 2018). Plants lacking in proper PHT4;1 function have reduced levels of ATP and more acidic thylakoids, suggesting that ATP synthase relies on the proper functioning of PHT4;1, and that a lack of proper P assimilation results in more acidic leaf tissue (Karlsson et al., 2015). Phosphate transporter 2;1 (PHT2;1; AT3G26570) also resides within

the chloroplast and increases the amount of phosphate in plant leaf tissue under CTL/P-fed conditions (Versaw & Harrison, 2002). Plants with deficiencies in ATP synthase fare poorly by having defects in male and female gametophyte tissue (Geisler et al., 2012). Further, acidic cellular conditions causes an increase in ROS accumulation in plants (Hasanuzzaman et al., 2020). Plants function optimally at a pH of 7 to 7.5 (Shavrukov & Hirai, 2015), where more acidic conditions disrupts cellular protein function (Kochian et al., 2015). Thus, with greater accumulations of TCA intermediates in the chloroplast, it is plausible that due to a disruption of ATP synthase activity or an overaccumulation of ROS, that *prr5-11 prr7-11* plants would fare worse under P-stress (**Figure 21**).

3.4.4 Plants with *prr5-11 prr7-11* and *prr5-11* alleles fare worse under S stress due to increases in fatty acids and differential pools of select amino acids

Plants with *prr5-11 prr7-11* and *prr5-11* alleles have shorter primary roots under S stress, which is not detected in *prr7-11* plants (**Figure 21**). To elucidate why PRR5-lacking plants fare worse under -S, I characterized the changing metabolome of *prr5-11 prr7-11* and *prr5-11* plants to elucidate the underlying metabolic changes at the cellular level (**Figure 26**). Both *prr5-11 prr7-11* and *prr5-11* show an increase in oleic acid (OA; an unsaturated fatty acid) pools from ZT0 (Log_2FC = -0.71 and -0.77, respectively) to ZT8 (Log_2FC = 2.94 and 2.81, respectively) under S stress. Plant organic fatty acids are synthesized in response to stress (He & Ding, 2020). OA is required for the activation of JA-mediated responses in Arabidopsis (Wasternack & Hause, 2002), such that a reduction in OA is met with a corresponding reduction in JA-derived responses (Gao et al., 2010; Kachroo et al., 2003). Plants with improper fatty acid desaturation enzymes such as FATTY ACID BIOSYNTHESIS 2 (FAB2; AT2G43710), when treated with exogenous OA, showed a restored JA response (Kachroo et al., 2001), illustrating how essential OA is to JA-mediated processes.

JA and sulfur metabolism are intimately linked, such that key sulfur-metabolizing enzymes also regulate JA pools in Arabidopsis. MYB DOMAIN PROTEIN (MYB) 34 (AT5G60890), 51 (AT1G18570), and 122 (AT1G74080) regulate glucosinolate biosynthesis in Arabidopsis, wherein MYB-lacking plants (*myb34 myb51 myb122*) have lower glucosinolate pools (Frerigmann & Gigolashvili, 2014). Glucosinolates are S-rich metabolites that function as stores of bioavailable sulfate in plants, which are remobilized in response to S-deficiency (Aarabi et al., 2020). Plants

treated with exogenous JA have decreased expression of *MYB51*, suggesting that JA preferentially decreases glucosinolate biosynthesis (Frerigmann & Gigolashvili, 2014). JA has been shown to mediate the primary root length by decreasing the number or size of cells in the elongation zone, restricting root elongation (Valenzuela et al., 2016; Yang et al., 2016). More directly, the exogenous application of OA results in shorter plant roots in Arabidopsis (Di Fino et al., 2020). It is possible that *prr5-11 prr7-11* and *prr5-11* plants have shorter roots under S stress, due to an increased amount of OA later in the day, which may be the result of S-compound breakdown and S remobilisation (**Figure 21**; **Figure 26**; Aarabi et al., 2020).

Subjecting *prr5-11 prr7-11* and *prr5-11* plants to S-stress also causes serine pools to decrease late in the day from ZT8 (Log_2FC = 0.55 and 0.51, respectively) to ZT12 (Log_2FC = -1.01 and -1.02, respectively) (**Figure 26**). Serine is an important amino acid, in that it is a precursor for cysteine: under S-stress, pools of cysteine are consumed, leading to the breakdown of N-rich proteins to biosynthesize S-containing compounds (Nikiforova et al., 2006). Under S-stress, Arabidopsis plants seem to maintain elevated pools of cysteine-intermediate metabolite O-acetylserine in leaf tissue (Krueger et al., 2010). Plants subjected to S-deficient conditions also have increased activity of acetylserine sulfhydrylase, the enzyme responsible for catalyzing the production of cysteine, suggesting that the biosynthesis of cysteine from serine is induced under S-stress (León et al., 1988). Cysteine is also a key player in sulfur metabolism, being the only amino acid capable of forming the disulfide bridge in proteins (Wiedemann et al., 2020). The application of exogenous cysteine results in shorter plant roots in a concentration-dependent manner, where higher amounts of cysteine inhibit root elongation through auxin accumulation in the root tips (Wang et al., 2014). Overall, my data suggests that *prr5-11 prr7-11* and *prr5-11* plants have shorter roots under S stress, due to decreased amounts of serine late in the day, which might contribute to the increased levels of cysteine biosynthesis under S stress (**Figure 21**; **Figure 26**; Hu et al., 2014).

Summary

Together, the data presented within **chapter 3** suggests that the clock is intimately involved in nutrient stress responses in Arabidopsis (**Figure 21**). More specifically, it seems that plants with *prr5-11 prr7-11* alleles fare worse under N-stress, P-stress, and S-stress, while plants lacking in *prr5-11* seem to fare worse under S-stress, suggesting that PRR5-controlled proteins are especially

impacted by S-stress (**Figure 26**), consistent with my phenotyping screen (**Figure 21**). I have shown that under N-stress, *prr5-11 prr7-11* plants increase the pools of glycine, asparagine, and BA, which all partially explain the shorter root phenotype that is observed (**Figure 24**). I have illustrated that under P stress, *prr5-11 prr7-11* plants increase the concentration of glycerol and FA, which might explain the root truncation that is observed (**Figure 25**). I have shown that *prr5-11 prr7-11* and *prr5-11* plants increase and decrease the amount of OA and serine, respectively at selected time-points which could partially explain the phenotypes observed (**Figure 26**). Since *prr5-11 prr7-11* fare worse under N-stress, P-stress, and S-stress, a closer look at the impact of each stressor needs to be explored to understand precisely which regulatory processes are at play to confer the reported phenotypes. This will allow for the complete characterization of how *prr5-11 prr7-11* plants operate under diverse nutrient stressors, while providing new insights as to the full impact of nutrient stress on plant homeostasis.

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Chapter 4:

Overarching Perspectives, Conclusions, and Future Directions

4.1 Summary and significance

As the circadian clock regulates approximately 40% of the genes in *Arabidopsis* (Romanowski et al., 2020), understanding the impact of different inputs on the clock is of paramount importance in the field of plant chronobiology. In **Chapter 2**, I analyzed the role of REVIELLE proteins (the sole elucidated activator of the circadian clock) in the osmoregulatory process in *Arabidopsis* using *rve 4 6 8* knockout lines (Hsu et al., 2013). I subjected WT and *rve 4 6 8* plants to osmotic and salt stress to see if I could detect phenotypic differences under drought-like stress (**Chapter 2**). I showed that plants lacking in RVE8-like proteins fare worse when subjected to osmotic or salt stress (**Chapter 2**).

I then looked at the changing proteome of WT and *rve 4 6 8* whole seedlings at ZT11 and ZT23 under CTL conditions (**Chapter 2**). I found that proteins which confer osmotolerance were differentially abundant in WT (**Chapter 2**), illustrating that plants which lack RVE8-like proteins fare worse under drought-like stress due to a lack of osmoprotection-conferring proteins. Given this encouraging set of results under CTL conditions, I then analyzed the proteome differences between WT and *rve 4 6 8* seedlings under osmotic and salt stress (**Chapter 2**). Under osmotic stress, I showed that tryptophan biosynthesis proteins were differentially abundant in WT plants (**Chapter 2**). This suggests that plants lacking in RVE8-like proteins could have lower pools of the osmoprotecting osmolyte, melatonin due to lower tryptophan pools (Chen et al., 2009; Mannino et al., 2021), partially explaining why WT do better under mannitol (**Chapter 2**).

Under salinity stress, I saw that JA biosynthetic proteins were differentially abundant in WT, relative to *rve 4 6 8*, indicating that plants lacking in RVE8-like proteins might have lower pools of endogenous JA (**Chapter 2**). This difference in JA biosynthesis between WT and *rve 4 6 8* may explain why the difference in root length under mannitol between WT and RVE8-lacking proteins is more exaggerated, as opposed to the root truncation observed under salt stress (**Chapter 2**). JA partially mediates primary root truncation, by decreasing the number or size of cells in the elongation zone (Valenzuela et al., 2016), such that higher levels of JA biosynthesis might partially result in shorter primary roots.

I then went on to discover differences in Glutathione transferase (GST) enzymes under CTL, mannitol and NaCl (**Chapter 2**). Here, I found that under CTL conditions, there is a

differential abundance of the GST supergene family proteins in WT, which could contribute to the elevated osmotolerant response of WT under abiotic stress (**Chapter 2**). GST proteins catalyze the fusion of glutathione metabolites to toxins for vacuolar isolation (Mauch & Dudler, 1993), preventing toxic compounds from causing irreparable damage to the cell. I show that the pools of GST enzymes remain differentially abundant in WT under mannitol stress, which might directly, partially confer osmotolerance under osmotic stress (**Chapter 2**). I also show that the pools of GST enzymes become differentially abundant in *rve 4 6 8* under salt stress (**Chapter 2**). Given this, I make the case that RVE8-lacking plants seem to fare better under salt stress, as opposed to mannitol stress, because of a greater abundance of GST enzymes under salt stress in *rve 4 6 8* (**Chapter 2**).

In **Chapter 3**, I explore the role of the clock in the N, P, and S plant nutrition processes. I screen a large number of clock deficient plant lines for their growth under each nutrient deficient condition (**Chapter 3**). Here, I found that the morning loop deficient mutants were susceptible to N, P, and S deficient conditions, while the evening loop was more susceptible to P stress (**Chapter 3**). In my phenotyping screen, I measured changes in both root and hypocotyl elongation (**Chapter 3**). Based on this screen, I then went on to further examine *prr5* and *prr7* deficient plants in more detail by using *prr5-11*, *prr7-11* and *prr5-1* mutant lines (**Chapter 3**). These series of phenomic results suggested that plants that lack both PRR5 and PRR7 proteins fare worse than plants which lack either PRR5 or PRR7 proteins, while also suggesting that PRR5 and PRR7 proteins seem to have disparate roles in nutrient related phenotype processes (**Chapter 3**).

After doing these sets of experiments, I attempted to elucidate the roles of PRR5 and PRR7 proteins in the N, P, and S metabolic response by using time-of-day GC-MS metabolomics (**Chapter 3**). In these series of experiments, I looked at the changing metabolome of *prr5-11 prr7-11*, *prr5-11*, and *prr7-11* plants under CTL and -N, -P, or -S conditions at ZT 0, ZT 4, ZT 8, and ZT 12, as the PRR proteins are expressed between ZT 0 and ZT 12 (**Chapter 3**; Nakamichi et al., 2010). I was able to show that, while metabolite pools tend to be the highest at ZT0 and the lowest at ZT12 under CTL conditions, under -P and -S stress, nearly all mutants had the highest concentration of metabolites at ZT8 (**Chapter 3**). Further, I was also able to detect nuanced differences in metabolite profiles between CTL and -N conditions, where the overall pool of glycine amino acid metabolites remained significantly elevated in *prr5-11 prr7-11* at all time-points tested (**Chapter 3**). I go on to show that the metabolite profiles between *prr5-11 prr7-11*

and *prr5-11* seem to trend similarly under -S conditions, illustrating congruency between the phenotyping data and the metabolomics results obtained (**Chapter 3**).

Overall, the results I present throughout my MSc. thesis functions as a foundation, from which future analyses concerning the interplay between the clock and osmoregulatory or nutrient-dependent processes can be explored. Given this, I outline a selected number of projects which would serve as logical next steps in elucidating the interplay between the circadian circuit and drought-like or nutrient-related responses in *Arabidopsis*.

4.2 Unraveling the role of RVE8-like proteins in the regulation of osmolyte pools under drought-like stress

Overall, In **Chapter 2**, I use proteomics to illustrate why WT plants do better than RVE8-lacking plants under drought-like conditions. Although it does appear that WT plants have more osmoprotection-conferring proteins, elucidating how the metabolite pool differs between WT and *rve 4 6 8* under mannitol and salt is certainly of interest (**Chapter 2**). I have shown that WT plants seem to have higher pools of JA-biosynthesizing proteins, proline-synthesizing proteins, and tryptophan biosynthesis proteins, thus, it would be interesting to elucidate how the pools of osmoprotecting osmolytes differs between WT and *rve 4 6 8* under drought-like stress (**Chapter 2**).

4.3 Phenotyping of *PRR9*-deficient seedlings and plants with improper *PRR5*, *PRR7*, and *PRR9* expression under nutrient stress

In **Chapter 3**, I attempt to parse out the effects of PRR5 and/or PRR7 proteins through phenotyping screens and subsequent GC-MS metabolomics (**Chapter 3**). While conducting my preliminary phenomic screen, I found that plants deficient in PRR7 and PRR9 proteins have a very clear hypocotyl phenotype under -N conditions (**Chapter 3**). Further, metabolomic analyses of *prr7-3 prr9-1* rosette leaves under diel conditions have unveiled an excess of organic acid metabolites between ZT 6 and ZT 16 (Flis et al., 2019). I have analyzed the metabolome of PRR7-lacking plants under diel light and could not report an excess of organic acids, which might indicate that PRR9 could be regulating TCA acid intermediates throughout the day (**Chapter 3**). Thus, I believe that the examination of PRR9-deficient plants under nutrient stress is certainly of interest to further understand how PRR-deficient plants cope under nutrient stress. Prior metabolomic

experiments have shown that plants void of proper *PRR5*, *PRR7*, and *PRR9* expression present with an inability to regulate metabolite profiles throughout the day, such that *prr5-11 prr7-11 prr9-10* plants present with elevated pools of TCA cycle intermediates at all time-points throughout the day under diel light (Fukushima et al., 2009). Thus, it would certainly be interesting to unveil how plants with perturbed *PRR5*, *PRR7*, and *PRR9* expression would function under nutrient stress. The phenotyping of *prr9-1* and *prr5-11 prr7-11 prr9-10* plants would allow us to parse out the effects of PRR5, PRR7 and/or PRR9 in nutrient assimilation, extending the story of how PRR proteins regulate plant nutrition responses.

4.4 Time-of-day quantitative proteomics analysis of PRR-deficient seedlings under nutrient stress

After conducting the last phenotyping screens of PRR-lacking plants under nutrient stress, uncovering which proteins are differentially abundant under nutrient stress would most certainly be of interest. Time-of-day quantitative proteomics at ZTs 0, 4, 8, and 12 of PRR-deficient plants would illustrate which proteins are involved in conferring the differing phenotypes. I show that PRR5 and 7-deficient plants fare worse (to differing degrees) under disparate nutrient stress conditions (**Chapter 3**), however, elucidating which protein groups are involved in conferring the diverging phenomic and metabolomic responses would provide agrobiotechnologists with targets for knockdown or overexpression. Further, by conducting a time-course total proteome experiment, we would be able to uncover why PRR-lacking plants fare worse under nutrient stress, which would provide new insights into the interplay between the clock and nutrient metabolism. This experiment would also allow us to directly implicate the clock in nutrient-mediated metabolism, which has not been established.

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