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ORIGINAL ARTICLE

Dynamic physiological and transcriptome changes reveal a potential relationship between the circadian clock and salt stress response in *Ulmus pumila*

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Abstract

Despite the important role the circadian clock plays in numerous critical physiological responses in plants, such as hypocotyl elongation, leaf movement, stomatal opening, flowering, and stress responses, there have been no investigations into the effect of the circadian clock on physiological and transcriptional networks under salt stress. *Ulmus pumila* L. has been reported to tolerate 100–150 mM NaCl treatment. We measured the diurnal variation in photosynthesis and chlorophyll fluorescence parameters and performed a time-course transcriptome analysis of 2-years-old *U. pumila* seedlings under salt treatment to dissect the physiological regulation and potential relationship between the circadian network and the salt stress response. Seedlings in 150 mM NaCl treatment exhibited salt-induced physiological enhancement compared to the control group. A total of 7009 differentially expressed unigenes (DEGs) were identified under salt stress, of which 16 DEGs were identified as circadian rhythm-related DEGs (crDEGs). Further analysis of dynamic expression changes revealed that DEGs involved in four crucial pathways—photosynthesis, thiamine metabolism, abscisic acid synthesis and metabolism, and the hormone-MAPK signal crosstalk pathway—are closely related to the circadian clock. Finally, we constructed a co-expression network between the circadian clock and these four crucial pathways. Our results help shed light on the molecular link between the circadian network and salt stress tolerance in *U. pumila*.

Keywords Circadian network · Salt · Expression profile · Co-expression network

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Introduction

The circadian clock is an endogenous regulatory system. Its ~ 24-h cycle plays roles in myriad metabolic and physiological processes to ensure that plants adjust to the day–night cycle caused by the rotation of the Earth (Greenham and McClung 2015; Sanchez and Kay 2016). The first circadian clock gene cloned in plants was *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*). *CCA1* encodes a MYB-related transcription factor involved in the phytochrome induction of the light-harvesting chlorophyll a/b-protein (Lhcb) gene (Wang and Tobin 1998). With the discovery of luciferase and the maturity of the plant transgenic technology system (Welsh et al. 2005), researchers have found a large number of circadian clock components in *Arabidopsis* whose functions are relatively conservative (De Caluwé et al. 2016). These circadian clock components interact to form a complex interlock feedback circuit at the transcription level. The core oscillator of the plant circadian clock

contains three feedback loops: (1) the core circuit, composed of *CCA1*, *LATE ELONGATED HYPOCOTYL (LHY)*, and *TIMING OF CAB EXPRESSION 1* (*TOC1*); (2) the morning loop, composed of *PSEUDORESPONSE REGULATOR 5/7/9* (*PRR5/7/9*); and (3) the evening loop, composed of *EARLY FLOWERING 3* (*ELF3*), *EARLY FLOWERING 4* (*ELF4*), *GIGANTEA* (*GI*), and *LUX ARRHYTHMO* (*LUX*) (Romanowski and Yanovsky 2015).

By providing time-of-day information, the circadian clock enables plants to synchronize their endogenous physiology in anticipation of daily and seasonal fluctuations under various external environmental conditions, thereby enhancing fitness (Dodd et al. 2005). The circadian clock is highly relevant to numerous critical physiological responses in plants, including metabolism, hypocotyl elongation, leaf movement, stomatal opening, photoperiodic control of flowering, and stress responses (Greenham and McClung 2015; Seung et al. 2012; Kong et al. 2020). Over the past few years, intensive research has established that the circadian clock plays an important role in regulating plant responses to biotic and abiotic stresses. In Arabidopsis, the loss of circadian function due to a *CCA1* mutation compromises resistance to downy mildew, whereas *CCA1* overexpression (OE) enhances resistance to the same pathogen (Wang et al. 2011). The expression of four *GmLHYs* was all induced by drought in soybean, and quadruple mutants of *GmLHYs* demonstrated significantly improved drought tolerance. Functional characterization of *LHY1a* and *LHY1b* in Arabidopsis and soybean have further demonstrated that *GmLHYs* can maintain cellular homeostasis through the abscisic acid (ABA) signaling pathway under drought stress (Wang et al. 2020a, b). The red light receptor PHYTOCHROME B (PhyB) was noted for its major nighttime thermosensory role in Arabidopsis (Jung et al. 2016), and its ortholog was demonstrated to negatively regulate chilling tolerance in rice. PhyB deficiency in rice, which led to a more stabilized chloroplast structure and a higher unsaturated fatty acid content in membrane lipids, could alleviate chilling-induced photoinhibition (Yang et al. 2013; He et al. 2016). In addition, a circadian clock component is also involved in the adaptation to salt stress. *OsPRR73* has been shown to specifically confer salt tolerance in rice. The grain size and yield of *osprr73* null mutants were significantly decreased under salt stress, with higher accumulated levels of reactive oxygen species and sodium ions (Na^+). Salt-induced *OsPRR73* expression confers salt tolerance by recruiting histone deacetylase (HDAC10) to form a complex that represses high-affinity sodium transporter (OsHKT2; 1) transcription by altering its promoter chromatin status, thus reducing cellular Na^+ accumulation (Wei et al. 2020).

Salt stress is one of the major abiotic stresses that limits plant growth and development (Zhao et al. 2016; Cristiano et al. 2016). It leads to significant losses in agricultural production and restricts afforestation (Herrero and Pérez-Coveta 2005; Rozema and Flowers 2008). Salinity stress inhibits plant growth and development by imposing several constraints, such as sequential osmotic stress, oxidative stress, nutritional stress, and ionic imbalance (Deinlein et al. 2014; Ismail et al. 2014; Yang and Guo 2018). High salt concentrations damage the semipermeability of the plasma membrane, thus disrupting interstitial ionic homeostasis. It also reduces chlorophyll content, damages the chloroplast structure, and decreases photosynthesis, all of which lead to reduced growth and development and even death (Tavakkoli et al. 2011; Ma et al. 2012). Salt tolerance in plants is complex and involves the coordination of various genes. It involves stomatal water loss, ion selective absorption, metabolism, permeability, and regulation of the antioxidant defense system (Abogadallah 2010). Many salinity-induced genes are part of a complicated cascade of molecular networks and are thought to play crucial roles in responding to salt stress. However, details about the transcript regulation network involved in these important salt stress-induced pathways associated with the circadian clock are still unclear.

Ulmus pumila L., a major tree species used for timber, shelter, fodder, medicine, food, and ecological protection, has been reported to be able to tolerate 100–150 mM NaCl treatment (Feng et al. 2014). In recent years, several groups have studied the physiology and biochemistry of *U. pumila* under salt stress (Liu et al. 2012; Zhu et al. 2016), but none has investigated the effect of the circadian clock on the transcriptional regulatory network under salt stress in *U. pumila*. Here, we performed a time-course physiological and transcriptome analysis of *U. pumila* under salt stress to dissect the physiological regulation and expression of genes related to the circadian clock. Short Time-Series Expression Miner (STEM) and Weighted Gene Co-expression Network Analysis (WGCNA) based on salt-induced differentially expressed genes (DEGs) were used to identify crucial metabolic pathways and construct a co-expression network involving circadian rhythm-related genes and DEGs, helping to shed light on the molecular link between the circadian network and salt stress tolerance in *U. pumila*.

Materials and methods

Plant materials and salt stress treatment

Two-year-old *U. pumila* seedlings were cultured in plastic pots filled with a sandy soil and vermiculite mixture (1:1 v/v) in a greenhouse with day/night temperatures of 26 °C/18 °C, relative air humidity of 50–60%, and natural light. After 30 days of further growth (April 1, 2019), plant seedlings with similar growth status were treated with 0 mM

(control group, CT) or 150 mM NaCl solution (salt treatment group, ST).

Diurnal variation in photosynthesis and chlorophyll fluorescence parameters

After 90 days of treatment, three *U. pumila* seedlings from each group were examined to record photosynthesis and chlorophyll fluorescence parameters. Daily changes in the net photosynthetic rate (Pn), transpiration rate (Tr), intercellular CO_2 concentration (Ci), and stomatal conductance (Gs) were measured in mature leaves using the LI-6400 portable photosynthesis system (LI-COR Corp., Lincoln, NE, USA) on a sunny day in July. The measurements were performed every 3 h from 06:00 to 18:00. The corresponding measurement time points were 06:00 (T06), 09:00 (T09), 12:00 (T12), 15:00 (T15), and 18:00 (T18).

Chlorophyll fluorescence parameters were measured using a MultispeQ device (beta version) linked to the PhotosynQ platform (http://www.photosynq.org). These parameters were: minimum variable fluorescence (Fo), maximum variable fluorescence (Fm), actual photochemical efficiency of photosystem II (ΦII), quantum yield of non-photochemical quenching (ΦNPQ), quantum yield of non-regulated energy dissipation (ΦNO), and leaf temperature (Tleaf). All chlorophyll fluorescence parameters were measured using the same leaf at T06, T09, T12, T15, T18, and T21. Meanwhile, mature leaves from *U. pumila* seedlings at each time point in the CT and ST groups were collected and immediately frozen in liquid nitrogen, then stored at − 80 °C.

RNA isolation, library preparation, and RNA sequencing

The leaf samples collected from T09 to T21 in the CT and ST groups were used for transcriptome sequencing. Total RNA was extracted following the Plant Qiagen RNeasy kit protocol (Qiagen China, Shanghai, China). The purity and quality of the RNA was evaluated using agarose gel electrophoresis and the NanoPhotometer® spectrophotometer (IM-PLEN, Westlake Village, CA, USA). Purified RNAs were taken to generate strand-specific sequencing libraries using the NEBNext® Ultra™ Directional RNA Library Prep kit (NEB, Ipswich, MA, USA). These libraries were then sequenced on an Illumina Hiseq2500 platform (San Diego, CA, USA).

Unigene functional annotation and expression analysis

After low-quality read filtration and transcriptome assembly, six complementary approaches were employed to annotate the assembled unigenes. All unigenes underwent a BLAST search with the following databases: Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Groups of proteins (KOG/COG), National Center for Biotechnology Information's non-redundant protein sequences database (Nr), a protein family database (Pfam), and a manually annotated and reviewed protein sequence database (Swiss-Prot).

To estimate gene expression levels, the transcriptome sequences obtained via Trinity (Grabherr et al. 2011) splicing were used as reference sequences. The read count number compared to each reference gene was then obtained after mapping the clean reads to the reference sequences. The expected number of fragments per kilobase of transcript per million mapped reads (FPKM) was used to estimate gene expression levels. Unigenes with q-value < 0.005 and |log (foldchange)|> 1 were identified as statistically significant DEGs.

Quantitative real-time reverse transcription PCR

To validate the RNA sequencing results, total RNA from each sample was isolated using the same method described above. Reverse transcription reactions were performed using a PrimeScriptTM RT Reagent Kit (+rDNA Eraser) (TaKaRa, Shiga, Japan) with 2 μg of total RNA, according to the manufacturer's instructions. A total of eight unigenes were selected for quantitative real-time reverse transcription PCR (qRT-PCR). Gene-specific primers were designed using Primer Premier v6.0 software (gene-specific primers are listed in Supplementary Table S1). qRT-PCR was performed using a qPCR System (M× 3005P, Agilent, Santa Clara, CA, USA) with the QuantiNova SYBR® Green PCR kit (Qiagen, Hilden, Germany). The relative expression level of each unigene was normalized based on the corresponding amount of 18 s rRNA, and data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Temporal expression pattern analysis and functional enrichment analysis

To evaluate the rhythm-dependent transcriptome variations of *U. pumila* in the CT and ST groups, we performed STEM clustering analysis using STEM software (Ernst and Bar-Joseph 2006) to detect dynamic trends of temporal expression changes in DEGs at five time points. The maximum number of model profiles was set to 50, and the minimum ratio of DEG fold-change was set to 2.0. The expression profiles of DEGs would be significantly enriched by comparing the distributions in observed groups with those expected from random permutations. In addition, KEGG pathway enrichment analysis was performed on DEGs with similar expression tendencies in specific clusters.

Construction of interactive network

Co-expression networks of DEGs in *U. pumila* in the ST group were constructed using the WGCNA (v1.6.6) package in R software (v3.4.4). DEGs differentially expressed at least one time point were used to construct a gene co-expression network. The FPKM values of DEGs were input into WGCNA. Modules were obtained using the automatic network construction function blockwiseModules with default settings. To identify hub genes within the co-expressed modules, the internal gene connectivity was calculated based on Pearson correlation coefficients. The resulting regulatory networks were visualized and represented as graph networks using Cytoscape 3.6 software (http://cytoscape.org/).

Statistical analysis

Each experiment was carried out using at least three biological replicates. Statistical analyses were performed using ANOVA tests in SPSS 22.0, and the mean values of three replicates in each treatment group were compared, with $P \leq 0.05$ or 0.01 indicating significance.

Results

Diurnal variation in photosynthesis

To characterize the effects of salt stress on photosynthesis in *U. pumila*, the Pn, Tr, Ci, and Gs of seedlings in the ST and CT groups were examined every 3 h from 06:00 (T06) to 18:00 (T18). Pn, Tr, and Gs, but not Ci, exhibited a typical bimodal curve, peaking at T09 and T15 (Fig. 1a–d). The ST seedlings maintained a higher photosynthetic capacity. Pn in the ST seedlings increased by 13.89%, 12.65%, and 15.80% at T09, T12, and T15, respectively. Similarly, Gs and Tr in the ST seedlings were also promoted and significantly higher than those in the CT seedlings (19.23% and 20% higher, respectively) at T15. Ci in ST seedlings significantly decreased by 23.81% at T15. It is indicative that salt stress promote photosynthesis in *U. pumila.*

Diurnal variation in chlorophyll fluorescence

To explore the physiological regulation of photosynthetic activity, changes in chlorophyll fluorescence parameters were measured simultaneously. There was no difference in Fo between the ST and CT groups (Fig. 1e). Fm in ST seedlings increased markedly at T09 and T15 (23.5% and 20.5% higher, respectively, than in the CT group) (Fig. 1f), indicating a higher electron transfer rate in the PSII reaction center under ST. The parameter ΦII directly reflects the ability to

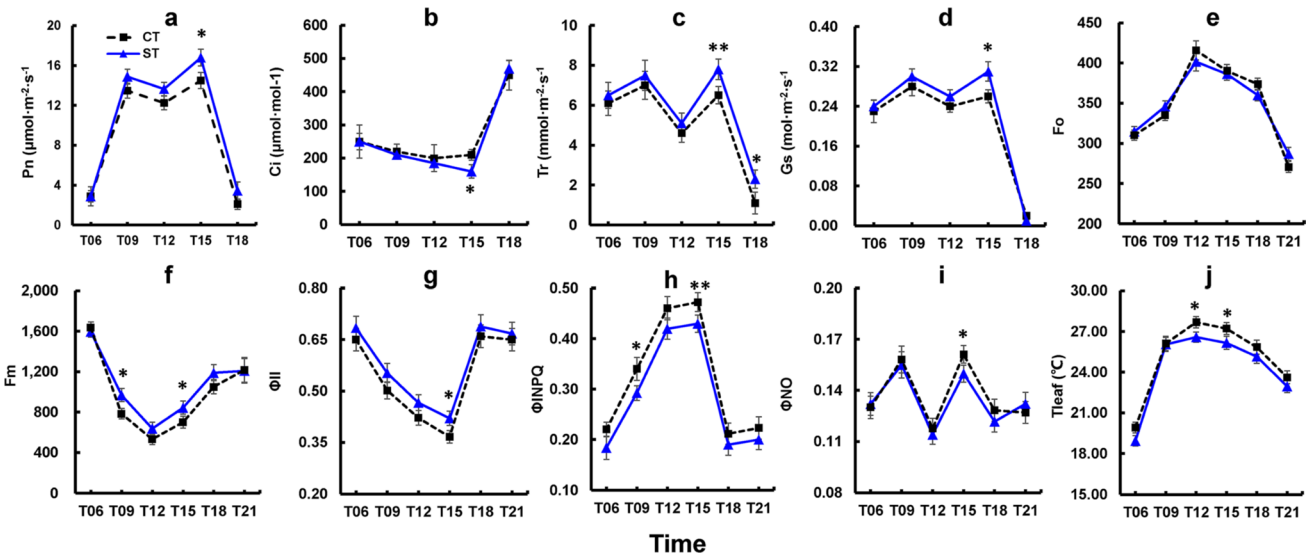

Fig. 1 Diurnal variation of photosynthesis and chlorophyll fluorescence parameters of *U. pumila* seedlings in response to salt treatment. **a**–**d** Changes of photosynthesis under control or NaCl treatment. **a** Net photosynthetic rate (Pn); **b** intercellular CO_2 concentration (Ci); **c** transpiration rate (Tr); **d** stomatal conductance (Gs); **e**–**j** changes of chlorophyll fluorescence parameters under control or NaCl treatment. **e** Minimum variable fluorescence (F0); **f** maximum variable fluorescence (Fm); **g** actual photochemical efficiency of photosystem II (ΦII); **h** quantum yield of non-photochemical quenching (ΦNPQ); **i** quantum yield of non-regulated energy dissipation (ΦNO); **j** leaf temperature (T_{leaf}). Data are presented as means ± SD ($n = 3$), (*$P < 0.05$, **$P < 0.01$)

convert light energy into ATP and NADPH. Ultimately, synthesis sugar was significantly higher (14.7%) in ST seedlings than in CT seedlings at T15 (Fig. 1g). Meanwhile, the ΦNPQ and ΦNO of ST seedlings were significantly lower (9.0% and 7.0%, respectively) than in CT seedlings (Fig. 1h, i). These results indicate a higher light energy conversion efficiency in ST seedlings at T15. IN addition, the T_{leaf} of ST seedlings was significantly lower than that of CT seedlings at T12 and T15 (reduced by 1.1 °C and 1.06 °C, respectively) (Fig. 1j). T_{leaf} decreased with the increase of Gs and Tr which might benefits for weaken light inhibition of *U. pumila* under salt stress.

Transcriptomic analysis overview

To comprehensively characterize the gene expression dynamics, we used leaf samples from CT and ST seedlings collected at different time intervals (T09 to T21) for de novo transcriptome sequencing. Gene expression levels were generally higher in ST seedlings compared to CT seedlings (Fig. 2a). In total, we identified 7009 unigenes that were differentially expressed (DEGs, q-value < 0.005 and |log2 (foldchange)|> 1) between the ST and CT groups at least one time point, which included 1795 DEGs continuously up-regulated and 1929 DEGs continuously down-regulated from T09 to T21 (Supplementary Table S2). To confirm the differences in expression levels between ST and CT samples, we randomly selected eight DEGs to validate their time-course expression profiles using qRT-PCR. The results showed a consistent expression tendency in expression profiles between ST and CT samples, with some differences in expression levels (Supplementary Fig. S1). These results demonstrated the reliability of the sequencing data in our study.

Next, we calculated the Pearson correlation coefficient between each pair of samples using the expression data. The correlation results over consecutive time points showed that the unigenes were strongly affected by stress exposure and their expression exhibited time-dependent clustering (Fig. 2b). The 10 sampling time points were divided into two significantly different clusters (ST and CT) with high correlation values. Of the five CT-related time points, CT09 to CT21 were more strongly correlated, indicating a closer relationship. There were three similar expression modules according to the correlation relationship (Module I: CT09–CT12; Module II: CT12–CT18; Module III: CT15–CT21). Notably, the unigene transcription level of the ST samples exhibited a more obviously temporal transition relationship compared to the CT group. As shown in Fig. 2b**,** the unigene expression patterns at the five ST-related sampling time points can be distributed into four expression modules: Module IV (ST09 and ST12), Module V (ST12 and ST15), Module VI (ST15 and ST18), and Module VII (ST18 and ST21). ST09, ST15, and ST18 were identified as the transition points among the four expression modules. These results suggest that salt-induced regulatory changes at the transcription level are more obvious.

Identification of DEGs under salt treatment associated with the circadian network

To identify genes that could be both involved in salt stress responses and connected to the circadian network, we first annotated all unigenes using KEGG pathway enrichment analysis (Supplementary Table S3). As a result, 283 unigenes were identified as involved in the circadian rhythm pathway (Pathway ID: KO04712), 117 of which might participate in circadian clock feedback loops (Supplementary Table S4), including 4 homologues of *TOC1*, 14 homologues of *GI*, 16 homologues of *LHY*, 17 homologues of *PRR5*, 48 homologues of *PRR7* and 18 homologues of *ELF3*.. In circadian rhythm pathway feedback loop, the expression of *LHYs*

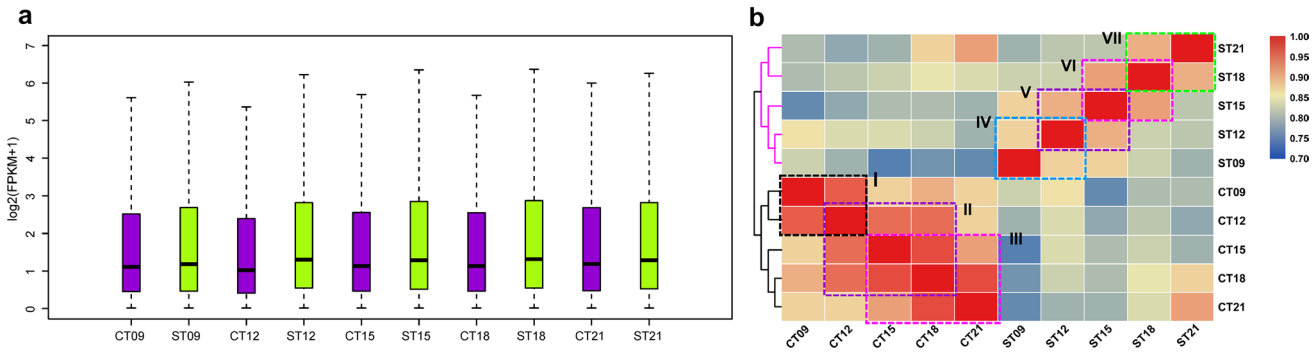

Fig. 2 Transcriptomic analysis of *U. pumila* seedlings under salt treatment. **a** Comparison of expression level of mRNAs between control and salt treatment group. The lines of the whiskers in the box represent the medians; **b** dynamic changes in expression profile of mRNAs. Modules are colored as in the legend, it represents the pairwise Pearson correlation. The correlation between each pair of samples was calculated by gene expression values (FPKM)

and *TOC1s* peaked at T09 under CT and ST (Supplementary Fig. S2a, b). In the morning loop, the expression of *PRR5s* and *PRR7s* peaked at T12 under CT, while their expression reached the peak at T15 under ST (Supplementary Fig. S2c, d). In the evening complex, the expression of *GIs* and *ELF3s* peaked at T15 and T18 under both treatment, respectively (Supplementary Fig. S2e, f). These results suggest that ST may changed the phase of circadian clock-related genes.

Among 283 unigenes involved in the circadian rhythm pathway, 16 were differentially expressed (circadian rhythm-related DEGs, or crDEGs) under ST, including 14 up-regulated genes and 2 down-regulated genes (Supplementary Fig. S2g, Supplementary Table S5). Among these crDEGs, two photoreceptor coding genes for cryptochromes (*CRYs*) were significantly expressed during the test period, one of which was significantly up-regulated at T15 and T18 (2.16- and 1.48-fold, respectively), whereas the other was down-regulated (2.89-fold) at T21 under ST. In the core circadian rhythm pathway feedback loop, *LHY* (Cluster-18787.78411) was significantly down-regulated at T09 (3.25-fold), and at least one of the three genes related to TOC1 biosynthesis was differentially expressed at T15 and T21. In the morning loop, *PRR5* and *PRR7* were induced by ST. Two *PRR5* genes were differentially up-regulated from T09 to T18, and the highest transcription level of *PRR5* occurred at T15 (the two genes were up-regulated 5.07- and 7.31-fold). Four *PRR7* genes were up-regulated (1.01- to 1.70-fold) from T15 to T21. In the evening complex, only *ELF3* was induced and significantly up-regulated (5.30-fold) at T18. In addition, *GI* genes, which play a crucial role in the induction of photoperiod flowering, were significantly up-regulated from T15 to T18. The F-box protein *ZEITLUPE* (*ZTL*), considered a new photoreceptor for regulating the photoperiodic response and biological rhythm, was induced by ST and up-regulated at T18. All these results showed that ST also changed the amplitude of circadian clock-related genes.

Pathways potentially regulated by circadian clock

To capture the dynamic variation in gene expression between CT and ST seedlings over the tested time series, the temporal expression patterns of DEGs were examined using STEM software. Each profile contained a cluster of multiple genes with similar expression change patterns in the CT and ST seedlings. All DEGs were successfully assigned to 50 different expression modules, and 16 and 18 significantly enriched clusters (Fig. 3a, b; Supplementary Fig. S3) were obtained ($P < 0.1$). Genes with similar expression tendencies in specific profiles were regarded as having the same biological function or regulatory mechanism. Therefore, there may be interactions among genes in a specific profile.

Of the 16 crDEGs identified in circadian rhythm pathway, 14 were assigned to 10 profiles under CT. Another two PRR5 genes were filtered out because no expression was detected in the CT seedlings (Fig. 3a; Supplementary Table S6), while all 16 crDEGs were assigned to 8 profiles under ST based on their expression tendency (Fig. 3b; Supplementary Table S7). There was a total of 1900 DEGs

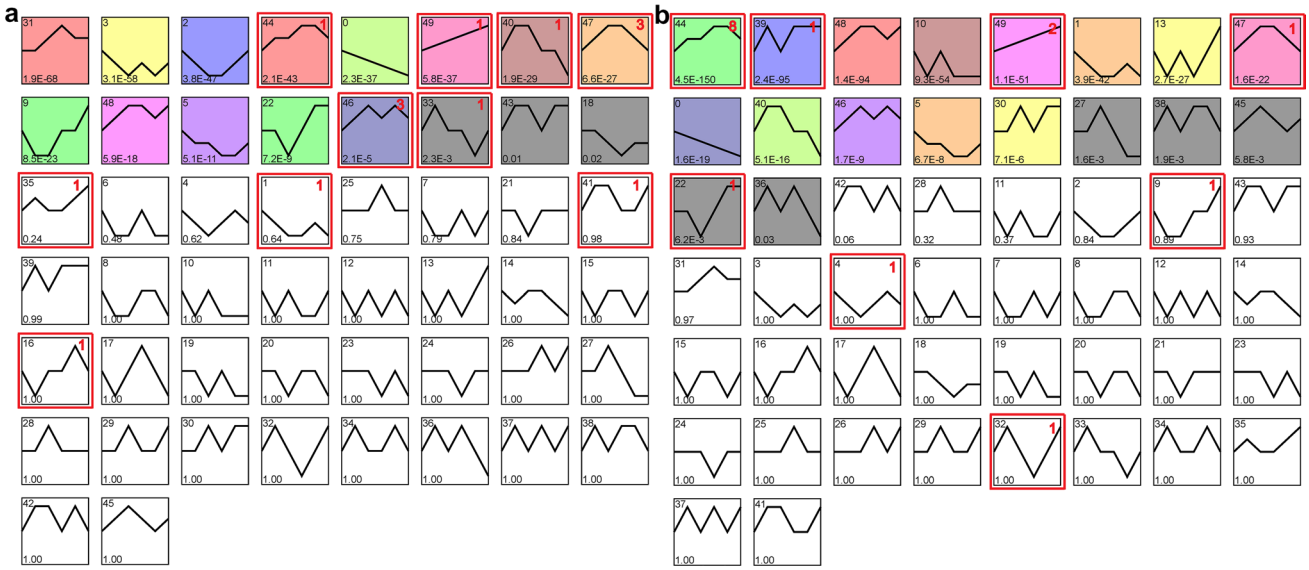

Fig. 3 Gene expression clustering analyzed by short time-series expression miner (STEM). **a** Expression profiles of DEGs under control; **b** expression profiles of DEGs under salt treatment. The profiles are ordered based on the *p*-value significance of number of genes assigned versus expected. The number in the upper left corner of each profile represents the serial number of the expression profile, the number in the lower left corner represents *P*-value, and red number in the upper right corner represents the number of crDEGs. The black line in the middle the overall trend of genes expression in this profile, Colored profiles are significantly enriched (*P*-value ≤ 0.1)

corresponding to 10 profiles for the CT seedlings and 1659 DEGs corresponding to 8 profiles for the ST seedlings. The Venn diagram for the ST and CT groups revealed that 1103 (36.7%) DEGs in the ST group were expressed in a manner similar to that of crDEGs under ST (Fig. 4a). We speculate that these DEGs might connect the circadian network with the salt stress response network in *U. pumila*. These 1103 DEGs were then used for KEGG pathway enrichment analysis. Notably, these DEGs were found to be mainly enriched in many important pathways, such as those involving photosynthesis, plant hormone signal transduction, carbon fixation in photosynthetic organisms, anthocyanin biosynthesis, MAPK signaling, and thiamine metabolism (Supplementary Fig. S4a, Supplementary Table S8). These results indicate that the DEGs involved in these pathways may be potentially regulated by circadian clock. We speculate that these pathways may be regarded as the most important pathways in the response to ST.

To further identify the circadian clock regulatory network, all DEGs in the ST and CT groups were used for WGCNA. The soft thresholding power was set to five and the minimodule size parameter was set to 30. We extracted DEGs that had a potential regulatory relationship with crDEGs (weight value > 0.3). As a result, 3540 DEGs were identified as interacting with 6 crDEGs, including 2 *CRY2* genes, 2 *PRR5* genes, *ELF3*, and ZTL (Supplementary Table S9). KEGG pathway enrichment analysis revealed that the DEGs were significantly enriched in photosynthesis, thiamine metabolism, plant hormone signal transduction, and MAPK signaling pathways (Supplementary Fig. S4b, Supplementary Table S10). These four pathways were also enriched in STEM analysis. Based on the WGCNA result, we visualized the relationship between crDEGs and the four pathways using Cytoscape (v3.5.1). As shown in the resulting network (Fig. 4b, Supplementary Table S11), *CRY2*, *ELF3*, *ZTL*, and *PRR5* were co-expressed with photosynthesis-related DEGs involved with *PSI* (*PHOTOSYSTEM I SUBUNIT* [*PSA*] *D*, *PSAG*, *PSAF*, *PSAL*, *PSAN*, and *PSAO*), *PSII* (*PHOTOSYSTEM II REACTION CENTER PROTEIN* [*PSB*] *E*, *PSBO*, *PSBP*, *PSBQ*, *PSBR*, and *PSBW*), the cytochrome b6/f complex and redox chain (*PHOTOSYNTHETIC ELECTRON TRANSFER* [*PET*] A, *PETC*, *PETE*, *PETF*, *PETH*, and *PETJ*), and *ATP SYNTHASE* (ATPF0B), with an edge weight up to 0.61 (between *psaF* and *CRY2*). *CRY2*, *ELF3*, and *PRR5* were shown to be co-expressed with AAO3 (edge weight values ranged from 0.30 to 0.39). *CRY2* and *ZTL* were co-expressed with *CYP707A1*. The former two genes regulate ABA synthesis and metabolism, respectively. *CRY2*, *ELF3*, *ZTL*, and *PRR5*, circadian clock-related genes, were co-expressed with *PYL*, *PP2C*, *SNRK2*, *ABF*, *CAT*, *ETR/ERS*, and *EIN3*—genes involved in ABA and ethylene signal transduction. In the thiamine metabolism pathway, *CRY2* was co-expressed with *THIC* under ST. These findings provide solid evidence for interactions between circadian network and the four pathways.

Circadian clock-regulated critical pathways in response to salt stress

A total of 671 unigenes were identified to be involved in the photosynthesis pathway, 22.61% (153 unigenes) of which were induced and differentially expressed under ST.

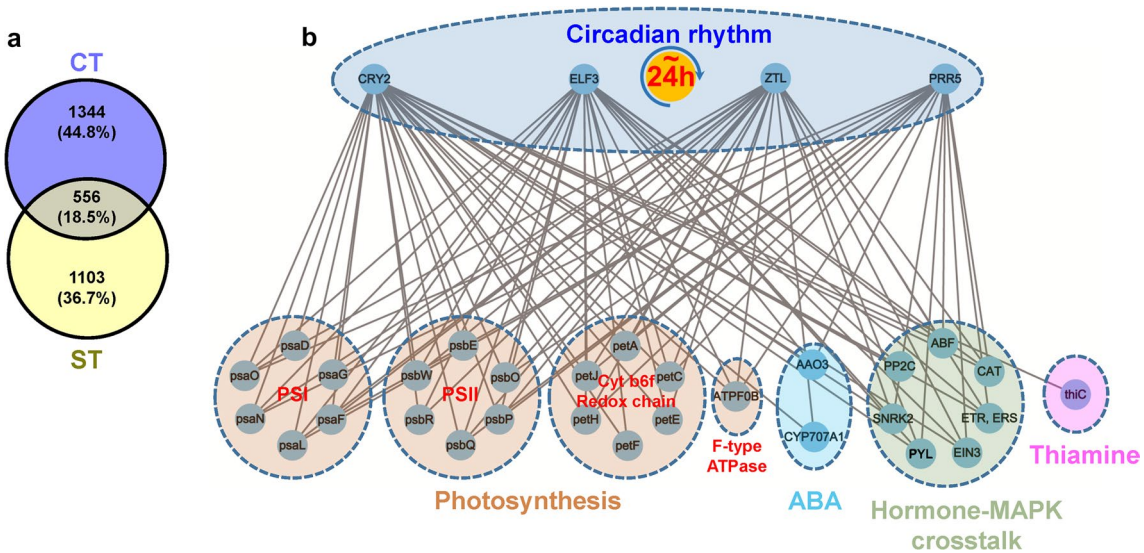

Fig. 4 Gene set screening and construction of co-expression network. **a** Venn diagram of DEGs with the same expression profile as crDEGs under control and salt treatment. **b** Gene regulatory network with potential interaction between crDEGs and DEGs involved in four crucial pathways

Twelve of these genes were up-regulated and 140 genes were down-regulated; one gene was both up- and down-regulated (Fig. 5a, Supplementary Table S12). In the PSII reaction center, five genes were significantly up-regulated (1.24–9.60-fold), including *PSBA*, *-E*, *-O,* and *-P* genes. In the cytochrome b6/f complex, *PETA* and *PETC* genes were differentially expressed under ST. Two *PETA* genes were up-regulated, but there was no difference at T12. One of the two *PETC* genes was down-regulated (1.77–2.17-fold) continuously from T09 to T21, whereas the other was only up-regulated at T09. This resulted in only one gene (*PETC*, Cluster-18787.83522) being exclusively down-regulated from T12 to T21, suggesting an activity reduction of the cytochrome b6/f complex at T12. In the PSI reaction center, *PSAF* and *PSAL* genes were significantly up-regulated under ST. In redox chain, one of *PETH* gene (Cluster-18787.85652) was down-regulated at T12 and subsequently up-regulated under salt stress, and another *PETH* gene (Cluster-18787.35014) was up-regulated continuously from T09 to T21. In addition, eight genes encoding F-type H^+/Na^+-transporting ATPase subunit delta (ATPF1D), beta (ATPF0B), and gamma (ATPF1G) were all significantly down-regulated under ST. Interestingly, all eight of these genes were not differentially expressed at T15, suggesting

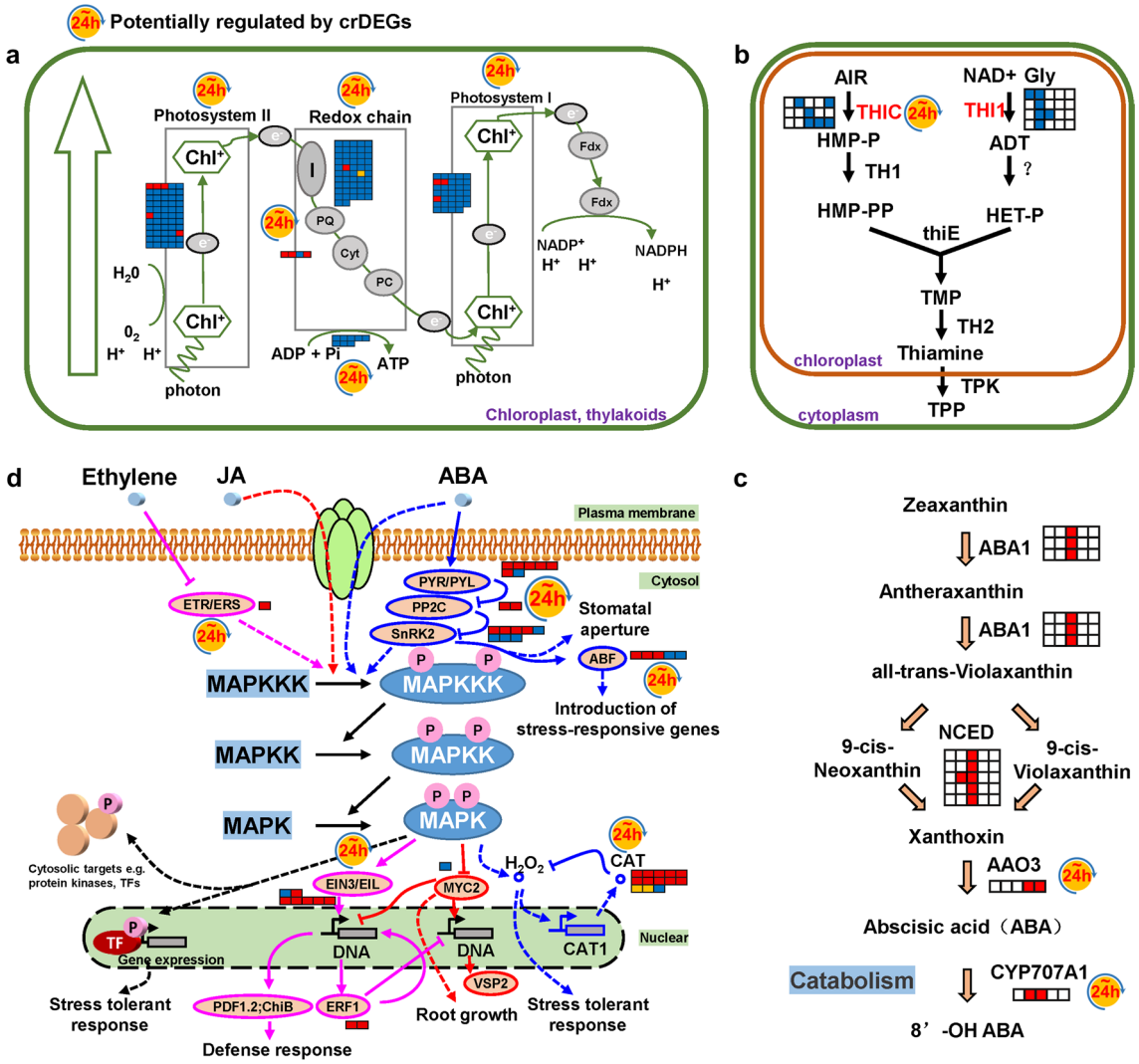

Fig. 5 Diagram of DEGs involved in crucial pathways under salt treatment. **a** Photosynthesis pathway; **b** thiamine metabolic pathway; **c** ABA synthesis and metabolism; **d** hormone-MAPK signal crosstalk pathway. The single rectangle in diagram **a** and **d** represent the whole time course (T09-T21/9:00–21:00), red rectangle represents the DEG was differentially up-regulated, blue rectangle represents the DEG was differentially down-regulated, and yellow rectangle represents the DEG was both differentially up-regulated and down-regulated under salt treatment in the whole time course under salt treatment. While the five rectangles in each row in diagram **b** and **c** correspond to five sampling time points (T09-T21/9:00–21:00) respectively, red rectangle represents the DEG was differentially up-regulated, and blue rectangle represents the DEG was differentially down-regulated at specific time points under salt treatment

that the rate of ATP synthesis decreased from T09 to T12 and T18 to T21, and ATP synthesis at T15 might not have been affected.

Phosphomethylpyrimidine synthase (THIC), thiamine phosphate synthase (TH1), thiazole biosynthetic enzyme (THI1), and thiamine pyrophosphokinase (TPK) are key enzymes in thiamine synthesis that play important roles in regulating the synthesis of thiamine in plants. In the thiamine metabolism pathway, we found that only seven genes, those synthesizing THIC and THI1, were significantly down-regulated under ST (Fig. 5b, Supplementary Table S13). Three *THIC* genes were significantly down-regulated (1.05–5.10-fold) after T09, whereas four *THI1* genes were significantly down-regulated (1.21–1.90-fold) from T09 to T15.

ABA is an important plant hormone that regulates plant growth and development. In our annotation results, 95 genes were found to be involved in ABA biosynthesis, 10.53% (10 genes) of which were differentially expressed. Of these 10 genes, four synthesize the key rate-limiting enzyme 9-cis-epoxycarotenoid dioxygenase (NCED), three genes code for zeaxanthin epoxidase (ABA1), one gene codes for abscisic-aldehyde oxidase (AAO3), and one gene codes for (+)-abscisic acid 8′-hydroxylase (CYP707A1) (Fig. 5c, Supplementary Table S14). Notably, all these genes were significantly up-regulated under ST, but at different time points. ABA1 coding genes were up-regulated (up to 1.62-fold) exclusively at T15, *NCEDs* were up-regulated from T12 to T15, *AAO3* was up-regulated from T18 to T21, and *CYP707A1* was up-regulated from T12 to T15. These results indicate that the biosynthesis of ABA was significantly enhanced and that the involved genes were regulated by circadian clock.

Plant hormones play key roles in plant responses to biotic and abiotic stress. They act on cells in the form of signal molecules, triggering major changes in gene expression and adaptive physiological responses, ultimately affecting and regulating plant growth and development. MAPK-mediated signal transduction plays an important role in the complex hormone signal network (hormone-MAPK signal crosstalk). According to our results, 214 genes were identified as synthetic genes of ABA signaling pathway related proteins, 22 of which were significantly induced (Fig. 5d, Supplementary Table S15). Of these 22 genes, 7 were PYL synthesis genes (1 significantly down-regulated at T21 and 6 significantly up-regulated at T09, T12, and T21), 2 were protein phosphatase 2C (PP2C) synthesis genes (significantly up-regulated from T18 to T21), 8 were serine/threonine-protein kinase SRK2 (SnRK2) synthesis genes (4 significantly up-regulated and 4 significantly down-regulated), 5 were ABA-responsive element-binding factor (ABF) synthesis genes (3 significantly up-regulated and 2 significantly down-regulated; ABFs induce stress responses by regulating downstream target genes). Furthermore, upstream ABA signals are transduced by the MAPK cascade signaling pathway and act on hydrogen peroxide, thereby causing a stress tolerance response, or induce *CATALASE 1* (*CAT1*) expression (differentially expressed before T21) to promote the decomposition and scavenging of reactive oxygen species. In the jasmonic acid signaling pathway (Fig. 5d, Supplementary Table S15), only the gene encoding the transcription factor MYC2 (*MYC2*) was down-regulated (2.16-fold) at T15. In addition, 10 genes involved in ethylene signal transduction were differentially expressed (Fig. 5d, Supplementary Table S15). *ETHYLENE RECEPTOR* (*ETR*/*ERS*) was continuously up-regulated. Five *ETHYLENE-INSENSITIVE PROTEIN 3* (*EIN3*/*EIL*) genes were significantly up-regulated, and another was down-regulated at T12 and T18. Two *ETHYLENE-RESPONSIVE TRANSCRIPTION FACTOR 1* (*ERF1*) genes were exclusively up-regulated at T09.

Discussion

Physiological regulation under ST

Plants suffer varying degrees of abiotic stress at different times during the day–night cycle. The biological clock enables the transfer of valuable energy between growth and stress responses by controlling various metabolic and physiological activities. The core of improving plant tolerance to abiotic stresses is the precise synchronization of circadian rhythm with the external environment. Internal circadian rhythms control gene expression, stomatal opening, and the timing component of photoperiodism (Yanovsky and Kay 2001; Michael et al. 2003). In *Arabidopsis thaliana*, the wild type and long- and short-circadian period mutant plants with a clock period matched to their environments contain more chlorophyll, have higher photosynthesis rates, grow faster, and survive better than do plants with circadian periods differing from their environments (Dodd et al. 2005). The *U. pumila* seedlings maintained higher photosynthetic capacity under ST. Pn at T09, T12, and T15 increased 13.89%, 12.65%, and 15.80%, respectively, in the CT group. An increase in Gs and Tr helps to reduce blade surface temperature and improve water efficiency. Ci in ST seedlings was significantly lower than that in CT seedling at T15, meaning that the photosynthetic activity of mesophyll cells increased at this time. Overall, the four parameters of photosynthesis all changed significantly at T15, which demonstrated that photosynthesis in *U. pumila* seedlings was promoted upon ST with 150 mM NaCl. The promotion of photosynthesis might have helped the *U. pumila* seedlings to accumulate energy and adapt to environmental salt stress.

Chlorophyll fluorescence is extremely sensitive to environmental changes and can accurately reflect the process of light energy absorption and transmission (Strasser et al.

1995). When plant photosynthesis is inhibited, PSII is the first to be affected (Rapacz 2007), including photosystem electron transfer reactions and the Calvin cycle (Steyn et al. 2002; Silveira and Carvalho 2016). Fm in ST seedlings increased markedly at T09 and T15, which was a notable sign of an enhanced electron transfer rate in the PSII reaction center. Moreover, ΦII was significantly higher (14.7%) in ST seedlings than in CT seedlings at T15, indicating that the ability of ST seedlings to convert light energy into ATP and NADPH (and synthesize sugar) increased. Enhanced Fm and ΦII would eventually lead to the improvement of photosynthetic capacity. In addition, the T_{leaf} of ST seedlings was lower compared to that in the CT group at T12 and T15, meaning that the ST seedlings might have reduced their T_{leaf} to weaken the strong light suppression that usually occurs at noon. These physiological changes might relieve light energy absorption and transmission of *U. pumila* seedlings in response to salt stress.

ST-induced crDEGs and their functions in stress tolerance

The transcriptome analysis identified 283 unigenes involved in circadian rhythm pathway. Among these, 16 (6.36%) crDEGs were induced by ST. CRY is an important blue light receptor found in *A. thaliana*, but is not a component of the circadian oscillator (Wang et al. 2014). CRY is widely distributed in microorganisms, animals, and plants, is mainly responsible for sensing external light in the environment. It has been found that cryptochromes can exhibit multiple biological functions, such as the regulation of stomatal opening and closing, leaf senescence, root growth, and more (Mao et al. 2005; Bae and Choi 2008; Briggs and Olney 2001; Ma et al. 2020). Time courses of whole-plant net carbon assimilation rate (A_{net}) and ΦII indicate that the contribution of blue light to Gs in *cry1* and *cry2* mutants is independent of A_{net}. The changing roles of cryptochromes throughout the day may allow more flexible coordination between Gs and A_{net} (Wang et al. 2020a, b). *CRYs* under ST were significantly up-regulated at T15 and T18 and down-regulated at T21. This suggests that the differential expression of *CRYs* may contribute to photosynthetic carbon assimilation and changes in Gs under salt stress. ZTL, considered a new photoreceptor for regulating the photoperiodic response and biological rhythm, was induced by ST and up-regulated at T18. Research has indicated that ZTL promotes hypocotyl extension in *Arabidopsis* embryos under white light and plays a positive role in hypocotyl extension induced by high temperature (Miyazaki et al. 2015). *ZTL* interacts with heat shock protein 90 (*HSP90*) to mediate the polyubiquitination of aggregated proteins, which leads to proteasomal degradation and the enhancement of the thermotolerance of plants growing at high temperatures (Gil et al. 2017).

In the core circadian clock feedback loop, *LHY* was induced by ST and significantly down-regulated. *LHY* encodes a putative MYB-related transcription factor involved in circadian rhythm along with another MYB transcription factor, *CCA1*. It has been shown to be involved in other biotic and abiotic stress responses. A *cca1-1* and *lhy* double mutant exhibited compromised nonhost resistance to *Pyricularia oryzae*, suggesting that both *CCA1* and *LHY* are involved in regulating penetration resistance in *A. thaliana*. The next analysis revealed that *LHY* regulates post-penetration resistance as a positive regulator (Yamaura et al. 2019). Four *GmLHYs* in soybean were all induced by drought, and quadruple mutants of *GmLHYs* exhibited significantly improved drought tolerance (Wang et al. 2020a, b). Wild-type *A. thaliana* and mutants of the circadian clock genes *CCA1*, *LHY*, *PRR7*, and *PRR9* were exposed to heat and moderate cold. Thousands of genes were differentially expressed in response to temperature, and the circadian clock, acting through *CCA1* and *LHY* (but not *PRR7/9*), appears to have a profound role in modulating heat stress responses during the day (Blair et al. 2019). In addition, three homologues of *TOC1* were differentially expressed at T15 and T21. TOC1 can bind to the regulatory elements of putative ABA receptors, thus regulating circadian expression. *TOC1*-suppressed lines of *Arabidopsis* exhibited more tolerance to drought. Consequently, the resistance of *TOC1*-OE lines to drought was significantly reduced. This indicates that *TOC1* is a transcriptional repressor of certain stress regulations (Castells et al. 2010; Legnaioli et al. 2009; Shen et al. 2006).

In the morning loop, *PRR5* and *PRR7* were induced by ST. Two homologues of *PRR5* were differentially up-regulated from T09 to T18. GO analysis was performed to explore the biological functions of the direct targets of *PRR5* based on significant enrichment. "Transcription factor activity," "response to salt stress," and "response to cadmium ion" were significantly enriched. The direct targets, three *AP2/EREBP* transcription factors, are involved in cold-stress responses, suggesting that *PRR5* controls diverse biological processes by regulating these target genes (Nakamichi et al. 2012). *PRR7* has been shown to be directly involved in the repression of master regulators of plant growth, light signaling, and stress responses. It has also been observed to be involved in the oxidative stress response and the regulation of stomatal conductance (Liu et al. 2013). Intriguingly, the triple mutant of *prr5/7/9* is more tolerant to salt stress (Nakamichi et al. 2009).

In the evening complex, only *ELF3* was induced and significantly up-regulated (5.30-fold) at T18. A previous study indicated that *ELF3*-OE lines are salt-tolerant, whereas *elf3* mutants exhibit more sensitivity to salt stress. The expression of many senescence- and salt stress-associated genes is altered in *ELF3*-OE and *elf3* mutant plants compared to

wild-type plants. During salt stress, *ELF3* suppresses factors that activate salt stress response pathways, mainly *GI* (at the post-translational level) and *PHYTOCHROME INTERACTING FACTOR 4* (*PIF4*; at the transcriptional level). PIF4 directly up-regulates the transcription of *ORESARA1* (*ORE1/ANAC092*) and *SAG29*—both positive regulators of salt stress response pathways (Sakuraba et al. 2017). In addition, two homologues of *GI*s were significantly up-regulated from T15 to T18. *GI* has been shown to regulate several developmental processes, such as photoperiod-mediated flowering, phyB signaling, the circadian clock, and carbohydrate metabolism (Nohales and Kay 2019). It has also been shown to be involved in abiotic stress responses. It can alter the transcription of drought-stress response genes, such as *EARLY RESPONSIVE TO DEHYDRATION 10* (*ERD10*) and *ERD7*, through an indirect mechanism mediated by *CYCLING DOF FACTORs* (*CDFs*) (Fornara et al. 2015). In a previous report, *gi-3* mutant plants exhibited an increased sensitivity to freezing stress in *Arabidopsis*. However, when the responses of wild-type and *gi-3* plants to cold stress were compared, no significant differences were detected in terms of transcript levels of *CBF* genes or their targeted genes. These results suggest that *GI* positively regulates cold tolerance via a *CBF*-independent pathway (Cao et al. 2005). GI is also involved in salt tolerance through physical interaction with SALT OVERLY SENSITIVE 2 (SOS2), a key positive regulator of salt tolerance (Kim et al. 2013).

Crucial pathways and their interplay with crDEGs

The molecular response to salinity stress involves interactions among multiple metabolic and signal transduction pathways (Manuka et al. 2018; Saddhe et al. 2019). Of the 7009 DEGs that responded to ST, 1103 (36.7%) were expressed especially similarly to crDEGs under ST. These DEGs are involved in many important pathways, such as photosynthesis, plant hormone signal transduction, carbon fixation in photosynthetic organisms, anthocyanin biosynthesis, MAPK signaling, and thiamine metabolism pathways, indicating that these pathways are potentially regulated by circadian clock. Further, WGCNA results showed that 3540 DEGs interacted with six crDEGs that were significantly enriched in pathways such as photosynthesis (93 DEGs), thiamine metabolism (1 DEG), ABA biosynthesis (2 DEGs), and hormone-MAPK signal crosstalk (22 DEGs) pathways. This is solid evidence for the interactions between circadian clock and these four pathways.

As shown in the co-expression network (Fig. 4), *CRY2*, *ELF3*, *ZTL*, and *PRR5* were co-expressed with DEGs involved with PSI (*PSAD*, *PSAG*, *PSAF*, *PSAL*, *PSAN*, and *PSAO*), PSII (*PSBE*, *PSBO*, *PSBP*, *PSBQ*, *PSBR*, and *PSBW*), the cytochrome b6/f complex (*PETA*, *PETC*, *PETE*, *PETF*, *PETH*, and *PETJ*), and ATP synthase (ATPF0B), with an edge weight up to 0.61 (between *PSAF* and *CRY2*). These results suggest that there is a complex relationship between the circadian clock and photosynthesis. The regulation of photosynthesis by the circadian clock has been well studied, showing that plants have evolved to track the highly predictable variation in solar radiation that results from the rotation of the Earth on its tilted axis (Müller et al. 2014). Photosynthesis is a complex process consisting of several interrelated molecular and physiological processes, with each part exhibiting strong circadian rhythm, such as those seen for the net carbon assimilation rate, chlorophyll content, chlorophyll fluorescence, and stomatal opening (Gorton et al. 1989; Hennessey and Field 1991; Rascher et al. 2001; Pan et al. 2015). Although the molecular mechanism remains relatively unknown, the circadian clock may play a role in photosynthesis by modulating responsiveness to changes in light (Hotta et al. 2007), shade avoidance (Salter et al. 2003), drought (Legnaioli et al. 2009), and cold (Dong et al. 2011). Stomatal responsiveness to light signals exhibits time-dependent patterns under a free-running condition that is regulated by the circadian clock (Gorton et al. 1993). Stomatal regulation by ABA signaling is mediated by TOC1 (Legnaioli et al. 2009; Lee et al. 2016). Since stomatal opening is essential for the assimilation of carbon, circadian gating of stomatal behavior can dramatically affect photosynthetic performance in nature.

ABA plays an important role in the regulation of plant growth and development, including seed maturation, seed dormancy, shoot elongation, root growth maintenance (Sharp and LeNoble 2002), and adaptive responses to abiotic stress (Nambara and Marion-Poll 2005). A previous study revealed that 40% of the ABA-responsive genes are regulated by the circadian clock in *A. thaliana*, demonstrating that the circadian clock is critical for stress responses (Covington et al. 2008). Transcriptome analysis has shown that several key enzymes involved in ABA biosynthesis, degradation, and transport are clock-regulated, including *ABA DEFICIENT 1* (*ABA1*), *ABA2*, *PHYTOENE SYNTHASE* (*PSY*), *CLOROPLASTOS ALTERADOS 1* (*CLA1*), and *NCED* (Seung et al. 2012; Covington et al. 2008). The *prr5/7/9* triple mutant exhibits an ABA-sensitive phenotype, demonstrating that these proteins function in the ABA synthesis and metabolism pathway (Liu et al. 2013). TOC1 can bind to the promoter of the *ABA-RELATED* (*ABAR*) gene and control its circadian expression. It was reported that *toc1-2* mutant plants are ABA-sensitive in stomatal movement, whereas *TOC1-OE* plants exhibit a reduced ABA response (Legnaioli et al. 2009). Recently, studies have reported that LHY is involved in ABA pathway regulation and that LHY can bind to the promoters of many ABA biosynthesis and signal transduction genes. *LHY*-OE plants accumulated lower contents of ABA under drought stress, and *lhy* mutant plants were more sensitive to ABA treatment during seed

germination (Adams et al. 2018). In this study, *CRY2*, *ELF3*, and *PRR5* were co-expressed with *AAO3* (edge weight values ranging from 0.30 to 0.39) and *CRY2* and *ZTL* were co-expressed with *CYP707A1*, indicating that the circadian clock may modulate the synthesis and metabolism of ABA by regulating *AAO3* and *CYP707A1* under salt stress in *U. pumila*.

MAPK-signaling pathways are common, versatile signaling components that lie downstream of second messengers and hormones, and they play central roles in plant responses to various stresses (Raja et al. 2017). In our study, circadian clock-related *CRY2*, *ELF3*, *ZTL*, and *PRR5* were co-expressed with *PYL*, *PP2C*, *SNRK2*, *ABF*, *CAT*, *ETR*/*ERS*, and *EIN3*—genes involved in ABA and ethylene signal transduction. ABA is perceived by the ABA receptors PYR/PYL, which leads to the inactivation of PP2C. This relieves the inhibition of *SnRK2*, which is a master regulator of the stomatal aperture and acts as a link between ABA signaling and guard cell movement (Mittler and Blumwald 2015). ABA-induced stomatal closure requires H_2O_2 (Zhang et al. 2001); *CAT* induced in the downstream pathway can inhibit the accumulation of H_2O_2 and ultimately improve plant antioxidant capacity. Some key components in the ABA signaling pathway exhibit robust diurnal oscillation, most likely due to clock regulation, such as *PYL3*, *RCAR1*, and *ABAR*/*CHLH*, as well as essential ABA signal transduction elements such as *SnRK2.6*, *HAB1*, *ABF3,* and *ABI1* [8]. Interestingly, the *TOC1* promoter region contains two ABA-responsive elements commonly associated with ABA-induced genes through the *PP2C*/*SnRK2* pathway (Yoshida et al. 2010; Nakashima and Yamaguchi-Shinozaki 2013). Ethylene is also an important phytohormone in plant growth, development, and stress responses. The emission of ethylene has been reported to be regulated by a light-entrained circadian clock in *Arabidopsis* (Thain et al. 2004). It has been demonstrated that ethylene can shorten the circadian period in *Arabidopsis*, requiring *GI* and conditional on the effects of sucrose (Haydon et al. 2017). The relationship between ethylene signaling and the biological clock, however, is unknown.

Thiamine is essential for the proper functioning of all living organisms because it plays important roles in the pentose phosphate pathway, glycolysis, and the tricarboxylic acid cycle as an enzymatic cofactor (Belanger et al. 1995; Goyer 2010). It has been well demonstrated that thiamine biosynthesis is promoted during plant adaptation responses to persistent abiotic stresses, such as heat (Ferreira et al. 2006), cold and drought (Wong et al. 2006), salinity and flooding (Ribeiro et al. 2005), and oxidative stress (Rapala-Kozik et al. 2008; Tunc-Ozdemir et al. 2009). Meanwhile, both *THIC* and *THI1* exhibited a response to various abiotic stresses and may enhance tolerance to mitochondrial DNA damage (Rapala-Kozik et al. 2012). In the thiamine metabolism pathway, *CRY2* was co-expressed with *THIC* under ST, showing that there is a hidden relationship between *CRY2* and *THIC*. In *CCA1*-OE plants, circadian expression of *THIC* and its splice variants was altered compared with the wild type, and the relative transcript levels of *THIC* and *CCA1* oscillated in opposite patterns. Subsequent electrophoretic mobility shift assay demonstrated that both the CCA1 and the LHY proteins were able to bind to the evening element in the *THIC* promoter in vitro (Bocobza et al. 2013). The expression of the *THIC* gene is also regulated by the level of free thiamine diphosphate through a riboswitch present in the 3′ untranslated region. The *THIC* promoter and thiamin-pyrophosphate riboswitch act simultaneously to tightly regulate thiamin biosynthesis in a circadian manner and consequently sense and control vital points of core cellular metabolism (Bocobza et al. 2013). However, the hidden relationship between *CRY2* and *THIC* requires further study.

Conclusions

Under salt stress, Tr, Gs and Pn in *U. pumila* increased, whereas Ci decreased, indicating that photosynthesis was enhanced. Chlorophyll fluorescence analysis showed that the electron transfer rate in the PSII reaction center and ΦII were enhanced in response to salt stress. This suggests that the up-regulation of photosynthesis may play an important role in *U. pumila's* accumulated energy and adaptation to salt stress. Transcriptome analysis showed that 16 crDEGs are involved in four crucial pathways: photosynthesis, thiamine metabolism, ABA synthesis and metabolism, and the hormone-MAPK signal crosstalk pathway. Finally, co-expression analysis showed that transcriptional regulatory network response to salt stress in *U. pumila* may be influenced by circadian clock. These results enhance our understanding of the molecular link between the circadian network and salt stress tolerance in *U. pumila*.

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Declarations

Conflict of interest All the authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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