



cis-Zeatin is an Active Cytokinin that Mediates Gene Expression and Delays Leaf Senescence in Tomato Under Salt Stress

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Abstract

Cytokinin (CK) plays a key role in responding to different abiotic stressors including salinity in delaying leaf senescence. *cis*-Zeatin (*cZ*) is a naturally occurring CK that has been less studied than its well-known isoform *trans*-Zeatin (*tZ*). Here, we investigate the function of *cZ* in regulating leaf senescence in *Solanum lycopersicum* (Tomato) using physiological and transcriptomic analyses. Mature leaves treated with *cZ* or *tZ* ± 150 mM NaCl were examined at early (2 h) and late (72 h) timepoints. Physiological analysis of Photosystem II efficiency (F_v/F_m) and chlorophyll content revealed that salt accelerates leaf senescence, but this effect can be delayed by both *cZ* and *tZ* in a dark-induced leaf senescence bioassay. Transcriptomic analysis indicated increasing number of differential expressed genes (DEGs) from early to late senescence under salt stress. *cZ* treatment uniquely regulates genes ± NaCl, including multiple signaling pathways and regulatory networks involved in salt stress. Weighted Gene Co-expression Network Analysis revealed modules with unique *cZ* elevated eigengene values, and correlations indicate a coordinated regulatory response to *cZ* and salt stress. Although *cZ* and *tZ* gene regulations largely overlapped in direction and response, some antagonistic patterns were observed, highlighting divergent CK functions in fine-tuning the salt stress response. Further organelle-level analysis of DEGs indicated distinct involvement of *cZ* and *tZ* in salt stress-induced senescence. Overall, our findings demonstrate that *cZ* is an active CK form that regulates gene expression and delays tomato leaf senescence under salinity. This reveals novel aspects of CK-mediated stress adaptation underscoring the importance of *cZ* in plant resilience strategies.

Keywords Tomato · Salt · Cytokinin · Transcriptome · Stress · Hormone

Abbreviations

CK Cytokinin
cZ *cis*-Zeatin
tZ *trans*-Zeatin

iP Isopentenyl adenine
 DHZ Dihydrozeatin
 NaCl Sodium Chloride
 DEGs Differential expressed Genes
 MT Micro-Tom
 GO Gene Ontology
 BP Biological Processes
 CC Cellular Components
 MF Molecular Functions
 DMSO Dimethyl Sulfoxide
 MES 2-(N-morpholino) ethane sulfonic acid
 DMAPP Dimethylallyl diphosphate
 MEP Methyl Erythritol Phosphate pathway
 MVA Mevalonate pathway
 YFP Yellow Fluorescence Protein
 F_v/F_m Photosystem II efficiency
 Log₂FC Log₂FoldChange
 TF Transcription Factor

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ABA	Abscisic acid
JA	Jasmonic Acid
SA	Salicylic Acid

Introduction

Tomato (*Solanum lycopersicum*) is one of the largest fresh and processed vegetable crops in the world and a member of the family Solanaceae. However, exposure to various abiotic stressors such as salinity plays a key role in reducing tomato crop production (Ben-Ari and Lavi 2012). Increased soil salinity occurs from excessive irrigation, improper use of fertilizer, and environmental factors including low rainfall and high evaporation that result in decline in crop production (Rietz and Haynes 2003). Salt affects plants in two phases: short-term exposure causes osmotic stress, while long-term exposure leads to ion toxicity due to accumulation of Na⁺ and Cl[−] ions in plant tissues (Hao et al. 2021).

Leaf senescence can be defined as the final stage of leaf development involving the coordinated function of cellular, tissue and organismal level actions. This process is critical for nutrient remobilization from senescing leaves to reproductive structures to maintain plant fitness (Quirino et al. 2000). Chlorophyll degradation is a notable feature of senescence, giving the characteristic change in leaf color from green to yellow (Hörtensteiner and Kräutler 2011). Unfortunately, exposure to abiotic stressors such as salt induces premature leaf senescence that adversely affects crop production (Wingler et al. 1998; Chaves et al. 2002; Keshishian et al. 2018).

Cytokinin (CK) is a well-known plant hormone involved in delaying leaf senescence as well as responding to stressors (Zwack and Rashotte 2015). CK in plants is composed of ~30 diverse compounds centered around modifications of four active base forms: Isopentenyl adenine (iP), *trans*-Zeatin (*tZ*), Dihydrozeatin (DHZ) and *cis*-zeatin (*cZ*) (Antoniadi et al. 2022). Studies have shown that CKs are produced from a common isoprenoid precursor: either isopentenyl diphosphate or dimethylallyl diphosphate (DMAPP) via Methyl Erythritol Phosphate (MEP) pathway or Mevalonate (MVA) pathway which occur in plastids and cytosol respectively (Kasahara et al. 2004). Although *cZ* and *tZ* are isomers, the MEP pathway produces iP, DHZ and *tZ* whereas MVA pathway mediates *cZ* biosynthesis with no interconversion between *tZ* and *cZ* (Kasahara et al. 2004; Kamada-Nobusada and Sakakibara 2009).

While *tZ* is considered a highly active and well-examined CK base form, limited studies have been conducted on *cZ*, in part due to reports of lower activity in classical CK bioassays and previous limitations in methodology to distinguish between *tZ* and *cZ* (Großkinsky et al. 2013). Additionally,

cZ shows lower ligand binding affinity for Arabidopsis CK receptors *AHK3* and *AHK4*, with similar findings for all *StHK2-4* CK receptors in potatoes. However, studies in rice and maize suggest that *cZ* binds with a similar affinity to *tZ* (Romanov et al. 2006; Stolz et al. 2011; Lomin et al. 2018). These findings suggest that the functional relevance of *cZ* may vary between species and should be investigated further.

CK is strongly linked to abiotic stressors from examinations of CK biosynthesis, degradation and Two-Component Signaling (TCS) pathway (Zwack and Rashotte 2015; Argueso and Kieber 2024). Salinity has also specifically been shown to alter endogenous CK levels by biosynthesis or degradation altering resistance to abiotic stresses such as salt (Nishiyama et al. 2011; Keshishian et al. 2018, 2022). There are studies supporting a role of *cZ* in different CK responses such as abiotic stress response, including exogenous *cZ* application that altered root growth similar to *tZ* and other CKs (Gajdošová et al. 2011; Schäfer et al. 2015; Silva-Navas et al. 2019; Antoniadi et al. 2022). Furthermore, exposure of tomato seedlings for 6 h to 150 mM Sodium Chloride (NaCl) resulted in a notable increase in *cZ* levels, suggesting *cZ* as a CK potentially connected to salt stress senescence (Keshishian et al. 2018). However, no extended studies have been conducted on mature leaves in response to *cZ* and salt stress in tomato.

Together this indicates a knowledge gap and need to examine *cZ*, and its role in salt stress-induced leaf senescence, which is addressed in this study. Here we take a physiological and transcriptomic (RNAseq) approach to understand gene expression changes in leaf senescence when exposed to salt stress and whether *cZ* plays a role in regulating salt stress response in tomatoes.

Methods and Materials

Plant Materials

Wildtype *Solanum lycopersicum* cv. Micro-Tom (MT) was used for all examinations except for *pTCSn::VENUS* lines for examination of CK induction which are in the M82 background. Seeds were grown in Promix BX soil in Percival growth chambers under a long day photoperiod (16 h, 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light/8 h dark cycle) with a temperature cycle of 26 °C (light) and 22 °C (dark).

Dark Induced Leaf Senescence Assay

Uniform-sized leaf discs (1.0 cm in diameter) were cut from fully expanded 65-day-old MT leaves, randomized, and floated in 2-(N-morpholino) ethane sulfonic acid (MES)

buffer with CK [1 nM, 100 nM and 1000 nM *tZ* or *cZ* or 0.1% Dimethyl Sulfoxide (DMSO) control]±salt [Sodium Chloride (NaCl) 150 mM]. To induce dark-mediated senescence plates were covered with aluminum foil to block light exposure. CKs were obtained from Olchemlm as base forms with 95%+purity.

Photosystem II Efficiency and Chlorophyll Content Measurement

Leaf discs for all treatments were dark-adapted 30 min prior to Photosystem II efficiency (F_v/F_m) measurement. F_v/F_m was recorded at 2 h and 72 h timepoints using the FluoroCam-photon System Instruments Handy FluoroCam (Photon System Instruments) SN-HFC042. After 2 h and 72 h of leaf senescence, leaf discs were placed in 1 mL pure methanol overnight at 4 °C for chlorophyll pigment extraction. Chlorophyll a, b, and total chlorophyll content were calculated from absorbance at 652 nm and 665 nm recorded using BECKMAN COULTER DU^R 730 Life Science UV/Vis Spectrophotometer (Warren 2008). Three biological replicates from each treatment were used.

Microscopic Analysis of CK Induction

Detached roots of *pTCSn::VENUS* 65-day-old M82 tomatoes were floated in MES buffer with CK [1 µM *tZ* or *cZ* or 0.1% DMSO control]±150 mM NaCl for 2 h or 72 h and viewed using Nikon Eclipse 80i microscope epifluorescence microscope with a yellow fluorescent protein (YFP) filter with 20 X magnification. Corresponding light microscopic images were taken from the same microscope using identical magnification setting. Three biological replicates for each treatment were used. Images were taken using a Qimaging Fast 1394 digital camera connected to the microscope, using QCapture pro 6.0 software. For figure presentation, brightness was uniformly adjusted using Microsoft PowerPoint (Windows 365) without altering the original image integrity or relative signal distribution.

RNA Extraction and Transcriptome Bioinformatics Analysis

After exposure to CK±NaCl treatment for 2 h and 72 h leaf discs from each treatment were flash frozen with liquid Nitrogen and stored in −80 °C until grinding where 30 mg of leaf powder was used for RNA isolation using RNeasy^R Plant Mini Kit according to manufacturer's instructions. Extracted RNA was sent to Novogene for cDNA library construction and sequencing [transcriptomic (RNAseq) analysis]. Three biological replicates for each treatment were used for the experiment. The library preparations were

sequenced on an Illumina Novaseq platform and 150 bp paired-end reads were generated. Raw RNA sequencing reads were processed through in-house scripts to obtain high quality clean transcript reads for all downstream analyses. The reference genome Heinz SL4.0 from Solgenomics.net (<https://solgenomics.net/>) was indexed and paired end clean reads were aligned and mapped using HISAT2 /2.2.0 (Pertea et al. 2016). Counting was done using StringTie/2.2.1 (Liao et al. 2014). Then differential expression analysis was conducted using DESeq2 R package v1.20.0 to obtain Differential Expressed Genes (DEGs) (Love et al. 2014).

Venny 2.1.0 (<https://bioinfogp.cnb.csic.es/tools/venny/>) was used to compare the RNAseq data and Gene Ontology (GO) statistical overrepresentation analysis was performed using PantherDB default parameters (<https://www.pantherdb.org/>).

Analysis of Transcript Levels by qRT-PCR

Similar RNA extraction procedure was used for the analysis of transcript levels by qRT-PCR. The total RNA concentration was determined using a Nanodrop Spectrophotometer (Thermo Scientific) upon the RNA extraction. cDNA synthesis was performed using qScript cDNA Synthesis Kit (QuantaBio) by utilizing equal concentration of starting RNA. qRT-PCR analysis was performed using Eppendorf Mastercycler epgradient realplex2 equipment. Thermal program utilized in qRT-PCR reaction included initial step of 2 min at 95 °C followed by 50 cycles of 15 s at 95 °C, 15 s at 60 °C, and 30 s at 68 °C, with the final step being a melting curve to check amplicon specificity. The 20 µL qRT-PCR reaction consisted of 10 µL PerfeCTa SYBR Green Fast-Mix (QuantaBio), 1 µL each of forward and reverse gene-specific primers (10 µM), 7 µL of sterile water, and 1 µL of cDNA template. All qRT-PCR reactions were performed in two technical replicates and three biological replicates. Samples were compared to *SITIP41* control gene utilizing the $\Delta\Delta C_t$ method. Results are presented as average of fold change relative to DMSO control (mean±Standard Error (SE)). Primer sequences utilized in this analysis are listed in Supplemental Table S1.

Weighted Gene Co-expression Network Analysis

Weighted Gene Co-expression Network Analysis (WGCNA) was conducted to identify the co-expression patterns of genes with CK [1 µM *tZ* or *cZ* or 0.1% DMSO control]±150 mM NaCl at 2 h and 72 h timepoints. Gene count matrix obtained from StringTie/2.2.1 was used to conduct WGCNA. Reads were filtered to remove the non-mapped reads and variance stabilizing transformation was conducted using DESeq2 R package v1.20.0 followed by WGCNA

package v1.72.5 to identify the co-expression pattern of genes (Langfelder and Horvath 2008). Further analysis was conducted as described in (Truong et al. 2022). Scale-free topology model fit (Signed R^2) was evaluated across a range of powers. A soft threshold power of 14 was identified as the lowest value at which the signed R^2 exceeds 0.8 indicating saturation. Using this power, we computed the adjacency matrix with normalized data to understand pairwise gene connections. The matrix was transformed into Topological Overlap Matrix (TOM) to understand both direct and shared connections between genes. TOM-based dissimilarity was calculated and used to perform hierarchical clustering of genes. Gene modules were identified and assigned a unique color for further analysis. Eigengene values for each module and individual gene membership were assigned, then highly correlated modules were merged using the default parameter of >75% relatedness based on eigengene dissimilarity clustering. The average eigengene value for each module was calculated from three biological replicates per sample. Module-to-module and module-to-trait (timepoint, NaCl, hormone) interactions were analyzed. For each sample z-score was calculated for each gene using the mapped read counts to standardize the expression levels across samples for further analysis.

Statistical Analysis

For all parameters, 18 leaf discs were utilized for a single biological replicate per treatment, with at least three biological replicates utilized for all measurements ($n=3$). All data are presented as the mean $\pm$ SE. Statistical analysis of F_v/F_m measurements and chlorophyll contents were done using student's t-test ($p<0.05$). P-values from the DEG analysis were adjusted using Benjamini and Hochberg's approach for controlling the False Discovery Rate (FDR). Genes with an adjusted P-value (padj) <0.05 found by DESeq2 were assigned as differentially expressed and used for further analysis. GO statistical overrepresentation analysis was performed using PantherDB with default parameters, including only the enriched GO terms in which the $\text{FDR}<0.05$. Pearson correlation was used to investigate the module-to-module interaction and module-to-trait interaction in WGCNA.

Results

cis-Zeatin Can Delay Tomato Leaf Senescence Under Salt Stress

To determine an effective cytokinin (CK) concentration under salt stress, 65-day-old MT leaves were treated with increasing concentrations of *cZ* and *tZ* (10 nM, 100 nM,

and 1000 nM) in the presence of 150 mM NaCl in a dark-induced leaf senescence assay for 72 h, after which photosystem II efficiency (F_v/F_m) was measured. A significant concentration-dependent increase in F_v/F_m was observed under salt stress, indicating a dose-responsive protective effect of CK against salt-induced senescence. The strongest and most consistent protective effect was observed at 1000 nM (1 μM), and this concentration was therefore selected for all subsequent experiments (Fig. 1A).

Using the selected concentration, we next examined whether *cZ* and *tZ* can mitigate salt stress induced senescence. Mature 65-day-old MT leaves were treated with [1 μM *cZ* or *tZ*] $\pm$ 150 mM NaCl under dark-induced senescence conditions for 72 h, followed by measurements of F_v/F_m and chlorophyll content. Salt treatment alone significantly reduced F_v/F_m (0.49) compared to the non-salt control (0.52), confirming that salt accelerates dark-induced leaf senescence (Fig. 1B; Supplemental File S1). In contrast, CK treatment maintained higher F_v/F_m values with $\pm$ salt. In the absence of salt, F_v/F_m values reached 0.68 and 0.64 for *cZ* and *tZ*, respectively, while under salt stress they remained elevated at 0.56 and 0.59. This suggests that CK can maintain higher F_v/F_m levels comparable to the non-salt control and indicates *cZ* and *tZ* ability to play a protective role in maintaining the photosynthetic efficiency even under salt stress.

Additionally, chlorophyll measurements at 72 h with [1 μM *cZ* or *tZ*] $\pm$ 150 mM NaCl indicated both *cZ* and *tZ* treatments preserve higher chlorophyll a, b, and total chlorophyll levels compared to control under salt stress conditions (Fig. 1C). Moreover, a comparison of *cZ* vs *tZ* treatment revealed that *cZ* maintains significantly higher chlorophyll levels than *tZ* treatment, suggesting that *cZ* is more effective in maintaining chlorophyll content in salt treated tomato leaves (Supplemental File S1). Both F_v/F_m and chlorophyll content measurements indicate that *cZ* and *tZ* have antisenesescence activity to regulate salt treated tomato leaves with *cZ* being more effective in retaining higher chlorophyll levels.

cis-Zeatin Induces Cytokinin Signaling Under Salt Stress

As a proxy for cytokinin (CK) response we further examined *pTCSn::VENUS* reporter line in primary root tips where its fluorescence is more easily detectable than in leaves and as previous work has shown that abiotic stress can reduce expression levels (Keshishian et al. 2018; Khanna et al. 2025; Mas-ud et al. 2025). *pTCSn::VENUS* plants were exposed to 1 μM *cZ* and *tZ* treatment with $\pm$ 150 mM NaCl treatment for 2 h and 72 h respectively and viewed for fluorescence changes. Salt treatment without CK application

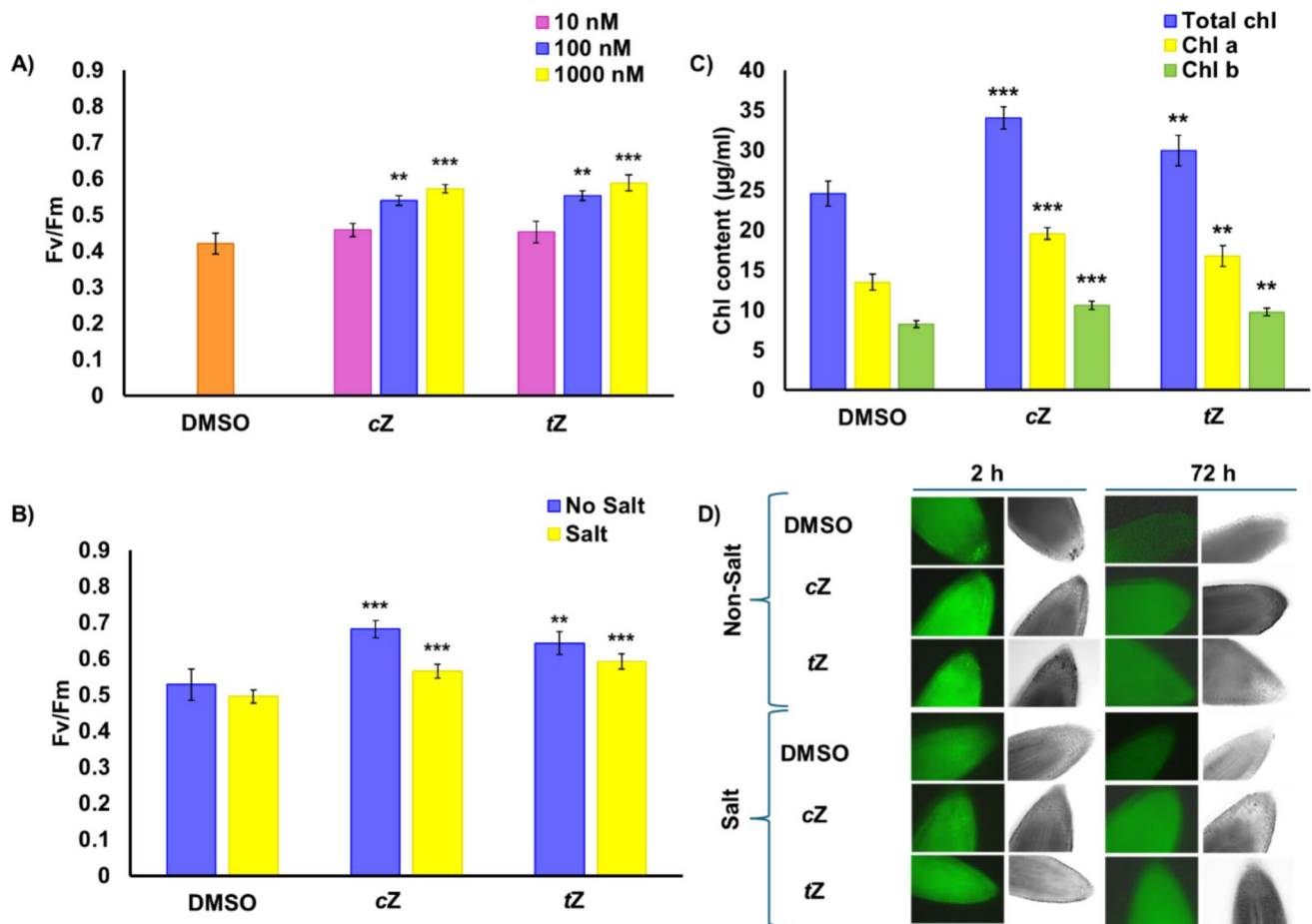


Fig. 1 Physiological response of tomato to *cis*-Zeatin (*cZ*) and *trans*-Zeatin (*tZ*) $\pm$ salt stress and dark induced senescence. **A** Dose response curves of *cZ* and *tZ* in 65-day-old tomato leaves exposed to 150 mM NaCl stress.—Photosystem II efficiency (F_v/F_m) was measured 72 h after treatment with 10 nM, 100 nM, or 1000 nM (1 μM) *cZ/tZ* treatment. Significant increasing trend towards photosystem II recovery by both *cZ* and *tZ* was observed upon increasing concentration. **B** F_v/F_m of 65-day-old tomato leaves treated with *cZ* and *tZ* $\pm$ 150 mM NaCl treatment—The exposure to salt stress reduces the F_v/F_m levels in leaves indicating a decline in photosynthetic efficiency. *cZ* and *tZ* mitigate this effect by preserving photosynthetic efficiency. **C** Total chlorophyll content, chlorophyll a and b in *cZ* and *tZ* treated tomato leaves exposed to 150 mM NaCl for 72 h—Salt stress declines the

chlorophyll contents of the plants under the salt stress but *cZ* and *tZ* maintain higher chlorophyll content compared to the control and thereby involving in delaying leaf senescence. Hormones were applied at 1 μM . Student T-test was used for statistical analysis. The error bars indicate the standard error (SE) of the mean. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ ($n = 3$). **D** Cytokinin (CK) responsive signaling in tomato roots visualized using *pTCSn::VENUS* reporter lines treated with *cZ* or *tZ* in the $\pm$ 150 mM NaCl. Representative fluorescent root tips are shown. Salt stress reduced CK responsiveness in control treatments over time, whereas exogenous *cZ* and *tZ* induced reporter expression at 2 h and maintained expression at 72 h under both control and salt stress conditions. Corresponding light microscopic images are shown alongside fluorescence images

had reduced CK fluorescence signal, indicating salt suppresses basal CK signaling activity in root tips over time. However, exogenous applications of *cZ* and *tZ* show comparable higher fluorescence plus salt across timepoints compared to the DMSO control, indicating both *cZ* and *tZ* show a similar regulation pattern in CK induction in roots (Fig. 1D). This positive *cZ* finding led us to further examine its effects during leaf senescence under salt stress.

Transcriptomic Analysis Patterns Reveal Strong Effects of Salt and Treatment Time

Transcriptomic (RNAseq) analysis was conducted from RNA in a split experimental design with the physiological bioassay utilizing tomato leaves of similar age and treatments with [1 μM *cZ* and *tZ*] $\pm$ 150 mM NaCl at 2 h and 72 h. cDNA library construction from extracted RNA was Illumina sequenced generating ~45 million raw reads per sample that mapped to the *Solanum lycopersicum* SL4.0 reference genome yielding 34,075 mapped genes (Supplemental File S2). From this DESeq2 was used to identify 27,395

Differentially Expressed Genes (DEGs) with adjusted P-value (p_{adj}) < 0.05 as significant between all treatments and controls (Supplemental File S3).

Examination of all transcriptome data for patterns by Principal Component Analysis (PCA) revealed four distinct groupings of samples by treatment length and presence or absence of salt: [2 h ± NaCl] and [72 h ± NaCl] (Fig. 2A). About 36% of the variance in PC1 appears related to duration of senescence, whereas about 25% of sample variance in PC2 appears related to presence or absence of salt stress. CK treatments of *cZ* and *tZ* were clustered within each of the four PCA groupings in a time and stress specific manner (Fig. 2A).

Effect of salt was more evident from the evaluation of DEG number; whereas expected, salt treatment increased total numbers of DEGs from 2 to 72 h (Fig. 2B). After 2 h of salt treatment there was a total of 1184 salt senescence regulated DEGs (616 upregulated, 568 downregulated) with a ~sixfold increase at 72 h to 7224 DEGs (3011 upregulated and 4213 downregulated) (Fig. 2B; Supplemental Table S2). An examination of salt (vs non-salt treated) specific senescence regulated DEGs at each timepoint revealed 573 uniquely regulated DEGs at 2 h and 6613 at 72 h or 7.3% and 84.8% of DEGs respectively. Furthermore, there were 611 or 7.8% DEGs regulated at both 2 h and 72 h suggesting a common subset of salt regulated DEGs (Fig. 2C). Unique salt related gene regulation at 2 h shows activation of potassium, sodium, sugar, phosphate transport, hormone signaling including Absciscic Acid (ABA) and Jasmonic Acid (JA) and redox reactions indicating the plant's ability to respond to the osmotic stress. Whereas at 72 h multiple transcription factors (TF) including *WRKY33*, *BHLH034*, *WRKY6*, *NAC16*, *HAT5*, *R2R3MYB31* are activated and salt regulating genes such as *SOS1*, *LT16b*, *TFT4*, *CASP1* are activated to respond to further ionic toxicity (Supplemental Table S3).

***cis*-Zeatin Regulates More DEGs Under Prolonged Exposure to Salt Stress**

Both *cZ* and *tZ* treatments increased numbers of DEGs over time ± salt treatment. Without salt, *tZ* regulated more DEGs than *cZ* at both timepoints, including 2.5-fold more at 72 h. Similarly, under salt treatment *tZ* also regulated more DEGs at 2 h, but at 72 h *cZ* regulated 407 more DEGs than *tZ* (Fig. 2B; Supplemental Table S2). This suggests that *tZ* acts as a strong regulator of gene expression during senescence ± NaCl treatment, yet so does *cZ*, especially under prolonged exposure to salt stress.

Interestingly, there is a notable shift in direction of gene regulation over time for *cZ* without salt treatment; about five times more DEGs were downregulated at 2 h, whereas approximately 2.4 more DEGs were upregulated at 72 h. A

similar pattern was observed just at 2 h with salt treatment for *cZ* with ~2.3-fold more upregulation of DEGs, although this pattern was not observed for *tZ* at any timepoint ± NaCl treatment (Fig. 2B; Supplemental Table S2). This suggests a distinct temporal reprogramming of gene expression associated with *cZ*.

***cis*-Zeatin Shows Both Unique and Common DEG Regulation with *trans*-Zeatin ± NaCl**

To study what underpins the role of *cZ* during senescence its DEGs were analyzed, revealing *cZ* unique and CK common regulation (Fig. 2D, E). Without salt *cZ* uniquely regulated 113 and 81 DEGs at 2 h and 72 h, whereas *tZ* uniquely regulated 149 and 516 DEGs, respectively (Fig. 2D). Furthermore, 22 and 145 DEGs were common between both CKs at 2 h and 72 h respectively. Interestingly there were 8 DEGs commonly regulated by both CKs at both timepoints, of which 4 are involved in Two Component Signaling (TCS) pathways of CK namely three Response Regulators (*RRs*); *SIRR1*, *SIRR3* and *SIRR5* (*Solyc05g006420*, *Solyc02g071220*, *Solyc10g079600*) and the Histidine Kinase receptor; *SIHK4* (*Solyc04g008110*) indicating a conserved core CK response (Fig. 2D; Supplemental Table S4).

During salt treated senescence, both *cZ* and *tZ* regulated larger sets of unique DEGs: with *cZ* regulating 61 and 2353 and *tZ* regulating 95 and 1901 at 2 h and 72 h respectively (Fig. 2E). 81 DEGs are unique to [*cZ* + NaCl] and [*tZ* + NaCl] at 2 h and 867 DEGs were unique to [*cZ* + NaCl] and [*tZ* + NaCl] at 72 h (Fig. 2E). Additionally, 18 DEGs were commonly regulated at both timepoints of [*cZ* + NaCl] and [*tZ* + NaCl] treatment (Supplemental Table S4). None of these 18 were classical CK-related genes suggesting that salt stress could alter the broader downstream CK regulatory network. TF such as *MYB16*, *TCP30*, *LBD4* (*LOB* domain-containing protein), and *KNOX2* (Homeobox protein knotted-1-like 1) were identified among these salt stress senescence shared DEGs, which have previous connections to CK and auxin potentially indicating their ability to integrate between the hormone signaling pathways and stress responses (Ma et al 2015; Ye et al 2021; Liu et al 2022a).

***cis*-Zeatin DEG Regulation Under Prolonged Exposure to Salt**

Considering the large numbers of DEG regulated at 72 h by both *cZ* and *tZ* when exposed to salt treatment we focused on the 72 h salt treatment to further investigate CK specific regulations in salt stress induced senescence (Fig. 2F). This revealed that there were 1371 and 1137 uniquely regulated DEGs by *cZ* and *tZ* respectively, suggesting that prolonged exposure to salt stress might regulate distinct

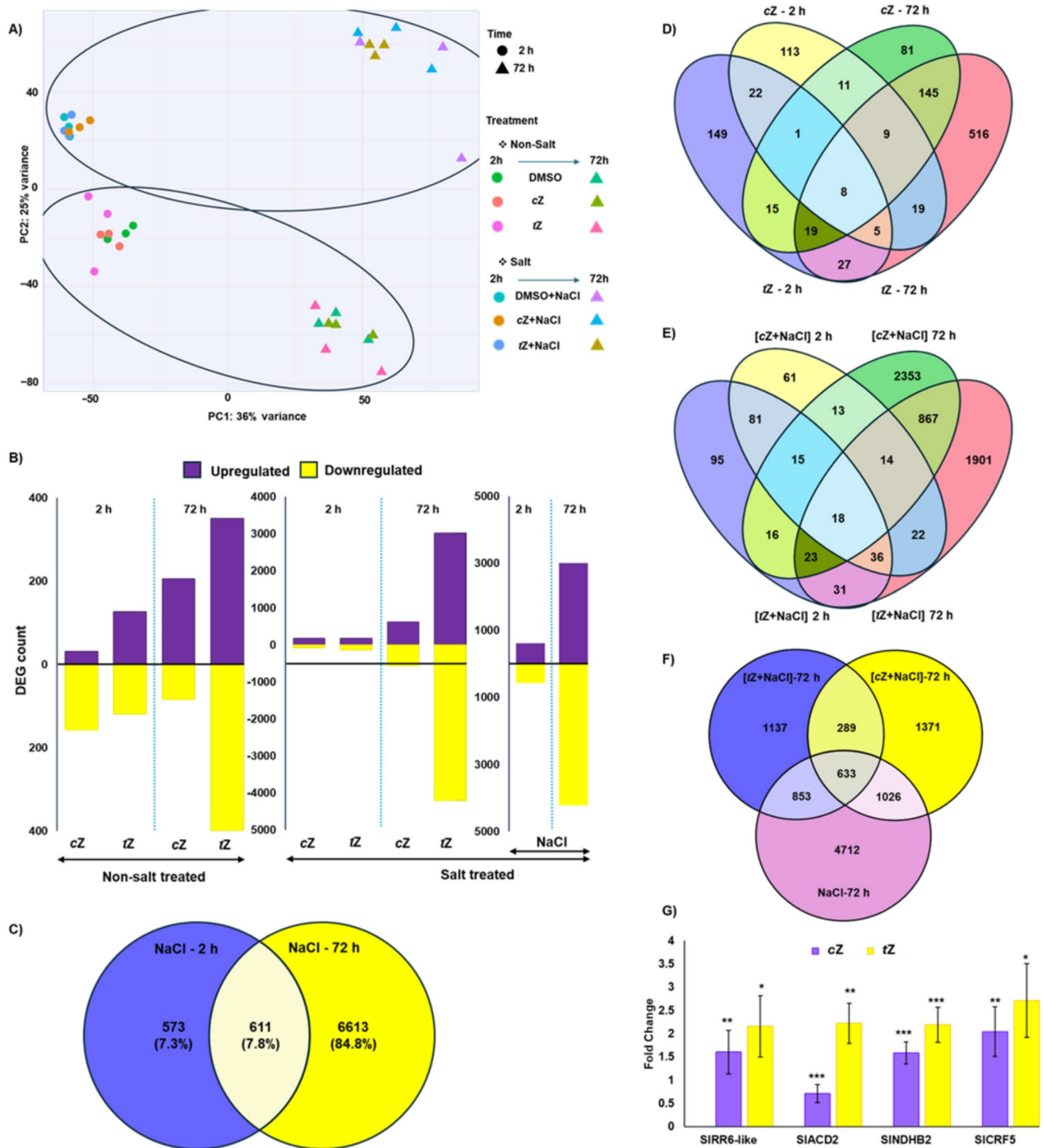


Fig. 2 Distribution of Differentially Expressed Gene (DEG) in response to *cis*-Zeatin (*cZ*), *trans*-Zeatin (*tZ*), and salt stress. **A** Principal Component Analysis (PCA) of transcriptomic profile for the tomato leaves treated with *cZ* and *tZ* $\pm$ 150 mM NaCl treatment—Each dot represents an individual sample and the samples with similar gene expression profiles cluster together while distinct clusters indicate a significant transcriptomic difference among timepoints and treatment. **B** Numbers of upregulated and downregulated DEGs in response to *cZ* and *tZ* at 2 h and 72 h $\pm$ NaCl treatment. **C** Progression of salt regulated DEGs over time, showing unique and shared NaCl-responsive genes at different time points relative to the DMSO control. **D** Overlap and

specificity of DEGs induced by *cZ* and *tZ* at 2 h and 72 h under non-salt conditions. **E** Overlap and specificity of DEGs induced by *cZ* and *tZ* at 2 h and 72 h exposed to 150 mM NaCl. **F** Salt regulated DEGs overlap between *cZ* and *tZ* at 72 h timepoint with unique and common DEGs when exposed to 150 mM NaCl treatment. **G** qRT-PCR validation of selected genes differentially expressed at 72 h under salt stress—Samples were compared to *SITIP41* control gene utilizing the $\Delta\Delta C_t$ method. Results are presented as average of fold change relative to DMSO control (mean $\pm$ Standard Error (SE)). Student's *t*-test was used for statistical analysis. Error bars represent the standard error (SE) of the mean. $P < 0.05$, $P < 0.01$, $P < 0.001$ ($n = 3$)

CK transcriptional responses. Similarly, 4712 DEGs were distinct from salt treatment without CK suggesting that salt treatment has a highly CK-independent stress response. Also, 289 DEGs have common CK regulations, while 853 and 1026 DEGs are regulated by [*tZ*+NaCl and NaCl] and [*cZ*+NaCl and NaCl] respectively. Further 633 DEGs were commonly regulated between all treatments and may be due to core gene regulation between CK induced salt regulation (Fig. 2F; Supplemental File S4). Manual examination for functions of unique and shared CK and salt-regulated DEGs indicated involvement in diverse roles such as hormone signaling, RNA binding, chlorophyll signaling, photosynthesis, ion transport, defense, protein sorting, and cell death (Supplemental File S4; Supplemental Table S5).

To validate the transcriptomic findings, qRT-PCR was performed on selected genes representing key functional categories affected by the treatment, including cytokinin signaling (*SIRR6*-like and *SICRF5*), photosynthesis-related processes (*SINDHB2*), and cell death regulation (*SIACD2*) (Fig. 2G). The expression trends observed by qRT-PCR

mirrored those obtained from RNA-seq, demonstrating the robustness and accuracy of the transcriptomic analysis.

***cis*-Zeatin Induces While Salt Treatment Represses CK Biosynthesis and Signaling Genes**

Expression of CK biosynthesis and signaling genes were affected by *cZ* and *tZ* exposure at 2 h and 72 h ± NaCl senescence treatment and aligns well with *pTCSn::VENUS* expression (Fig. 1D; Fig. 3). Under non-salt treated conditions *cZ* and *tZ* induce several TCS genes including *SIHK4*, *SIRR3* and *SIRR9*-like at both timepoints, *SIRR6*-like only by *tZ* at 72 h and *SIRR9* by *cZ* at both timepoints yet only by *tZ* at 72 h. Further CK oxidases (*CKXs*) *SICKX3*, and 4, were induced by *tZ* at 72 h, however, no *CKXs* were regulated by *cZ* without salt treatment (Supplemental File S5). Although induction of these CK-regulated genes would be expected for *tZ* based on prior work in tomato (Gupta et al. 2013; Shi et al. 2013), we believe these are the first such reports for *cZ* (Fig. 3).

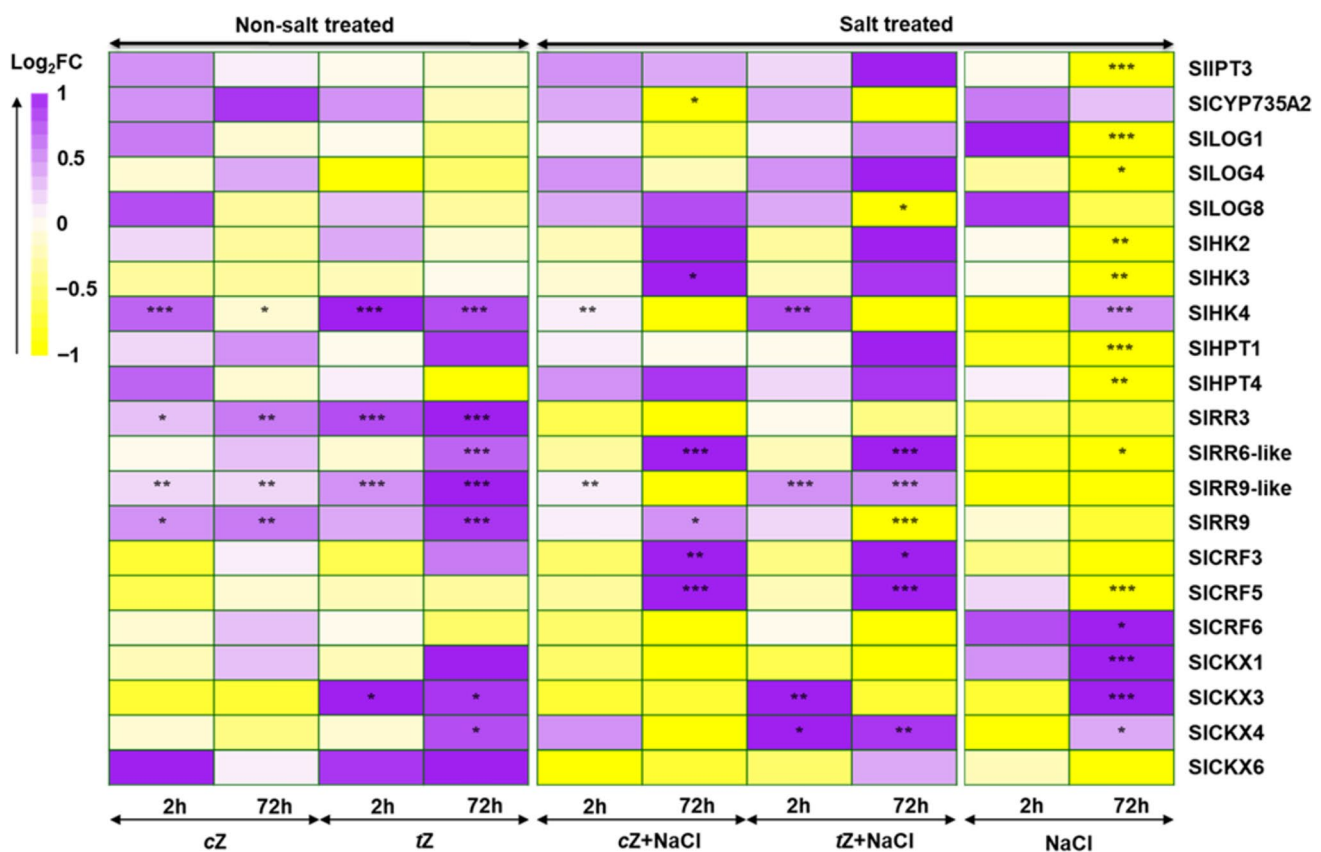


Fig. 3 Heatmap of Cytokinin (CK) related genes in response to *cis*-Zeatin (*cZ*) and *trans*-Zeatin (*tZ*) ± 150 mM NaCl treatment—Heatmap shows the expression patterns of CK biosynthesis, degradation and signaling related genes in tomatoes in response to *cZ* and *tZ* ± 150 mM NaCl treatment. Exposure to salt stress has significantly reduced the expression of the majority of CK biosynthetic genes and

increased expression of the CK degrading genes indicating an altered CK homeostasis when exposed to salt stress. Color intensity represents the Log₂ Fold Change (Log₂FC) indicating the level of gene expression changes. Asterisks (*) indicate the statistical significance (*P<0.05, **P<0.01, ***P<0.001)

Salt stress exposure under non-CK treated conditions also caused regulations of many CK-related genes. Repression of biosynthesis genes, isopentenyl transferase *SIIPT3* (-8.11 FC), *LONELY GUY*; *SILOG1* (-3.96 FC), *SILOG4* (-2.09 FC), and TCS genes, *SIHK2* (-1.47 FC), *SIHK3* (-1.3 FC), phosphotransferases; *SIHPt1* (-3.04 FC), *SIHPt4* (-7.85 FC), *RR*; *SIRR6*-like (-3.99 FC) and CK Response Factor (*CRF*) *SICRF5* (-15.93 FC). While induction of *SIHK4* (2.27 FC), *SICRF6* (5.07 FC) and *CXKs* such as *SICKX1* (3.76 FC), *SICKX3* (33.05 FC), and *SICKX4* (1.54 FC) by salt stress (Fig. 3; Supplemental File 5). Previous regulations of CK-related genes under salt stress including *SIIPT1* and *SIHPt4* repression and *SICKX1* induction were found despite that analysis of tomato seedlings for 6 h, potentially suggesting a conserved regulation across development (Keshishian et al. 2018).

Since many CK-related genes found to be induced by *cZ* and/or *tZ* are repressed by salt, it is not surprising that the combination of CK and salt treatment resulted in an intermediate FC level of regulation not significantly different than non-salt treatment (Fig. 3). However, for some genes induction by CK treatment with salt treatment still resulted in an increase in FC regulation. This was found specifically for *cZ* treatment where *SIHK3* was significantly upregulated 1.08 FC (Supplemental File 5). As previous work indicated that *SIHK3* is downregulated upon salt stress, this suggests *cZ* could act through this CK receptor to modulate CK response under salt stress (Yan et al. 2024). A similar pattern of induction by CK treatment overriding repression by salt treatment was also found for genes regulated by both *cZ* and *tZ* (*SIRR6*-like, *SIRR9*-like, *SIRR9*, *SICRF3*, and *SICRF5*) as well as just for *tZ* (*SICKX3*, *SICKX4* and *SICKX5*) (Fig. 3). Overall, this suggests that exogenous application of CK alters the CK-related gene regulation and in some cases can counter changes in regulation seen under salt stress.

Senescence Associated Genes (SAGs) are Oppositely Regulated by Salt and *cis*-Zeatin

To more broadly study *cZ* effects we examined expression of tomato orthologs of Senescence Associated Genes (SAGs) genes using the leaf senescence database from *Arabidopsis thaliana* (van der Graaff et al. 2006; Li et al. 2012). Gene functions of these identified SAGs were wide ranging and included transcription activity, RNA binding, ethylene, auxin, JA, Salicylic Acid, Brassinosteroid signaling, defense response, chlorophyll degradation, autophagy, redox regulation, protein trafficking and degradation, cell death, nutrient recycling, signal transduction, nucleic acid degradation and glucose signaling (Supplemental File S6).

Multiple SAGs that also have broad functional roles in delaying leaf senescence were found to be significantly

regulated with salt treatment including *CBF2* (8.35 FC), *SAUL1* (1.15 FC), *BAP2/BON/BAP1* (2.49 FC), *SEN4* (2.55 FC), *At2-MMP* (1.50 FC), and *APX4* (5.60 FC). Of these only *SEN4* (-3.17 FC) and *At2-MMP* (-1.28 FC) showed a significant counter effect to [*cZ*+NaCl] treatment. There were also several repressed SAG DEGs: *ACD2* (-22.57 FC), *AUX1* (-2.47 FC), *RCD1* (-0.95 FC), *SEN1* (-4.43 FC), *VTI11* (-0.52 FC), *D-2 HGDH* (-1.20 FC), *ATG6* (-0.93 FC). From these DEGs both *ACD2* (20.78 FC) and *RCD1* (1.02 FC) have significant counter expression under [*cZ*+NaCl] treatment. While no other SAG DEGs are significantly regulated by *cZ*, there appears to be a similar opposite trend in regulation between [*cZ*+NaCl] and NaCl treatment (Supplemental Figure S1A; Supplemental File S6).

Additionally, many SAGs known to promote leaf senescence with wide ranging roles were found as DEGs regulated under salt stress (Supplemental Figure S1B; Supplemental File S6). This includes the following DEGs that are induced: *WRKY6* (3.83 FC), *WRKY53* (4.38 FC), *BOT1* (2.64 FC), *SAG113* (2.71 FC), *Phl1;5* (1.65 FC), *ACS2* (3.12 FC). There also several DEGs that are repressed: *CLAVATA3* (-11.27 FC), *NAP* (-3.90 FC), *EIN2* (-1.39 FC), *PPDK* (-2.22 FC), *GBF1* (-0.73 FC), *YLS2* (-1.32 FC), *NOL* (-6.83 FC), *ESR2/ETR2* (-2.07 FC), *RAVI/EDF4* (-0.87 FC), *SAG24* (-0.89 FC), *YLS6* (-7.56 FC), *MPK6* (-1.29 FC), *HXK1/GIN2* (-1.71 FC), *NYC1* (-1.34 FC), *TCP4* (-5.07 FC), *COS1* (-2.84 FC). Of all those DEGs, *cZ* only uniquely regulates two SAGs namely *SAG101* (1.61 FC) and *SAG13* (-1.58 FC). Again, a trending opposing effect of *cZ* vs salt can be observed at 72 h for SAGs promoting leaf senescence, however it is not significant (Supplemental Figure S1B; Supplemental File S6). These results indicate that *cZ* delays salt induced leaf senescence by reprogramming transcriptional responses associated with stress, metabolism and cell death. In addition, even though not all changes that we observe were statistically significant, a consistent opposing trend was observed for photosynthesis, sugar transport, nutrient recycling suggesting a possible protective role of *cZ* in delaying stress induced leaf senescence and maintaining source-sink relationship.

cis-Zeatin has Unique Gene Regulation at 72 h of Salt Stress Senescence

Gene Ontology (GO) term enrichment analysis was conducted and compared between CK senescence treatments DEGs at 72 h under salt treatment revealing regulated Biological Processes (BP), Molecular Functions (MF) and Cellular Components (CC) (Fig. 4; Supplemental File S7). Interestingly, over 60% of enriched GO term functions were uniquely *cZ* regulated, in contrast to ~10% for *tZ* and ~25% shared between CKs (Fig. 4). Unique BPs regulated by *cZ*

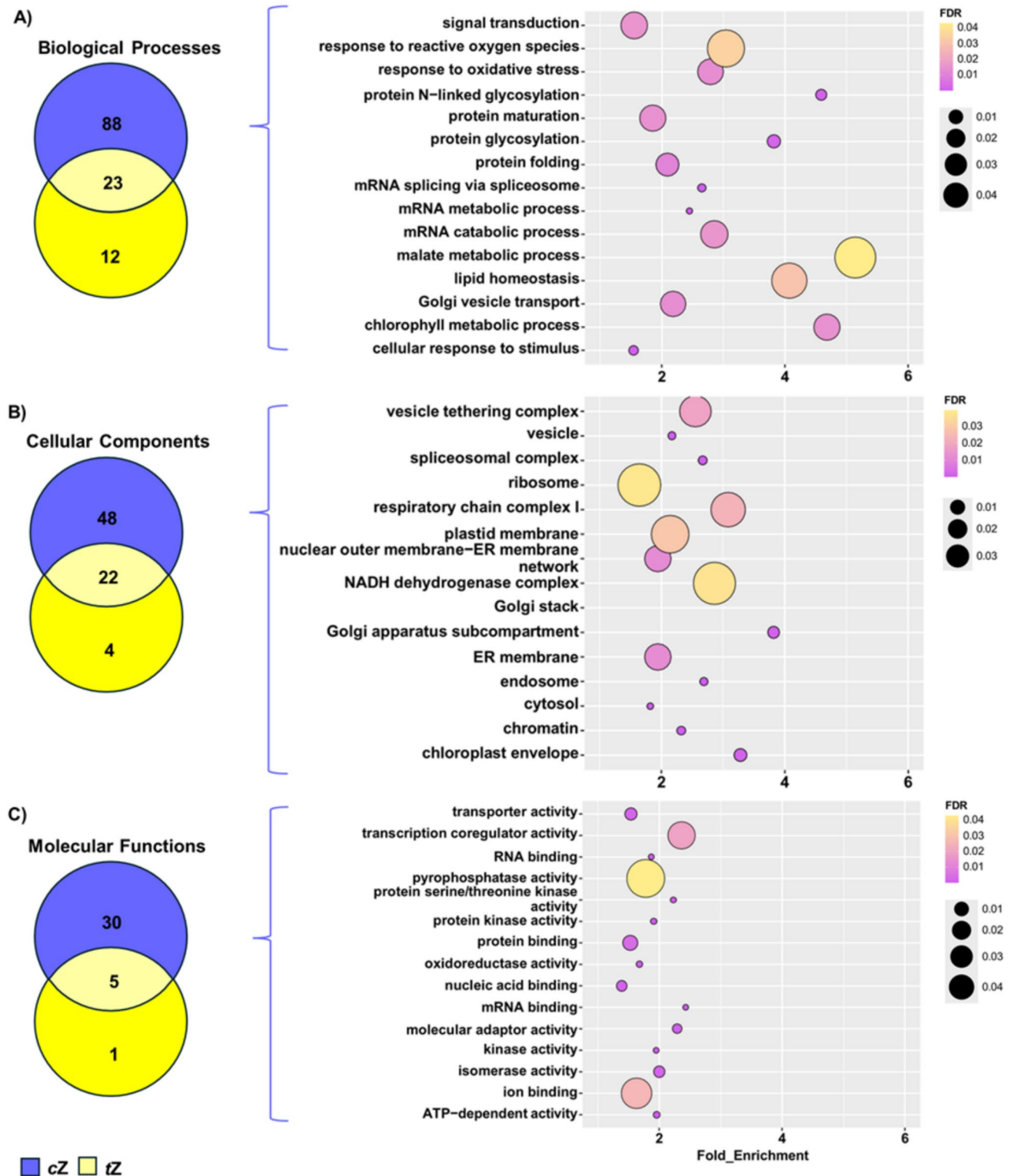


Fig. 4 Gene Ontology (GO) term analysis for *cis*-Zeatin (*cZ*) and *trans*-Zeatin (*tZ*) responsive genes at 72 h exposed to NaCl treatment—Bubble plots show GO term enrichment of Differentially Expressed Genes (DEGs) uniquely regulated by *cZ* at 72 h time point under NaCl treatment. Enriched GO terms are grouped into **A** Biological Process, **B** Cellular Component, and **C** Molecular Function categories. The size of the bubbles represents fold enrichment, and color intensity indicates

statistical significance. *cZ* uniquely regulated a substantially larger proportion of enriched GO terms (>50%) across all three categories compared with *tZ*, which showed relatively few uniquely enriched terms (<10%). This highlights the divergent functional responses of these cytokinin (CK) forms under salt stress. Enrichment significance was determined using a False Discovery Rate (FDR) threshold of <0.05 (Color figure online)

include: metabolic processes related to chlorophyll, malate, pyridine-containing compound, ROS, protein glycosylation, transport of proteins, intra-Golgi vesicle-mediated, nitrogen compounds; response to oxidative stress; mRNA processing, positive regulation of transcription by RNA polymerase II; cellular component biogenesis; nucleic acid biosynthetic process; protein localization and maturation and cellular component assembly (Supplemental File S7). Unique *cZ* regulated MFs included binding of RNA, mRNA, protein, nucleic acids and organic cyclic compounds; activity of phosphatases, kinases, transporters, oxidoreductases, ATP-dependent, and ribonucleoside triphosphate phosphatase. In contrast, *tZ* only regulated one MF, catalytic activity acting on a protein (Supplemental File S7). Together, this analysis suggests that *cZ* uniquely targets multiple signaling pathways and regulatory networks across the cell involved in salt stress response. However, *tZ* was also involved to a lesser extent in overlapping and its own specific salt stress regulation.

***cis*-Zeatin Uniquely Regulates DEGs Involving Photosynthetic and Hormone Signaling Pathways Under Salt Stress**

GO enrichment analysis indicated that majority of chloroplast-related and signaling and signal transduction terms were uniquely regulated by *cZ* therefore, we examined those DEGs (Fig. 4, 5; Supplemental File S7, S8). Again, counter regulatory effects of *cZ* vs salt treatment were found for most photosynthetic and chloroplast associated DEGs (Fig. 5A). A prominent example is *Solyc03g062720* Photosynthetic NDH subcomplex B 2- (*SINADHB2/SubB*) that is strongly repressed (-15.44 FC) under NaCl stress, while strongly induced (17.32 FC) with [*cZ*+NaCl] treatment (Fig. 5A; Supplemental file S8). Studies have indicated that *SubB* is an essential component for the interaction between PSI Light Harvesting Complex Gene 5 (*LHCA5*) and NDH complex (Otani et al. 2018). Similar significant opposing expression patterns were found for several similar genes *Solyc11g071710*, *Solyc07g039500*, *Solyc06g053980*, and *Solyc02g085940*. Whereas other DEGs also show opposite regulation but are repressed by salt and then induced with [*cZ*+NaCl] treatment, *Solyc07g066150* (Photosystem I reaction center subunit V) and *Solyc12g099650* (Photosystem II 5 kDa protein) (Fig. 5A; Supplemental file S8).

Different phytohormones are known to be involved in leaf senescence. While CK, auxin and Gibberellin (GA) are linked to delaying senescence, ethylene, ABA and JA are linked to inducing it under stress conditions (Huang et al. 2022). Our examination of *cZ* uniquely regulated phytohormone-related DEGs under salt stress show *Solyc01g099120* (Auxin response 4) and two GA-regulated

proteins, *Solyc03g113910* and *Solyc02g083880* with opposing *cZ* vs salt responses at 72 h (Fig. 5B; Supplemental File S8). Whereas for senescence inducing hormones we found several Ethylene-related genes *Solyc11g006180* (*ETR5*), *Solyc04g011440* (*HSP70*), *Solyc02g070040* (*ERT2*), and Ethylene-responsive TFs such as *Solyc06g063070*, *Solyc03g117130*, *Solyc11g042560*, *Solyc04g012050* show opposing *cZ* vs salt responses at 72 h. In addition, *Solyc06g061180* (ABA receptor *PYL1*) is repressed by [*cZ*+NaCl] at 72 h, while *Solyc09g015380* (ABA receptor *PYL5*) is upregulated by [*cZ*+NaCl] at 2 h (Fig. 5B; Supplemental File S8).

***cis*-Zeatin Can Show Antagonistic Gene Regulation to *trans*-Zeatin Under Salt Stress**

A comparison of DEGs regulated by *cZ* and *tZ* at different time points revealed interesting differences± salt treatment (Fig. 6). Regardless of salt at 2 h, all CK treated DEGs have a similar direction of expression. However, at 72 h of salt treatment there are ~80 antagonistically regulated DEGs (Supplemental Table S6). Of those 32 DEGs are upregulated by *cZ* and downregulated by *tZ*, while 46 DEGs are downregulated by *cZ* and upregulated by *tZ* (Supplemental Table S6). Interestingly, from those *cZ* up and *tZ* downregulated genes the CK-regulated *Solyc10g079700* (*SIRR9*) was identified. (Supplemental Table S6; Supplemental File S9). Overall, this suggests that *cZ* and *tZ* can produce antagonistic effects under extended salt treatment. Specifically, *cZ* is upregulating more TFs including *SCL3*, TF *GTE10-like*, and *GTE4-like*, and protein modifiers such as E3 ligase, F-box proteins, and ubiquitin hydrolase, and RNA processing components including *SNF2* helicase, polynucleotidyl transferase, translation initiation factor, suggesting *cZ* roles in complex regulation of plant responses (Supplemental File S9). This suggests that at later timepoints, *cZ* and *tZ* antagonistically action involves distinct roles for *cZ* and *tZ* in stress induced leaf senescence (Supplemental File S9).

Weighted Gene Co-expression Network Analysis

We expanded our RNAseq analysis using Weighted Gene Co-expression Network Analysis (WGCNA) to understand clusters of highly correlated CK-and salt-regulated genes during leaf senescence. A gene count matrix with 34,076 gene IDs were used for this analysis and 50 merged co-expression modules were obtained (Supplemental Figure S2A; Supplemental File S2; S10). Eigengene values indicated that MEdarkmagenta, MEwhite, MEgreenyellow, MEgreen, MELightsteelblue1, MEpink and MEsteelblue modules have higher values for *cZ* with 72 h salt treatment indicating that genes in those modules are collectively

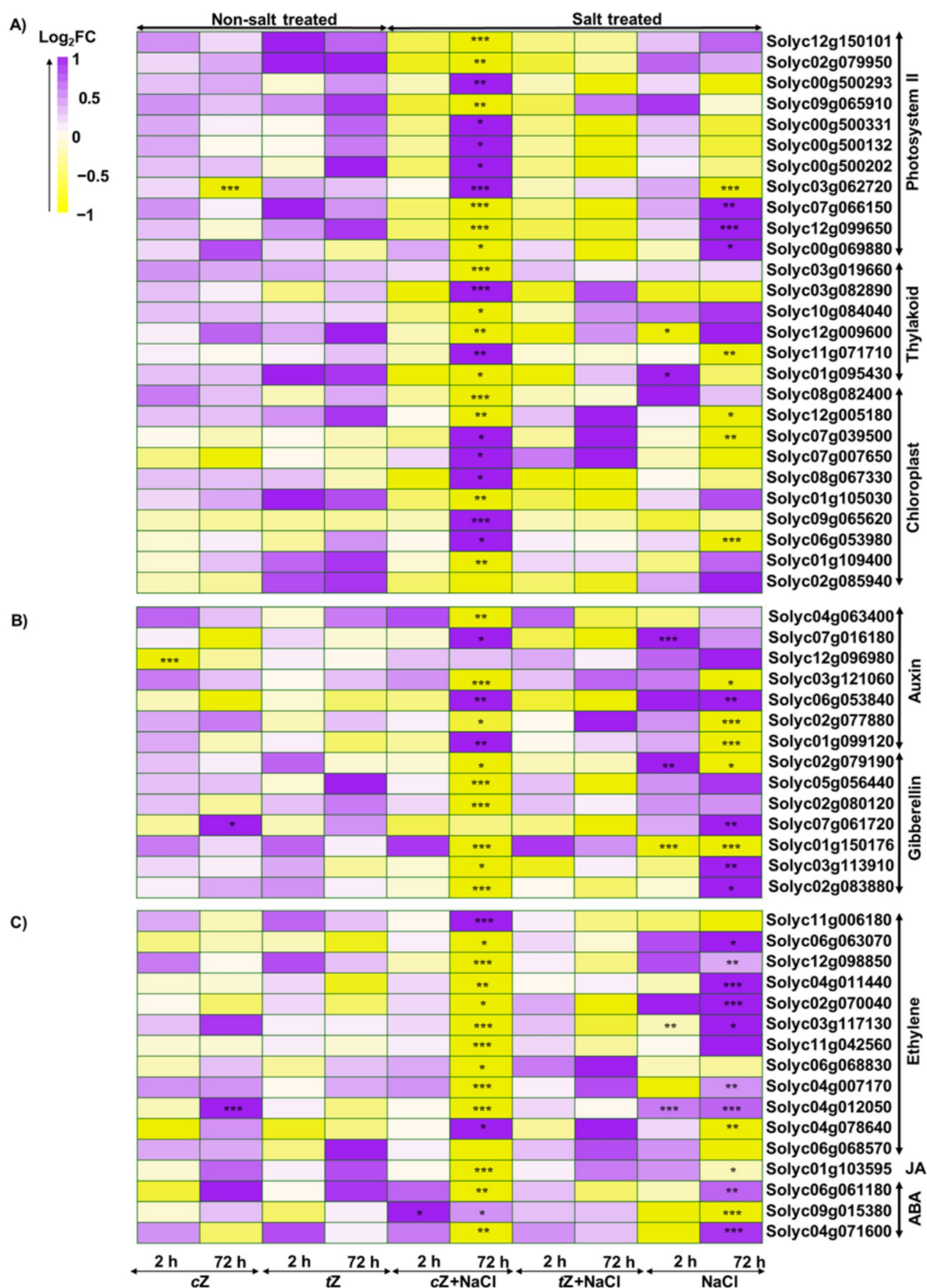


Fig. 5 Unique *cis*-Zeatin (*cZ*) regulated gene expression **A** photosystem II related genes—Uniquely regulated by *cZ* highlighting its potential role in maintaining photosynthetic activity when exposed to salt stress **B** hormone signaling genes associated with senescence-inhibiting pathways—Auxin and Gibberellin are generally linked to growth promotion and delayed senescence; differential regulation of these genes by *cZ* under salt stress suggests modulation of growth-related signaling pathways **C** hormone signaling genes associated with senescence-inducing pathways—ethylene, Jasmonic Acid (JA) and ABA signaling genes that contribute to stress-induced leaf senescence and adaptive to stress responses. Color intensity represents the Log₂ Fold Change (Log₂FC) indicating the level of gene expression changes. Asterisks (*) indicates the statistical significance (**P*<0.05, ***P*<0.01, ****P*<0.001) (Color figure online)

highly expressed. In contrast, *tZ* with 72 h salt treatment showed higher expression only in MEyellowgreen, MEroyalblue and MEdarkturquoise modules. MESaddlebrown, MEorange and MELightcyan showed similar expressions in both CKs at 72 h salt treatment (Supplemental Figure S2B).

Z-score values of genes in each module were plotted, and the overall expression pattern of modules were observed (Supplemental Figure S3). We observed that CK biosynthesis DEGs were distributed among 9 modules while TCS genes identified in a broader range of modules suggesting their widespread transcriptional regulation. However, CK biosynthesis and TCS genes were co-expressed mainly in MEbrown and MEblue modules. Photosynthesis related DEGs are mainly distributed in MEbrown, MEblue, MEgreenyellow and MELightcyan modules whereas 9 of 11 photosystem II associated genes belong to the MEblue module. Similarly, hormone-related genes are also distributed in MEbrown and MEblue modules (Fig. 5; Supplemental Figure S3). All identified SAGs are distributed in 17 different modules, notably in MEbrown, MEblue, and MEgreenyellow (Supplemental Figure S1; S3). Furthermore, unique salt-regulated DEGs at 72 h are also distributed in MEbrown, MEblue and greenyellow (Supplemental File S4; Supplemental Figure S3). These patterns indicate a coordinated transcriptional programming involving CK biosynthesis, signaling and its association in leaf senescence. MEblue and MEbrown modules have a similar z-score expression pattern and a positive Pearson correlation between each other, indicating these modules are key regulatory hubs in salt stress induced leaf senescence regulation under CK treatment (Fig. 7A; Supplemental Figure S3). Pearson correlation between module eigengene and traits; timepoint, NaCl and hormone treatment indicated that out of all 50 modules, MEgreenyellow, MEgreen, MELightsteelblue1 showed significant interaction between module to traits; timepoint, NaCl, and hormone respectively (Fig. 7B). These results indicate that *cZ* is playing a role in mediating *cZ* specific transcriptional response under prolonged exposure to salt stress thus, there is a potential co-regulation or

shared functional pathways in association to the hormone signaling and salt stress.

Discussion

Cytokinin Effects on Leaf Senescence Under Salt Stress are Dose-Dependent

Cytokinin (CK) effects on the leaf senescence response under salt stress are dose dependent and CK response to salt stress has been shown to act antagonistically in the regulation of Arabidopsis leaf senescence (Khanna et al. 2025). Accordingly, we performed a cytokinin dose–response analysis to identify an effective concentration for functional assays under salt stress in tomato leaves. We observed a concentration dependent increase of photosystem II efficiency (F_v/F_m) in salt [150 mM Sodium Chloride (NaCl)] treated leaves following CK application of 10 nM, 100 nM, and 1000 nM (1 μ M) *trans*-Zeatin (*tZ*) and *cis*-Zeatin (*cZ*), with the strongest and most reproducible effect observed at 1 μ M (Fig. 1A). Consistent with this, previous studies have shown similar concentration ranges of *tZ* to assess CK mediated regulation of senescence and stress responses and the leaf senescence recovery in tomato (Keshishian et al. 2018) and Arabidopsis (Khanna et al. 2025) well aligned with our data. Moreover, CK responsiveness has been robustly detected using micromolar hormone concentrations, including *CRF6::GUS* induction by 1 μ M 6-benzylaminopurine (BA) (Zwack et al. 2013). Together, these observations support the use of 1 μ M CK as a biologically effective for regulating CK signaling and its protective role during salt induced leaf senescence.

cis-Zeatin Delays Salt Accelerated Leaf Senescence Physiology

Physiological examinations of senescence and salt stress were conducted at 72 h using F_v/F_m measurements and chlorophyll deterioration as key indicators of senescence, widely used with CK treatments in dark induced leaf senescence bioassays (Keshishian et al. 2018, 2022). This bioassay revealed *cZ* functions to delay senescence in tomato leaves, similar to *tZ*. Moreover, *cZ* can also delay salt stress induced leaf senescence as seen by increased chlorophyll and F_v/F_m levels (Fig. 1B; C; Supplementary File S1). Retention of more chlorophyll by both *cZ* and *tZ* suggests the ability of these CKs to maintain photosynthesis even under salt stress. Overall, this physiology indicates that *cZ* functions as an active CK type in delaying leaf senescence in tomatoes.

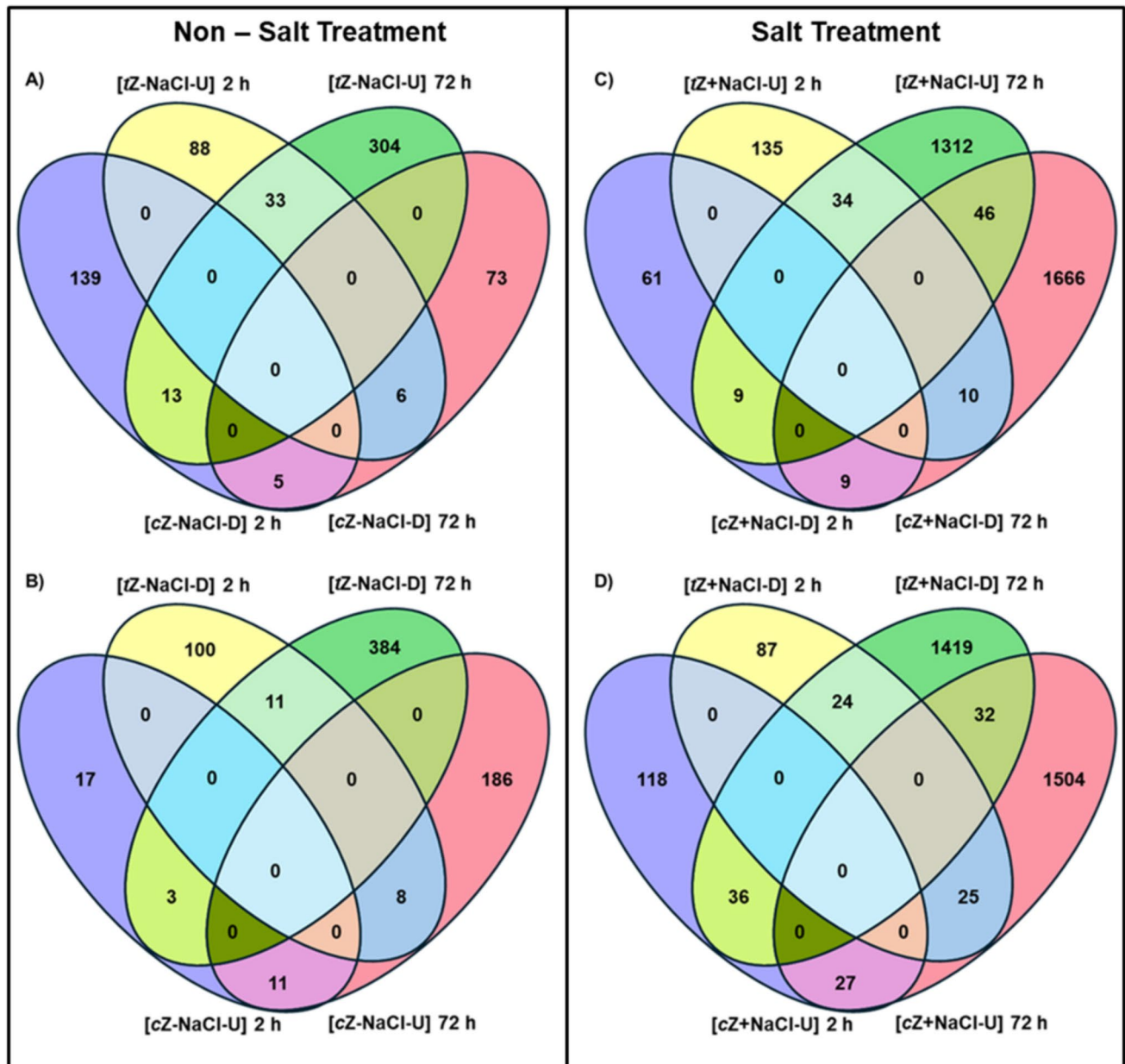


Fig. 6 Antagonistic transcriptional effects of *cis*-Zeatin (*cZ*) and *trans*-Zeatin (*tZ*) $\pm$ 150 mM NaCl treatment. **A** Overlap between genes upregulated by *tZ* and genes downregulated by *cZ* at 2 h and 72 h under non-salt conditions. **B** Overlap between genes downregulated by *tZ* and genes upregulated by *cZ* at 2 h and 72 h under non-salt conditions. **C** Overlap between genes upregulated by *tZ* and genes downreg-

ulated by *cZ* at 2 h and 72 h under NaCl treatment. **D** Overlap between genes downregulated by *tZ* and genes upregulated by *cZ* at 2 h and 72 h under NaCl treatment.—Antagonistic regulation between *cZ* and *tZ* was minimal under non-salt conditions but became apparent at the later time point under salt stress. “U” and “D” denote upregulated and downregulated genes, respectively

cZ Induces pTCSn Expression Upon Exposure to Salt Stress

Previous examinations of CK response have been observed using the *pTCSn::VENUS* reporter line, where moderate increases in fluorescent expression were seen in salt stress roots after 24 h, although greater expression occurs with the CKs (*tZ*) or 6-Benzylaminopurine (BA) application

(Keshishian et al. 2018; Steiner et al. 2020). To our knowledge *cis*-Zeatin (*cZ*) treatments have not been examined as a CK in this manner in tomato, which was checked here in roots revealing that *cZ* can induce CK response via pTCSn especially at 72 h (Fig. 1D), similar to that seen in Arabidopsis (Antoniadi et al. 2020, 2022). Further treatment of roots with 150 mM NaCl and CK treatment were conducted. While salt treatment appears to reduce pTCSn expression in

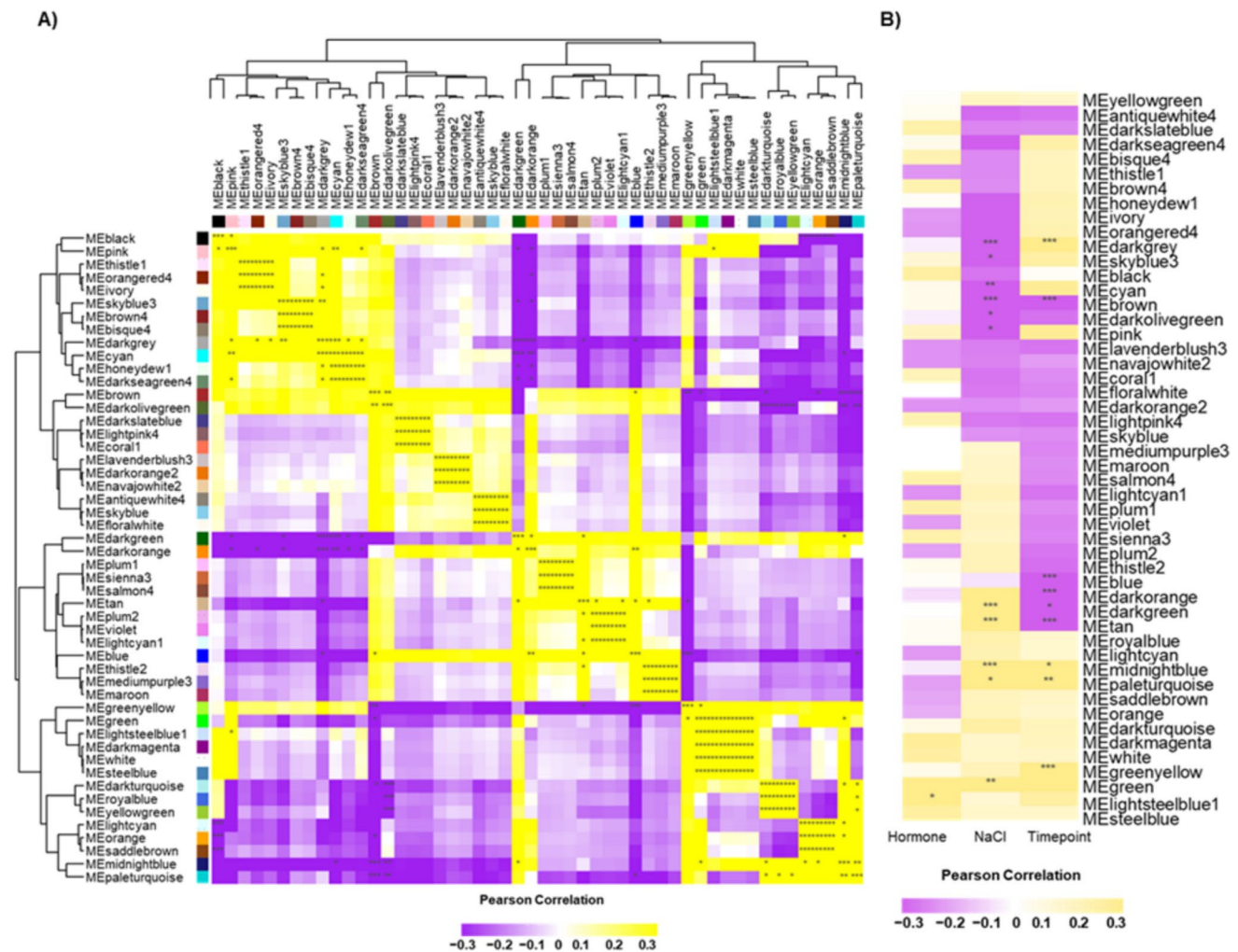


Fig. 7 Pearson correlation between modules from Weighted Gene Co-Expression Network Analysis (WGCNA) in response to *cis*-Zeatin (*cZ*) and *trans*-Zeatin (*tZ*) $\pm$ 150 mM NaCl treatment. **A** Module to module correlation—The heatmap shows the Pearson correlation among co-expressed modules. **B** Module trait correlation—The heatmap shows

the relationship between 50 co-expressed modules and experimental traits including *cZ* and *tZ* $\pm$ 150 mM NaCl treatment. Color intensity represents the $\log_2$ Fold Change ($\log_2FC$) indicating the level of gene expression changes. Asterisks (*) indicate the statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) (Color figure online)

the absence of CK, addition of either *cZ* or *tZ* yields high expression at 2 h that are maintained at 72 h, indicating that both *cZ* and *tZ* can counter salt effects. The high expression of *cZ* also indicates that this CK form can be functionally involved in CK responses similar to *tZ*, indicating further study of *cZ* effects on salt senescence of leaves should be examined.

Salt Stress Induces Time-Dependent Transcriptional Programs that Accelerate Leaf Senescence

Our transcriptomic analysis shows changes in gene expression within 2 h for both CK and salt, although this is most evident with salt increasing the number of DEGs at 72 h (Fig. 2). The number of DEGs increases ~ 6.5 fold with salt treatment, from 1184 at 2 h to 7724 at 72 h, highlighting

that salt accelerates leaf senescence from an early to later point where salt treated leaf senescence can be observed from physiological measurements (Figs. 1; 2; Supplemental Table S2). Manual examination of DEGs for gene function shows that there are unique types of gene regulation at each time point likely connected to osmotic stress and ionic toxicity from 2 to 72 h (Supplemental Table S3). During osmotic stress, cell turgor is maintained by regulating K^+ / Na^+ balance, synthesizing osmolytes including prolines and soluble sugars to respond as well as to regulate Reactive Oxygen Species (ROS) (Gamalero et al. 2020). Similarly, Abscissic acid (ABA) plays a key role in regulating transpiration rate upon the early exposure to stress (Fricke et al. 2004). Gene Ontology (GO) term analysis for 2 h salt treatment validates changes in the proline catabolic and metabolic processes, ABA activated signaling pathway, response

to water deprivation, transport, small molecule catabolic process, Jasmonic Acid (JA) metabolic process and amino acid catabolic process indicating the osmotic regulation (Supplemental File S7).

Upon longer exposure to salt stress ion toxicity occurs and salt is extruded out of cells by activation of Salt Overly Sensitive (SOS) pathway or vacuole sequestration (Ji et al. 2013). Our findings align well with salt regulation across timepoints, especially the activation of *Solyc01g005020* (*SOS1*) and salt stress regulated TFs *Solyc02g080890* (*WRKY6*) and *Solyc09g014990* (*WRKY33*) as seen in Arabidopsis or connected to salt tolerance in tomato (Khanna et al. 2025). Similarly, GO term analysis for 72 salt treatment revealed programmed cell death, spindle disassembly, RNA destabilization, mRNA destabilization, cellular component disassembly, autophagy, Golgi organization, glycosylation as effected processes involved in regulating ion toxicity, or cell death (Supplemental File S7). Senescence Associated Genes (SAGs) were also found here as both positively and negatively regulated by salt stress, indicating a complicated process of salt stress effects in leaf senescence (Supplemental Figure S1).

Number of *cis*-Zeatin Regulated DEGs is Highest at the Later Point of Salt Exposure

Similar analysis of DEGs with CK treatment $\pm$ salt indicated that overall numbers of DEGs increase with the duration of CK treatment (Fig. 2B). While *tZ* treatment has more DEGs during senescence without salt, *cZ* has ~1000 DEGs more than *tZ* at 72 h with salt: 3319 vs 2912 DEGs (Fig. 2B; Supplemental Table S2). This suggests for senescence without salt, *tZ* may play a prominent gene regulation. However, with salt, *cZ* plays a strong role at 72 h, suggesting it might be crucial in later stress response and adaptation in tomatoes, although the exact reason is unknown. Previous CK measurements of tomato seedlings and Arabidopsis leaves indicated *cZ* levels can increase after continued salt stress (Keshishian et al. 2022; Khanna et al. 2025). Potentially, this higher accumulation may trigger a large transcriptional activation in response to prolonged salt exposure. Interestingly, GO term analysis indicates that *cZ* regulates multiple regulatory network pathways compared to *tZ* (Fig. 4; Supplemental File S7), possibly suggesting a distinct *cZ* effect in regulating prolonged stress exposure.

cis-Zeatin and *trans*-Zeatin Regulate CK Signaling and Biosynthesis Genes in Opposing Manners Under Salt Stress

We found regulation of several tomato CK signaling and biosynthesis/degradation pathway genes (Fig. 3), which are

similar to the well described Arabidopsis system, although poorly characterized, particularly response to exogenous application of CK (*cZ*, *tZ*) and salt treatment (He et al. 2016; Žižková et al. 2015; Schäfer et al. 2015). We observed overlaps of *cZ* and *tZ* DEGs indicating functionally similar regulations by the two CKs. Previous studies from Arabidopsis revealed that various Two Component Signaling (TCS) mutants have different sensitivities towards Na^+ accumulation showing both positive and negative effects on salt tolerance, which is aligned with our transcriptome findings here (Mason et al. 2010).

We found the CK receptors are all significantly altered with salt treatment at 72 h: *SIHK2* and 3 repressed and *SIHK4* induced (Fig. 3). Interestingly, for all receptors with both CK and salt treatment expressions trend in the opposite direction, but are no longer significantly regulated, except for *SIHK3* regulation by *cZ* treatment (Fig. 3; Supplemental File S5). This could suggest that *cZ* may act through *SIHK3* to modulate CK response under salt stress, but greater examination of CK receptor binding and signaling is needed on this front. However, these findings suggest that each CK is playing a role in potentially modulating CK effects differently in the presence and absence of salt stress.

SICRF5 was also found to have opposing regulation under salt stress with/without CK treatment, being strongly repressed without CK under salt treatment at 72 h yet being strongly induced by both *cZ* and *tZ* with salt (Fig. 3). Interestingly, *SICRF5* has been previously shown as regulated by both CK (strongly induced) and various abiotic stresses, including a slight induction with salt in seedlings (Shi et al. 2012, 2013; Gupta and Rashotte 2014; Hughes et al. 2021). This shows another example of CK-related genes with opposing regulation by *cZ* and salt, validated here using qRT-PCR (Fig. 2G).

An additional example of a CK biosynthesis gene, *SIITP3* previously linked to salt stress (Žižková et al. 2015), was found to be significantly repressed under salt stress (72 h), but not with either *cZ* or *tZ*, suggesting that CK may be blocking that repression. *IPT* genes are CK synthesis genes involved in the initial step of either *cZ* or non *cZ*-CKs. In Arabidopsis, *AtIPT2*, 9 are specific for *cZ* biosynthesis, while it is unclear which tomato *IPTs* (*SIIPT1-SIIPT6*) are specified for that function (Žižková et al. 2015; Schäfer et al. 2015). We checked for *IPT* protein orthologs with (*SIIPT1-SIIPT6*) and (*AtIPT1-AtIPT9*) sequences utilizing ClustalW parameters in Clustal Omega Multiple Sequence Alignment (MSA) that produced a phylogenetic tree showing *SIIPT5* and *SIIPT6* as most closely related to *AtIPT2* and *AtIPT9* respectively (Supplemental Figure S4). However, we did not observe any induction of *SIIPT5* and *SIIPT6* (or any other *SIIPT*) with either *cZ* or *tZ*+NaCl treatment suggesting

differences in response between tomatoes and Arabidopsis (Fig. 3; Supplemental File S5).

***cis*-Zeatin and *trans*-Zeatin Antagonistically Regulate Genes Under Salt Stress**

While the majority of *cZ* and *tZ* DEGs show a similar direction of regulation, we did find antagonistic regulation of 80 DEGs under salt stress at 72 h. Manual examination of *cZ* up and *tZ* downregulated genes indicated involvement in flower development, protein turnover and ubiquitin pathway, vesicle trafficking and endomembrane system (Endoplasmic reticulum (ER) and Golgi), mitochondria and chloroplast related, transcriptional regulation and nucleic acid processing. In contrast, *tZ* up and *cZ* downregulated genes are mainly involved in DNA and RNA metabolism, transcriptional regulation, stress and defense response, cell wall remodeling, protein homeostasis, endomembrane and chloroplast related (Supplemental File S9).

Specific examples include *SIRR9*, a *RRA* negative regulator in CK signaling which is up by *cZ* while down by *tZ* under prolonged exposure to salt stress, whose rice ortholog *OsARR9* is linked to regulation of salt tolerance (Wang et al. 2019). *SCL3*, also up with *cZ* and down with *tZ*, which in Arabidopsis as a positive regulator of Gibberellin (GA) signaling (Zhang et al. 2011), with GA noted as a positive regulator in delaying leaf senescence (Huang et al. 2022). For up by *tZ* and down by *cZ*, *Solyc05g054710* (Beta-hexosaminidase) and ascorbate peroxidases (*APX*) which are involved in fruit ripening and oxidative damage protection were found (Supplemental File S9) (Preiss 2010; Irfan et al. 2022). Overall this shows that different CKs can have opposing effects on leaf senescence, although clearly more studies are needed to further investigate these effects.

WGCNA analysis indicated that *tZ* up, *cZ* down DEGs are found in 4 modules, primarily in MEdarkgrey, and MEbrown, while *cZ* up, *tZ* down DEGs are in 7 non-overlapping modules prominently MEgreen, MEgreenyellow, and MEbrown, indicating unique CK module regulation. GO term analysis indicated that MEgreen and MEgreenyellow modules are centered around processes related to nucleus regulation and eigengene values for these modules are collectively highly expressed in *cZ* with 72 h salt treatment with a positive Pearson correlation between module eigengene and traits (Supplemental Figure S2; Fig. 7B). In contrast, GO term analysis of MEdarkgrey module indicated broader processes including cellular response to nutrient levels, protein ubiquitination, nucleic acid catabolism, and micro and macroautophagy, well aligned with the manual observation of antagonistically regulated DEGs functions. Additionally, the MEdarkgrey module showed a negative Pearson correlation to the MEgreen module (Fig. 7A), further supporting

that *cZ* and *tZ* activate distinct and opposite transcriptional regulations including developmental processes, and stress response.

Unique Transcriptional Regulation of *cZ* Towards Leaf Senescence

CK-regulated TFs in Arabidopsis have been well examined, but effects of different CK types on tomatoes TF have not. Here we observed that *type B SLARR1* is positively regulated by [*cZ*+NaCl] at 72 h paralleling finding of *AtARR1* (Fig. 3; Supplemental File S5) (Mason et al 2010). Similarly, multiple *SICRFs* (*SICRF1,3,5,6* and 9) were regulated under salt stress, including upregulation of *SICRF6* and 9, and one negative regulation for *SICRF1* [*cZ*+NaCl] treatment at 72 h (Supplemental File S5). While previous work showed *AtCRF1* and *AtCRF2* were salt stress regulated (Hallmark and Rashotte 2019).

Few salt or senescence regulated TFs are known in tomato, however, one such TF, *Solyc01g079260* (*SIWRKY23*) has been identified as induced by salt stress in tomato (Singh et al. 2023). Our findings also showed a similar induction by salt treatment, while only *cZ* downregulates the expression under longer salt stress. Another *NAC TF Solyc04g005610* (*SINAP2*) is downregulated by salt stress, but uniquely counter regulated by the *cZ* under the salt stress (Supplemental File S3). Studies indicated that *SINAP2* activates *SISAG113* and promotes leaf senescence, however, we found regulation of *SISAG113* only from *tZ* with prolonged salt stress (Ma et al. 2018).

A higher positive Pearson correlation was observed between MEgreen module, which carries *SIWRKY71* and *SIWRKY40*, to NaCl interaction along with a higher eigengene expression of *cZ* at 72 h salt treatment. Expression values indicate that these WRKY genes are significant positive regulators under salt stress (Fig. 7B; Supplemental File S3; S10). Overall, this suggests that salt stress can trigger hormonal signaling pathways and transcriptional changes during salt stress senescence progression.

***cis*-Zeatin Uniquely Regulates Photosynthetic and Hormone Signaling Genes**

GO enrichment analysis of *cZ* DEGs indicates unique regulation of Biological Processes such as chlorophyll, malate, and metabolic processes (Fig. 4, 8; Supplemental File S7). Similarly, numerous Cellular Components terms related to plastid or chloroplast membranes are uniquely regulated by *cZ* (Supplemental File S7). One example is *Solyc03g062720* (Photosynthetic NDH subcomplex B 2(*SINDHB2*) -*SubB*) which is strongly repressed by salt stress (-15.44 FC), while strongly induced by *cZ* with salt (17.32 FC) (Fig. 5A;

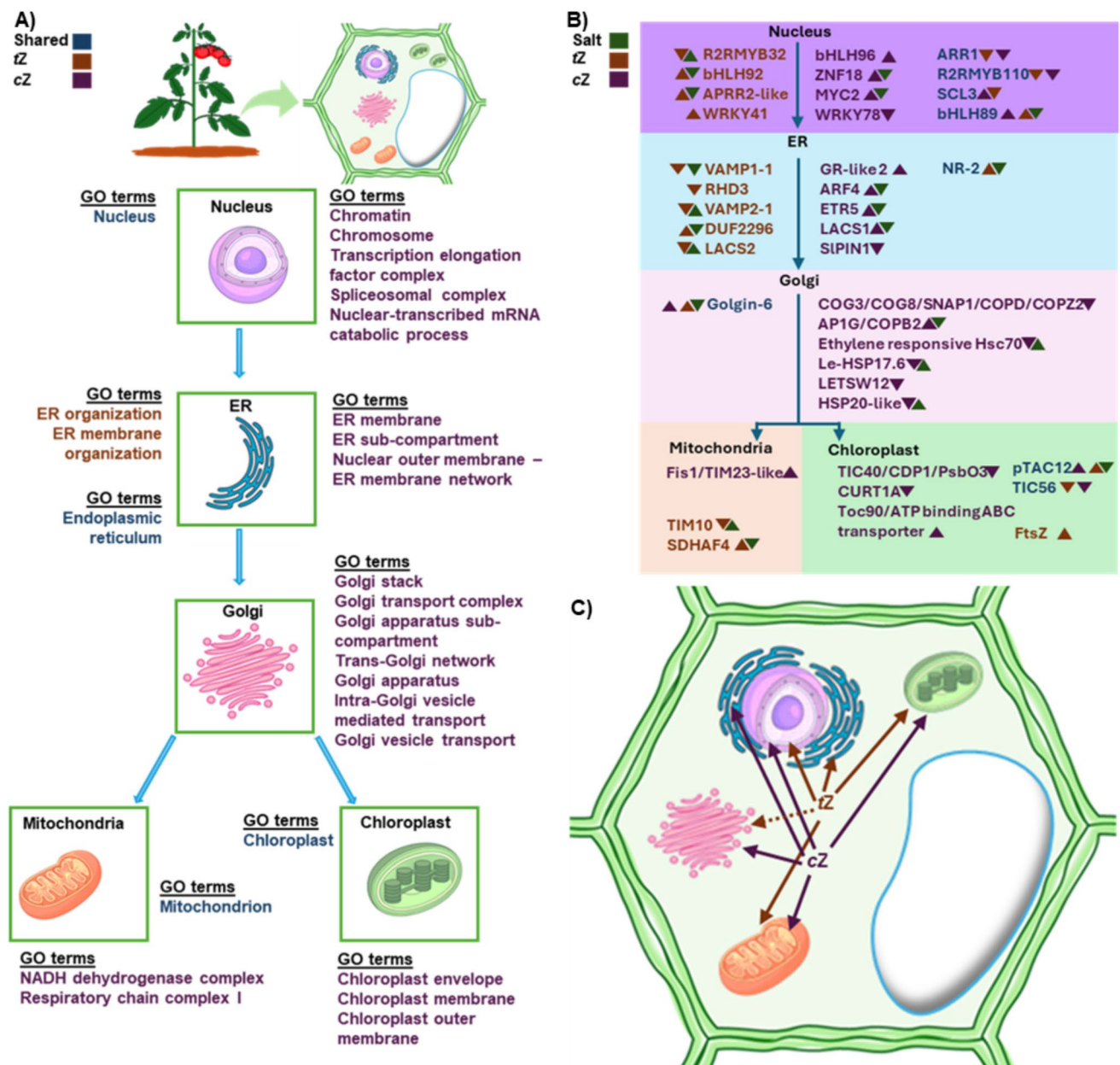


Fig. 8 Intracellular cytokinin (CK) transcriptome effects of *cis*-Zeatin (*cZ*) and *trans*-Zeatin (*tZ*). **A** Gene Ontology (GO) term enrichment for organelle-specific genes, including nucleus, Endoplasmic Reticulum (ER), chloroplast, and plastids—Text color indicates GO terms shared between *cZ* and *tZ* (blue) or uniquely associated with *tZ* (brown) or *cZ* (purple). **B** Distribution of unique and shared differentially expressed genes (DEGs) regulated by *cZ* and *tZ* within each organelle—Upward

triangles indicate upregulated genes, and downward triangles indicate downregulated genes. **C** Conceptual model of intracellular CK regulation—Solid arrows represent regulation supported by GO term enrichment in this study, whereas dashed arrows indicate potential regulatory interactions not directly identified from the current GO analysis (Color figure online)

Supplemental file S8). *SubB* is an essential component for interaction between PSI Light Harvesting Complex Gene 5 (*LHCA5*) and NDH complex (Otani et al. 2018). While no previous work connects *cZ* to *Solyc03g062720*, *Solyc06g053980* (*Chlorophyllase 2*) shows a similar repression by salt (−5.52 FC) but induction by [*cZ*+NaCl] (4.39 FC) (Fig. 5A; Supplemental File S8). Previous examination of *Solyc06g053980* indicates transcripts are significantly

decreased in aging leaves (Liu et al. 2022b). Although no link between *Arabidopsis chlorophyllase 1* and *chlorophyllase 2* to senescence has been seen (Schenk et al. 2007). Despite the difference between tomato and *Arabidopsis* findings, our data suggests that *Solyc06g053980* has a developmental role in chlorophyll regulation and *cZ* is uniquely regulating that effect.

cZ also shows unique regulation of hormone genes other than CK under salt stress (Fig. 5; Supplemental File S7). This includes several ethylene related genes: Ethylene-responsive *HSP70 Solyc04g011440*, ethylene-responsive nuclear protein *ERT2*, and ethylene TFs *Solyc03g117130*, *Solyc11g042560* (Fig. 5B; Supplemental File S8). There is similar induction by only *cZ* under salt stress for JA *ZIM* domain protein 1, ABA receptors *PYL1* and 5, and ABA stress ripening 5. Previous studies have indicated that ABA, JA and ethylene are all stress-related, and overexpression of *PYL5* in rice and *AtPYL5* Arabidopsis enhanced drought tolerance and resistance.

Distinct *cis*-Zeatin and *trans*-Zeatin Regulation of Cell Organelles; Nucleus, Endomembrane System and Plastids

GO term examination for CK regulation of the organelles revealed that *tZ* and *cZ* have a shared regulation for nucleus, ER, mitochondria and chloroplast (Fig. 8A; Supplementary File S7; S11). TF regulation is nuclear, and several TCS TFs are regulated by both CKs, while other TFs show specificity to *tZ/cZ* and salt stress (Fig. 8B). ER organization and membrane organization were the only *tZ* unique regulations. In contrast, *cZ* GO terms are widely distributed across organelles, with unique connections to the Golgi (Fig. 8A; Supplementary File S7; S11). Intercellular CK distribution indicated *tZ* is predominantly in ER, while *cZ* is mainly in the cytoplasm. Overall ER regulation is shared between both CKs, while each CK form has unique ER terms, as noted above for *tZ*. Clearly CK is important for ER gene regulation as its receptors are localized there, yet specific CK forms might play unique roles in organizing ER membranes to cope with the stress regulation or with nuclear membrane and Golgi network trafficking (Fig. 8B) (Hoyerová and Hošek 2020; Hu and Shani 2023).

cZ has unique GO enrichment for most Golgi terms (Fig. 8; Supplementary File S7). Few studies have examined the association of Golgi in leaf senescence. Although, the *trans*-Golgi localized phosphate transporter *PHT4;6* in Arabidopsis is critical for leaf senescence and mutants accelerate dark induced leaf senescence and CK acts as an upstream regulator (Hassler et al. 2016). Also, mutations in Conserved Oligomeric Golgi 7 (*COG7*), a subunit in retrograde trafficking of golgi localized glycosylation enzymes causes accelerated dark induced senescence under sugar deprivation (Choi et al. 2023). Furthermore, Golgi localized *SAG113* is a protein phosphatase induced by ABA and negatively regulates ABA mediated stomatal closure that accelerates leaf senescence (Zhang et al. 2012). Interestingly, another study indicated that *GnTII* which catalyzes glycosylation of proteins by addition of a N-glycan in the

Golgi, has a significantly reduced CK signal in the mutant during dark induced leaf senescence, suggesting this N-glycan plays an essential role in proper expression TCS components (Yoo et al. 2021). This potentially links *cZ* to a crucial role in glycosylation processes to delay the leaf senescence and overall indicates that *cZ* is associated with Golgi apparatus during leaf senescence.

The chloroplast has an overall shared regulation as might be expected since this organelle is a key component in regulating leaf senescence as it holds the chlorophyll pigments and conducts photosynthesis. There are multiple chloroplast specific GO terms that are only regulated by *cZ* involving the membrane and envelope, possibly suggesting *cZ* has a larger role in regulating this organelle (Fig. 8A; Supplemental File S7). The Mitochondria findings are similar to the chloroplast in that there is shared CK overall regulation, and only *cZ* specific distinct GO terms. While there are some links between CK and mitochondria function, including mitochondrial retrograde signaling via *AtCRF6* (Zwack et al. 2016) this general connection is not well explored.

Interestingly, CK intracellular measurements in Arabidopsis indicate a high *cZ/tZ* type ratio of 75–175 fold for each examined organelle: nucleus, ER, chloroplast, and Mitochondria (Skalický et al. 2023), which parallel our findings of abundant *cZ* GO term distribution. Unfortunately, quantification of CK in Golgi remains undetermined, but it would seem that similar high *cZ* levels would be found there. Although vacuolar CK has been determined and is ~300 fold, no vacuolar specific GO terms were identified in this study.

Proposed Model for CK Diversification Through Signal Duration

Although *cZ* and *tZ* are perceived through same CK receptors in the TCS pathway (Fig. 3), our transcriptomic data indicates distinct downstream responses across time points upon the exposure to salt stress. Therefore, we propose a mechanistic model wherein these CK response differences may result from isoform stability rather than receptor affinity (Supplemental Figure S5). Although not examined in tomato, previous studies in Arabidopsis and potato have indicated that *tZ* has a higher receptor affinity than *cZ*, while similar receptor affinities between these isoforms have been observed in maize and rice (Romanov et al. 2006; Stolz et al. 2011; Lomin et al. 2018). This indicates that rates of rapid CK signaling may depend on species of plant examined. In addition, previous studies indicated that *tZ* is predominantly degraded by *CKX1*, 5, and 7 under non salt conditions, whereas *CKX3* and 4 significantly degrade *tZ* under salt treatment (Khanna et al. 2025). Similarly, our transcriptomic data indicates that FC levels of *SICKXs* are generally

higher in *tZ* compared to *cZ* treatment (Supplemental Figure S5). Although in this study CK concentrations or enzymatic activities were not measured, it is possible that there is a rapid degradation happening for *tZ* compared to *cZ* CK isoforms, resulting in *cZ* having greater stability that enables a sustained transcription over a longer time scale. This prolonged response could regulate multiple regulatory pathways including organelle remodelling and other metabolic processes taking place in the plant when exposed to salt stress.

While further, biochemical and genetic analysis are needed, to validate this model, transcriptomic data from this study supports the concept in which *cZ* and *tZ* may act through shared receptors but drive functionally distinct transcriptional programs via differences in signal duration due to the degradation response by *SICKXs*.

Conclusion

Overall, we found that *cZ* plays a crucial role in delaying leaf senescence in tomatoes under salt stress. First, *cZ* can delay leaf senescence with or without salt, indicating overall importance to the regulation of this process. Moreover, transcriptomic analysis shows that *cZ* uniquely regulated genes involved in senescence regulation $\pm$ NaCl treatment. Greatest effects were at a later senescence phase (72 h) where *cZ* appears to target genes connected to multiple salt stress signaling and regulatory networks. While *cZ* shows some overlapping regulation, it often regulates genes as a CK, distinct from *tZ*, including with some antagonistic regulation. Intracellular regulation by CK indicates that *cZ* is involved in more specific organelle processes than *tZ*, with unique regulation in Golgi revealing that underexplored *cZ* is broadly important to regulation of leaf senescence under salt stress.

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Author contributions M.T and A.M.R designed the experiments, analyzed the results, and wrote the manuscript. M.T., R.R.K. and S.S performed the experiments.

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Availability of data and materials All data generated in this study is provided within the text or as additional supplemental data, tables, and figures that are available as Online Resources to article. In addition, RNA sequencing transcriptomic data has been deposited in NCBI as BioProject: PRJNA1128586. Pipeline used for the transcriptomic analysis is available in <https://github.com/mzw0135/Transcriptomic-Analysis>

Declarations

Competing interests The authors declare they have no competing interests.

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

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