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Cytokinins act synergistically with heat acclimation to enhance rice thermotolerance affecting hormonal dynamics, gene expression and volatile emission

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ABSTRACT

Heat stress is a frequent environmental constraint. Phytohormones can significantly affect plant thermotolerance. This study compares the effects of exogenous cytokinin *meta*-topolin-9-(tetrahydropyran-2-yl)purine (mT9THP) on rice (*Oryza sativa*) under control conditions, after acclimation by moderate temperature (A; 37 °C, 2h), heat stress (HS; 45 °C, 6h) and their combination (AHS). mT9THP is a stable cytokinin derivative that releases active *meta*-topolin gradually, preventing the rapid deactivation reported after exogenous cytokinin application. Under control conditions, mT9THP negatively affected jasmonic acid in leaves and abscisic and salicylic acids in crowns (meristematic tissue crucial for tillering). Exogenous cytokinin stimulated the emission of volatile organic compounds (VOC), especially 2,3-butanediol. Acclimation upregulated *trans*-zeatin, expression of stress- and hormone-related genes, and VOC emission. The combination of acclimation and mT9THP promoted the expression of stress markers and antioxidant enzymes and moderately increased VOC emission, including 2-ethylhexyl salicylate or furanones. AHS and HS responses shared some common features, namely, increase of ethylene precursor aminocyclopropane-1-carboxylic acid (ACC), *cis*-zeatin and cytokinin methylthio derivatives, as well as the expression of heat shock proteins, alternative oxidases, and superoxide dismutases. AHS specifically induced jasmonic acid and auxin indole-3-acetic acid levels, diacylglycerolipids with fewer double bonds, and VOC emissions [e.g., acetamide, lipoxygenase (LOX)-derived volatiles]. Under direct HS, exogenous cytokinin mimicked some positive acclimation effects. The combination of mT9THP and AHS had the strongest thermo-protective effect, including a strong stimulation of VOC emissions (including LOX-derived ones). These results demonstrate for the first time the crucial contribution of volatiles to the beneficial effects of cytokinin and AHS on rice thermotolerance.

1. Introduction

Heat stress (HS) is a common environmental factor that significantly affects many physiological and biochemical processes in plants. Plants have evolved complex systems to cope with HS, including transcriptome and metabolic changes, phytohormonal signaling and volatile emissions (Hayes et al., 2021). Responses to HS include synthesis of heat shock proteins (HSPs) which act as chaperones, stabilizing the structures of proteins sensitive to HS (Hayes et al., 2021). HSPs are crucial for tolerance to high temperatures and interact with phytohormonal pathways (Prerostova and Vankova, 2023). In contrast, knowledge about the

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Abbreviations

A	acclimation
ABA	abscisic acid
ABI5	abscisic acid insensitive 5
ACC	aminocyclopropane-1-carboxylic acid
AHS	acclimation combined with heat stress
AOX	alternative oxidase
CK	cytokinin
CKX	cytokinin oxidase/dehydrogenase
cZ	*cis*-zeatin
DGDG	digalactosyl diacylglycerol
HS	heat stress
HSF	heat shock factor
HSP	heat shock protein
IAA	indole-3-acetic acid
iPR	isopentenyladenosine;
IPT	isopentenyl transferase
JA	jasmonic acid
JA-Ile	jasmonoyl-isoleucine;
LOX	lipoxygenase
MGDG	monogalactosyl diacylglycerol
mT9THP	*meta*-topolin-9-(tetrahydropyran-2-yl)purine;
NCED	9-cis-epoxycarotenoid dioxygenase
PAA	phenylacetic acid
RR	response regulator
SA	salicylic acid
SIG5	sigma factor 5
SOD	superoxide dismutase
tZ	*trans*-zeatin
tZR	*trans*-zeatin riboside;
tZRMP	*trans*-zeatin riboside monophosphate
VOC	volatile organic compound

emission of volatile organic compounds (VOC) under stress conditions is limited, although they can move more rapidly than vascular signals (Kleist et al., 2012; Loreto and Schnitzler, 2010; Dani and Loreto, 2022). There is growing evidence of their role in defense against biotic stresses, but also in abiotic stress responses affecting phytohormones, antioxidant capacity, and thylakoid membrane stability, diminishing suppression of photosynthesis (Loreto and Schnitzler, 2010; Shi et al., 2018; Dani and Loreto, 2022).

Acclimation to HS caused by moderate stress (priming) can significantly increase plant tolerance through a series of changes that persist for some time after stress release (stress memory) (Charng et al., 2023). Mechanisms of acclimation include transcriptomic, metabolomic as well as epigenetic changes, and altogether are not well understood. However, there is a direct link between acclimation and the expression of HSPs and heat shock factors (HSFs) (Friedrich et al., 2021).

Due to climate change and sustainable agriculture practices, new ways to improve crop thermotolerance are becoming increasingly important. One promising approach is to manipulate cytokinin (CK) levels, as these regulate nearly all aspects of plant growth and development, as well as interactions with the environment (Hwang et al., 2012). These phytohormones have the potential to increase HS tolerance and stimulate growth during the recovery phase, as demonstrated after the application of exogenous CK (Jespersen et al., 2015), an inhibitor of CK degradation (Prerostova et al., 2020) or by the use of CK biosynthetic gene transformants (Xing et al., 2009). Černý et al. (2014) found high similarity between the proteomes of HS and CK treated plants. Expected CK effects in HS include stabilization of the photosynthesis machinery (Veerasamy et al., 2007), stimulation of the antioxidant system (Xing et al., 2009), HSP up-regulation (Černý et al., 2014), and modulation of membrane composition (Kobayashi et al., 2009) associated with a reduction in lipid peroxidation (Liu and Huang, 2002), all of which may influence HS tolerance. CKs stimulate the synthesis of the lipids monogalactosyl diacylglycerols (MGDG) which constitute 50% of chloroplast membrane lipids and are important for photosynthesis and stress tolerance (Kobayashi et al., 2014). MGDG interact with the light-harvesting complex II (LHCII) and their conformational changes ensure stability under HS conditions (Dlouhý et al., 2020).

Promotion of stress tolerance through the use of CK is complicated by the fact that CK levels are dynamically regulated during the stress responses. A cold shock is associated with rapid CK down-regulation (which stops growth), followed by an increase in CK levels during acclimation (Kosová et al., 2012). In contrast, heat shock is associated with a transient CK increase (which promotes transpiration – the primary leaf cooling mechanism), followed by CK suppression (Dobrá et al., 2015). Timing of application may therefore be of significance. The effects of CK also depend on the severity and duration of the stress.

The choice of CK used for exogenous application is of great importance. Unlike isoprenoid CKs, the most abundant endogenous CK forms, aromatic CKs are resistant to degradation by the enzyme cytokinin oxidase/dehydrogenase (CKX) and are therefore more suitable for long-term applications (Ahmad and Strnad, 2021). The CK derivative ***meta***--topolin-9-(tetrahydropyran-2-yl)purine (mT9THP), used in this study, is based on the aromatic CK ***meta***-topolin structure. It appears to be a promising compound (Podlešáková et al., 2012). Substitution with a tetrahydropyran-2-yl (THP) group at the ***N9***-position of the purine ring prevents deactivation by ***N***-glucosylation (Ahmad and Strnad, 2021). The resulting derivative is reversibly ***O***-glucosylated by the plant, which allows a gradual release of the active compound. ***O***-glucosylation of the side chain can be prevented by its methoxy derivatization. The THP derivatives were found to be transported by maize xylem much faster (ca. 10-fold) than free bases (Podlešáková et al., 2012). They gradually release the active CK base, bypassing the negative effects on the expression of the CK biosynthetic genes ***IPT*** (isopentenyl transferase) and delaying the stimulation of the expression of ***CKX*** genes that occurs in response to the application of active exogenous CKs.

The aim of this study was to elucidate the mechanisms by which CKs can enhance plant thermotolerance. Particular attention was paid to the link between CKs and acclimation, as both have been reported to promote HS responses. Stable exogenous CK derivative mT9THP was used. As CKs exhibit intensive cross-talk with other hormones, the levels of endogenous phytohormones, including ABA, jasmonic acid (JA), salicylic acid (SA), ethylene, and auxin were followed. In order to characterize the stress strength, the expression of selected stress-related genes was determined. Considering the crucial role of CKs and ABA in the response to HS, the expression of genes related to these hormones was followed. The levels of membrane lipids MGDG and digalactosyl diacylglycerol (DGDG) under stress conditions were examined. Special attention was paid to the production of VOCs to reveal their role in HS and to elucidate their association with CKs.

2. Materials and methods

2.1. Plant cultivation and experimental setup

Seeds of rice (*Oryza sativa* ssp. *japonica*, variety Kitaake, wild type; University of North Carolina, USA) were soaked for 1 day in distilled water, and then transferred to an Araponics hydroponic system (Araponics, Belgium) with ¼ Hoagland solution (18 plants per 1.7 L vessel). Plants were grown in Sanyo MLR-350H climate chambers (Sanyo Electric Co., Japan) using a 14/10 h light/dark photoperiod, an optimal light

intensity of 150 μmol m^{-2} s^{-1}, temperatures 25/20 °C, and ca. 70% relative humidity.

Two synthetic CKs, *meta*-methoxytopolin-9-(tetrahydropyran-2-yl) purine (mMeTH9THP) and *meta*-topolin-9-(tetrahydropyran-2-yl)purine (mT9THP), were tested in preliminary experiments in concentration range 10 nM–5 μM. mT9THP at 10 nM concentration was selected for further experiments as a highly active CK with positive effect on plant growth (Fig. 1, S1, S2). mT9THP was applied 24 h before HS.

The scheme of the experimental setup is shown in Fig. 1A. Two parallel sets of 15-day old plants at the two-leaf stage with or without CK (+CK and -CK) were acclimated (A; 37 °C, 2 h), starting 2 h after dawn, then returned to control conditions for 2 h. After this priming, some of the acclimated plants (variants A, A-CK) and controls that remained at control conditions (C1, C1-CK; corresponding to 6 h after dawn) were sampled, while others were subjected to heat stress (45 °C for 6 h, with medium pre-heated to 45 °C; variants AHS, AHS-CK). Simultaneously, HS was directly applied to plants maintained until that time at control conditions (HS, HS-CK). At the same time-point, controls kept for the whole time in control conditions were collected (C2; corresponding to 12 h after dawn). At the time corresponding to the beginning of HS, the medium in all variants (stressed and controls) was changed and the CK was no longer added to the media of +CK variants.

The experiments were repeated three times. Samples of middle sections of the second (younger) and first (older) leaves (without bases and tips), crowns (meristematic tissues at the border between shoots and roots) and roots were collected, frozen in liquid nitrogen, and stored at −80 °C until analysis.

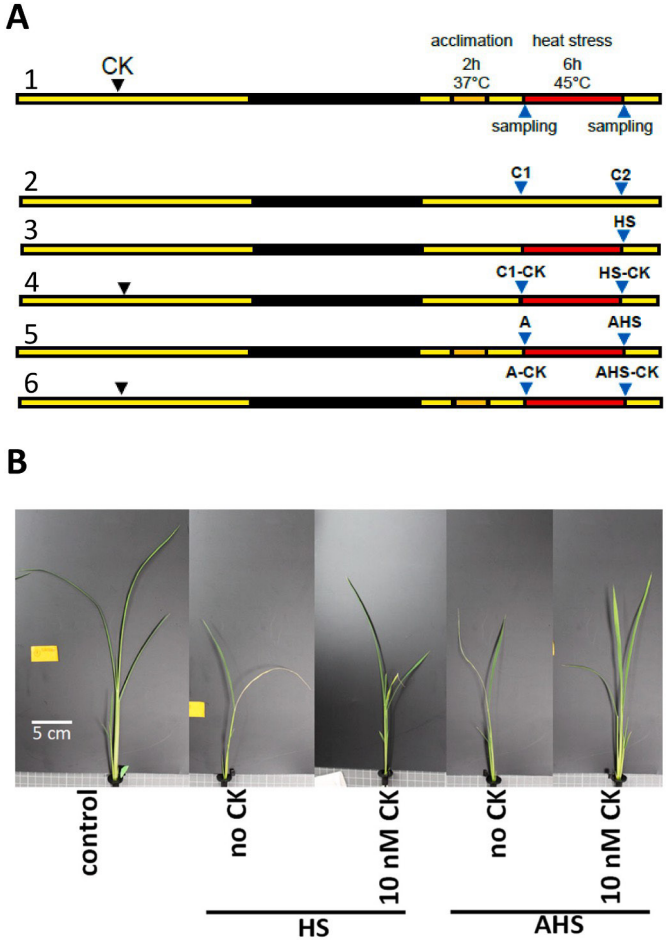

Fig. 1. (A) Scheme of the experimental set-up. 1 – timing of exogenous cytokinin *meta*-topolin-9-(tetrahydropyran-2-yl)purine (mT9THP = CK – black triangle) application and specification of acclimation (A – orange; 37 °C 2 h followed by 2 h at control conditions – yellow) and heat stress (HS – red; 45 °C 6 h) conditions; 2 – C1, C2 = controls collected at the time-points corresponding to variants before and after heat stress, respectively; 3 – HS = samples after direct heat stress; 4 – C1-CK = application of CK at control conditions sampled at the time-point before HS initiation, HS-CK = samples of heat-stressed and CK pre-treated plants; 5 – A = samples after acclimation followed by 2 h at control conditions, AHS = samples of heat-stressed pre-acclimated plants; 6 – A-CK = samples after acclimation of CK pre-treated plants and after 2 h at control conditions, AHS-CK = samples of heat-stressed pre-acclimated and CK pre-treated plants Yellow and black fields indicate light and dark periods at control conditions (14/10 h at 25/20 °C). Sampling time is indicated by blue triangles. (B) Illustrative photos of rice seedlings exposed to heat stress (HS) or heat stress with previous acclimation (AHS). Plants treated with 10 nM mT9THP are indicated as CK. Photos were made after 5-day recovery. Scale bar is 5 cm.

2.2. Phytohormone analysis

Phytohormones were analyzed according to Prerostova et al. (2020). Briefly, samples (ca. 10 mg FW; two biological samples per experiment, giving six repetitions in total) were homogenized with zirconia balls in a FastPrep-24 5G homogenizer (MP Biomedicals, CA, USA) for 40 s at 6 m s^{-1} and extracted twice with cold (−20 °C) methanol/water/formic acid (15/4/1, v/v/v). The following isotope-labeled standards were added at 10 pmol per sample: $^{13}C_6$-IAA (Cambridge Isotope Laboratories, MA, USA); $^{2}H_4$-SA (Sigma-Aldrich, MO, USA); $^{2}H_3$-PA, $^{2}H_3$-DPA (NRC-PBI, Canada); $^{2}H_6$-ABA, $^{2}H_5$-JA, $^{2}H_5$-tZ, $^{2}H_5$-tZR, $^{2}H_5$-tZRMP, $^{2}H_5$-tZ7G, $^{2}H_5$-tZ9G, $^{2}H_5$-tZOG, $^{2}H_5$-tZROG, $^{15}N_4$-cZ, $^{2}H_3$-DZ, $^{2}H_3$-DZR, $^{2}H_3$-DZ9G, $^{2}H_3$-DZRMP, $^{2}H_7$-DZOG, $^{2}H_6$-iP, $^{2}H_6$-iPR, $^{2}H_6$-iP7G, $^{2}H_6$-iP9G, $^{2}H_6$-iPRMP, $^{2}H_2$-GA_{19}, ($^{2}H_5$)($^{15}N_1$)-IAA-Asp and ($^{2}H_5$)($^{15}N_1$)-IAA-Glu (Olchemim, Czech Republic). The extracts were centrifuged for 20 min at 4 °C and 17,000×*g* then the supernatants were concentrated using an Alpha RVC vacuum evaporator (Christ, Germany; 40 °C, 15 mbar, 1.5 h). Phytohormones were separated using a reverse-phase–cation exchange SPE column (Oasis-MCX, Waters, MA, USA), yielding an acid fraction eluted with methanol and a basic fraction eluted with 0.35 M NH_4OH in 60% methanol. The acid and basic fractions were dried in a vacuum evaporator and resuspended in 30 μL portions of 15% acetonitrile and 5% methanol, respectively. Hormone contents were then analyzed using an Ultimate 3000 HPLC system (Dionex, CA, USA) coupled to a 3200 Q TRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems, MA, USA). Hormone metabolites were quantified using the isotope dilution method with multilevel calibration curves (r^2 > 0.99) and the Analyst 1.5 software package (Applied Biosystems, MA, USA).

2.3. RT-qPCR

Samples (ca. 100 mg FW; two biological samples per experiment, giving six replicates in total) were homogenized with zirconia balls in a cooled MM301 homogenizer (Retsch, Germany) for 150 s at 25 Hz. RNA was isolated using a FavorPrep™ Plant Total RNA Mini Kit (Favorgen, Austria) and treated with DNase I recombinant (Roche Applied Science, Germany) in accordance with the manufacturer's instructions. Total mRNA was translated to cDNA using M-MLV Reverse Transcriptase (RNase-H Minus, Point Mutant, Promega, WI, USA), random hexamers, and Protector RNase Inhibitor (Roche Applied Science, Germany). The resulting cDNA (diluted 10-fold) was mixed with 5 μL GoTaq qPCR Master Mix (Promega, WI, USA) and specific primers (Table S1; Jain et al., 2006; Chauhan et al., 2011; Li et al., 2013; Xu et al., 2015; Zhang et al., 2016; Ghosh et al., 2018; Kumar et al., 2018) to a final volume of 10 μL. Target sequences were amplified by PCR with cycles of 10 s at 95 °C for primer denaturation and 30 s at 60 °C for annealing and elongation using Light Cycler 480 (Roche Applied Science, Germany). The eukaryotic translation initiation factor *eIF5A* and ubiquitin *UBQ5* were selected as reference genes (Prerostova et al., 2022). Relative RNA contents were calculated according to Pfaffl (2001).

2.4. Volatile compound analysis

Volatile emissions from five plants subjected to each treatment were measured in hydroponic vessels, simultaneously with control plants or variant missing CK or A in a parallel trapping system with air Zero plus supplies (purity 4.8, from Air Products, UK). Thus, there were twelve experimental variants measured in six treatments, with five biological repetitions each. Detail description of the procedure is in Supplementary Data S1. Emitted volatiles were trapped (for 10 min) using two programmable Apex Air Sampling Pumps equipped with Low Flow Adaptors (Casella CEL, MA, USA) and Tenax TA sorbent tubes (Gerstel GmbH and Co. KG, Germany), after a 2 min air wash and 10 min equilibration. In each case, volatiles in 1 L of headspace were sampled by trapping for 10 min with a 100 mL min^{-1} sampling flow. The volatiles were analyzed using LECO Pegasus 4D GC × GC-TOFMS system (Leco Corporation, MI, USA) containing Agilent 7890 gas chromatograph (Agilent Technologies, CA, USA) equipped with a LECO quad-jet dual stage thermal modulator; Gerstel MultiPurpose Sampler (MPS), Gerstel Thermal Desorption Unit (TDU) and temperature programmed CIS4 inlet (Gerstel GmbH and Co. KG, Germany). Peaks were identified using MetaboAnalyst 5.0. Approximately 3000 peaks were detected in each chromatogram at a signal-to-noise threshold ratio of 10. After alignment of all chromatograms and preliminary statistical analysis (based on F-ratios) these peaks were filtered and 151 of the most variable among treatments were selected for further data processing. A final set of 127 peaks was selected for detailed statistical analysis, and identified based on comparison of mass spectra with entries in available spectral databases.

2.5. Membrane lipids analysis

Membrane galactolipids were extracted from at least four independent biological samples in three technical repetitions per treatment for galactolipid analysis following Welti et al. (2002) with slight modifications. Briefly, frozen tissue samples were extracted by isopropanol with 0.01% BHT at 75 °C for 15 min in 35 ml screw glass tube with Teflon sealed cap. Then 1.5 mL $CHCl_3$ and 0.6 mL H_2O were added to each tube and the resulting mixtures were shaken for 1 h. The extracts were collected and the tissue samples were re-extracted four times with 3 mL of $CHCl_3$/MeOH (2:1 v/v) with 0.01% BHT by shaking for 30 min. The combined extracts were washed with 1 mL 1 M KCl followed by 2 mL deionized water. The organic layers were evaporated under a stream of nitrogen and kept at −80 °C. Solid residues after extraction were dried and weighed for calculating concentrations per dry weight (DW). Two technical blanks were analyzed together with each extraction series. Immediately before analysis, the samples were diluted in 1 mL $CHCl_3$, and 80 μL of each resulting solution was added to 390 μL $CHCl_3$ with 10 μL of internal standard [hydrogenated monogalactosyl diacylglycerols MGDG 34:0 (27.06 μg) and digalactosyl diacylglycerols DGDG 36:0 (23.76 μg) in $CHCl_3$; Merck, Germany]. Shot-gun lipidomic analysis of both MGDG and DGDG was performed using direct infusion by syringe pump at a flow rate of 25 μL/min for 5 min. Samples were analyzed using a 4000 Q-TRAP hybrid triple quadrupole/linear ion trap mass spectrometer (AB Sciex, MA, USA) operating with ESI^+ ionization in precursor mode (precursor ions $[M+Na]^+$ of 243^+ *m/z*). Centroid spectra were processed and normalized to internal standards and DW.

2.6. Statistics

The significance of between-treatment differences in measured variables was assessed by t-tests ($p < 0.05$) using Prism 8 (GraphPad, CA, USA). Effects of the CK treatment, acclimation and high temperature in each organ were evaluated using controls collected at corresponding time-points (C1 before HS, or C2 after HS). Numbers of independent biological replicates in each analysis are specified above.

3. Results and discussion

This study has been focused on elucidation of the role of CK in HS responses. As the timing of CK application prior to HS can substantially affect their impact on plants, the schedule optimized in Prerostova et al. (2020) was used for both treatments. Evaluation of plant growth after a 5-day recovery period showed that plants treated directly with HS (45 °C, 6 h) suffered severely, whereas acclimated plants (exposed to 37 °C for 2 h before HS) effectively renewed their growth (Fig. 1B). Exogenous CK mT9THP promoted tillering and recovery from crowns, and suppressed leaf senescence after direct HS.

3.1. Effects of exogenous cytokinin under control conditions

Control plants exposed to exogenous CK mT9THP (for 24 h) at 10 nM concentration did not exhibit significant disruption of CK homeostasis, probably due to the gradual release of the active compound (Podlešáková et al., 2012). Plants were able to balance it largely through fine regulation of signaling (decreased expression of the positive CK response regulator *RR26* in younger leaves and increased expression of the negative regulator *RR4* in roots; Fig. 3). Despite the weak effects on endogenous CKs, exogenous CK mT9THP was able to significantly down-regulate ABA (Fig. 2A) along with its metabolites phaseic acid and ABA-glucose ester (Table S2), and SA (Fig. S4) in crowns. This suggests suppression of growth inhibitors in order to stimulate growth and tillering from this meristematic tissue (Fig. S1; Sharma et al., 2020). At the same time, the levels of JA and JA-Ile were diminished in leaves (Fig. 2B, S3). This is consistent with the generally antagonistic relationship between 'growth-promoting hormones' and 'stress hormones' under optimal conditions (Hwang et al., 2012). Exogenous CK also diminished the inactivation of auxin indole-3-acetic acid (IAA, another 'growth-promoting hormone'; Fig. 2D) to oxo-IAA in all organs tested (Table S2).

In agreement with the known CK promotive effect on plant antioxidant systems (Xing et al., 2009), exogenous CK positively stimulated dismutase *FSD1* expression in older leaves and crowns (Fig. 3). The effect of the CK application on the thylakoid membrane of plastids, in which the glycolipids MGDG and DGDG are the major lipids (Kobayashi et al., 2014), was evaluated by lipid analysis. Exogenous application of CK resulted in more significant changes (reduction) in DGDG composition compared to MGDG species, reducing their content in younger leaves and roots (Fig. 4), which partially correlates with the previously found stimulatory effect of CK on MGDG accumulation (Kobayashi et al., 2014). Higher ratio of MGDG glycolipids indicates enhanced photosynthesis (Dlouhý et al., 2020).

Exogenous CK also affected the production of VOCs, which might, however, depend not only on the duration of CK treatment but also on the circadian rhythm (Fig. 5; Singh and Mas, 2018). CK increased emissions of 2,3-butanediol (Fig. 5A). Previously, exogenous butanediol was found to stimulate the expression of genes related to cell elongation, carbon and nitrogen metabolism as well as CK-related genes, including genes encoding type-A response regulator and adenine phosphoribosyl transferase, under moderate HS in *Agrostis stolonifera* (Shi et al., 2018). Butanediol has been reported to stimulate the expression of lipoxygenase (LOX), an enzyme involved in the biosynthesis of fatty acid derived volatiles and JA (Shi et al., 2018), to activate systemic resistance to biotic stresses (Ryu et al., 2004) and to enhance tolerance to abiotic stresses, including drought (Cho et al., 2008a), salinity (Cho et al., 2008b), and HS (Shi et al., 2018). Our results suggest a reciprocal interaction between CKs and butanediol. The data indicate that CK may also be involved in stress tolerance via stimulation of VOC production.

Exogenous CK elevated production of 2-ethylhexyl salicylate (Fig. 5B) linking CK to SA metabolism and stress responses (Nawrocka et al., 2018). Strong emission was also observed for indole derivatives such as 1-H-indole-2,3-dione (isatin) which can elicit an auxin response (Applewhite et al., 1994), sesquiterpene δ-cadinene, and fatty acid

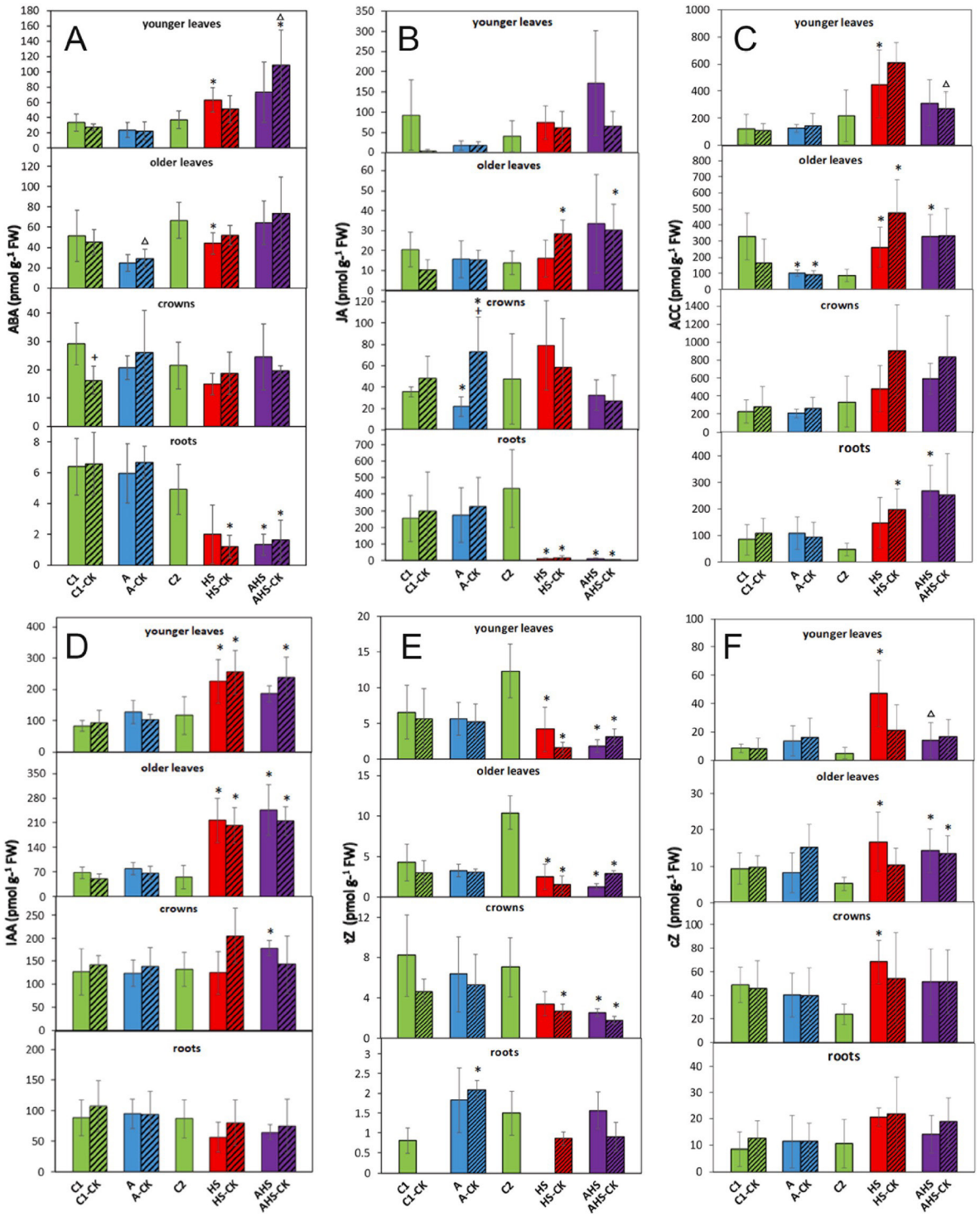

Fig. 2. Contents of abscisic acid (ABA) – A, jasmonic acid (JA) – B, ethylene precursor aminocyclopropane carboxylic acid (ACC) – C, auxin indole-3-acetic acid (IAA) – D, cytokinin *trans*-zeatin (tZ) – E, cytokinin *cis*-zeatin (cZ) – F in younger and older leaves, crowns and roots of rice plants subjected to acclimation (A), heat stress (HS) or combined acclimation with heat stress (AHS), with or without exogenous application of cytokinin mT9THP (CK) 24 h before heat stress. Samples were collected before and after heat stress (with corresponding controls designated C1 and C2, respectively). Student's t-test ($p < 0.05$, $n = 6$) was used to detect significant differences between plants subjected to the treatments and controls at each time-point (indicated by asterisks, *), CK-treated vs. non-CK-treated plants (indicated by crosses, +), acclimated vs. non-acclimated plants (AHS vs. HS, AHS-CK vs. HS-CK, and A-CK vs. C1-CK treated plants; indicated by triangles, Δ).

derivative dodecanoic acid (lauric acid; Table S5). Emission of another 18 detected compounds increased at least twofold (Table S5). In contrast, CK-treated plants did not release geranyl acetone (Fig. 5F), heptyl ester of cyclobutanecarboxylic acid, and 3,3′-oxy-bis-propanenitrile as much as controls (Table S5). Emissions of 20 other detected VOCs decreased at least twofold after the CK treatment (Table S5).

Summary of the most important effects of CK treatment is shown in Fig. 6.

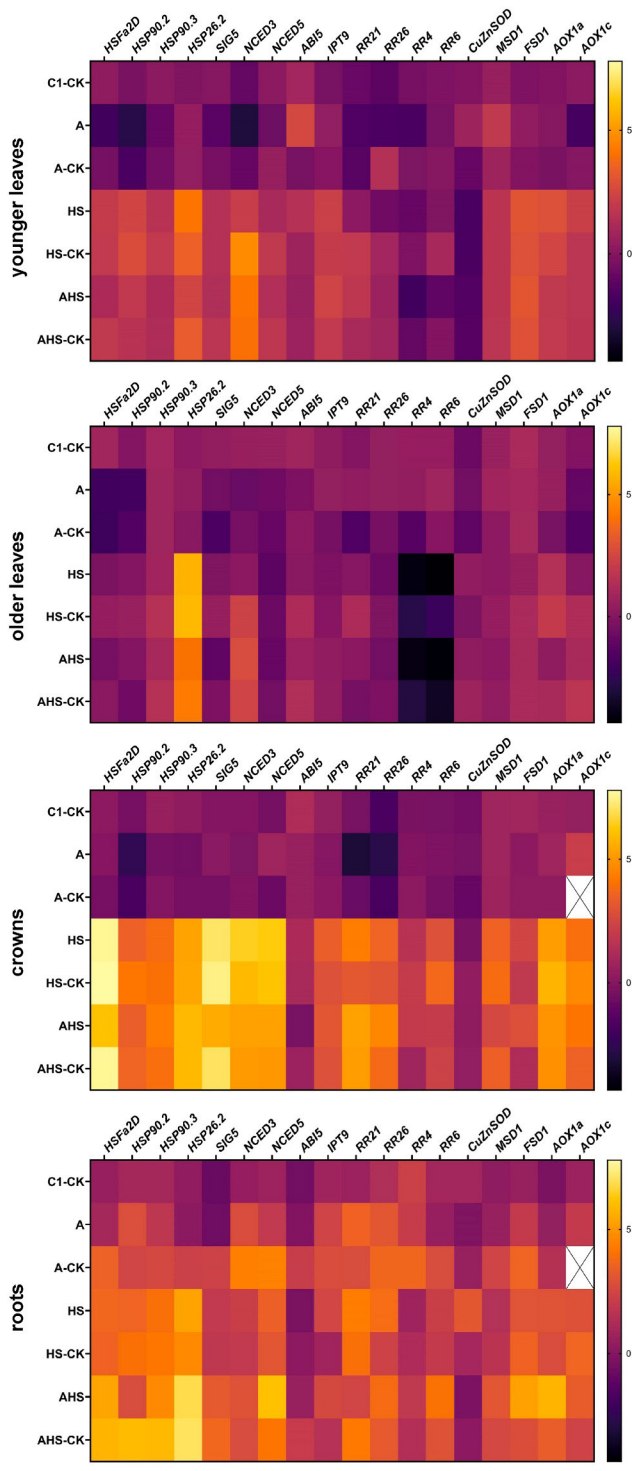

Fig. 3. Heatmap of changes in transcript levels of selected stress- and phytohormone-related genes in younger and older leaves, crowns and roots of rice plants subjected to acclimation (A), heat stress (HS) or combined acclimation with heat stress (AHS), with exogenous application of the cytokinin mT9THP (CK) 24 h before heat stress. Samples were collected before and after heat stress (with corresponding controls designated C1 and C2, respectively). Six independent biological samples per treatment were evaluated. Data in the heatmap are normalized against control levels separately for each tissue and time-point (C1 or C2). The mean values are presented in $\log_{10}$ color scale. Means ± SD and other statistics are shown in Table S3. The crosses indicate the values under the detection limit.

3.2. Acclimation

Heat acclimation (priming) is known to enhance tolerance to subsequent severe stresses. It activates low energy-demanding adaptive and defense responses that allow for more rapid stimulation of defenses against subsequent stresses (Martinez-Medina et al., 2016). The acclimation treatment (37 °C, 2 h) applied in this study had a modest negative effect on ABA content in older leaves but no impact on the other organs (Fig. 2A). Despite negligible changes in ABA content, up-regulation of the ABA biosynthetic gene *NCED3* (9-*cis*-epoxycarotenoid dioxygenase) expression and accumulation of ABA-glucose ester in roots were detected, indicating intensive ABA biosynthesis and storage of its reversible conjugate in this organ, allowing rapid response to potential future stress (Fig. 3, Table S2). Acclimation resulted in a decrease of JA level in crowns and of the ethylene precursor ACC level in older leaves (Fig. 2B and C).

Acclimation was also associated with an increase in the most physiologically active CK *trans*-zeatin (tZ), its riboside (tZR), and its precursor tZR monophosphate (tZRMP), as well as up-regulated expression of the CK biosynthetic gene *IPT9* in roots, and increased levels of isopentenyladenosine and its precursor isopentenyladenosine monophosphate in leaves, which seems to reflect an up-regulation of the biosynthesis of endogenous CKs (Fig. 2E and 3, Table S2). This could contribute to improved plant fitness after acclimation, as tZR is the major CK form transported to aboveground tissues, where released tZ can stabilize the photosynthetic apparatus (Kieber and Schaller, 2014). Fine-tuning of CK signaling during acclimation is indicated by down-regulation of positive type-B response regulators (*RR21* and *RR26*) in leaves and crowns, but up-regulation in roots, as well as down-regulation of the negative type-A response regulator *RR4* in leaves (Fig. 3). These changes reflect the important role of CKs in stress tolerance (Prerostova et al., 2020). Further evidence of stimulation of the stress protection machinery was the increase in transcription of genes encoding antioxidant enzymes: mitochondrial (*MSD1*) and plastid (*FSD1*) superoxide dismutases, and the alternative oxidase *AOX1c* (Fig. 3).

Lipid analysis revealed only slight restructuring of individual species of MGDG (higher content of the 36:4 moiety in roots) and DGDG (higher content of moieties with 38 carbon acyl groups in younger leaves) suggesting a slight increase in membrane fluidity during acclimation (Fig. 4; Hara, 2020).

Acclimation was associated with increased emission of a fairly limited number of volatiles (Table S5), consistent with the results of a study on the effects of heat priming on *Achillea millefolium* (Liu et al., 2021). The strongest change was an increase in the emission of the long-chain unsaturated alcohol 2-ethyl-2-hexen-1-ol and 2-methyl-2--propenoic acid (methacrylic acid), which can potentially stimulate plant growth (Naznin et al., 2013). Acclimation significantly increased the emission of a number of other organic acids, such as heptanoic acid, octanoic (caprylic) acid, nonanoic (pelargonic) acid, n-decanoic (capric) acid and benzoic acid, which could have an effect on membrane stability or antioxidant capacity, most of these compounds being known as herbicides (Li et al., 2018). In addition to emission of the unsaturated alcohol (*Z*)-2-penten-1-ol, moderate stimulation of LOX pathway volatiles (also known as C6 volatiles or 'green leaf volatiles', GLVs) formed via a pathway partially shared with JA was also detected (particularly (*Z*)-3-hexen-1-ol). After LOX-catalyzed hydroperoxidation of linolenic acid to 13-hydroperoxylinolenic acid, either (*Z*)-3-hexenal is cleaved off or JA is formed by epoxidation and three β-oxidation steps (Wasternack and Hause, 2013). Reduction of JA contents in crowns by acclimation (Fig. 2B) possibly indicates preferential production of volatiles. The (*Z*)-3-hexenal could be further metabolized to (*Z*)-3-hexen-1-ol or isomerized to (*E*)-3-hexenal and (*E*)-3-hexen-1-ol. These compounds have been found to alter membrane potential and calcium signaling and stimulate mitogen activated protein kinase pathway (Cofer et al., 2018). In contrast to the reported responses of *A. millefolium* (Liu et al., 2021),

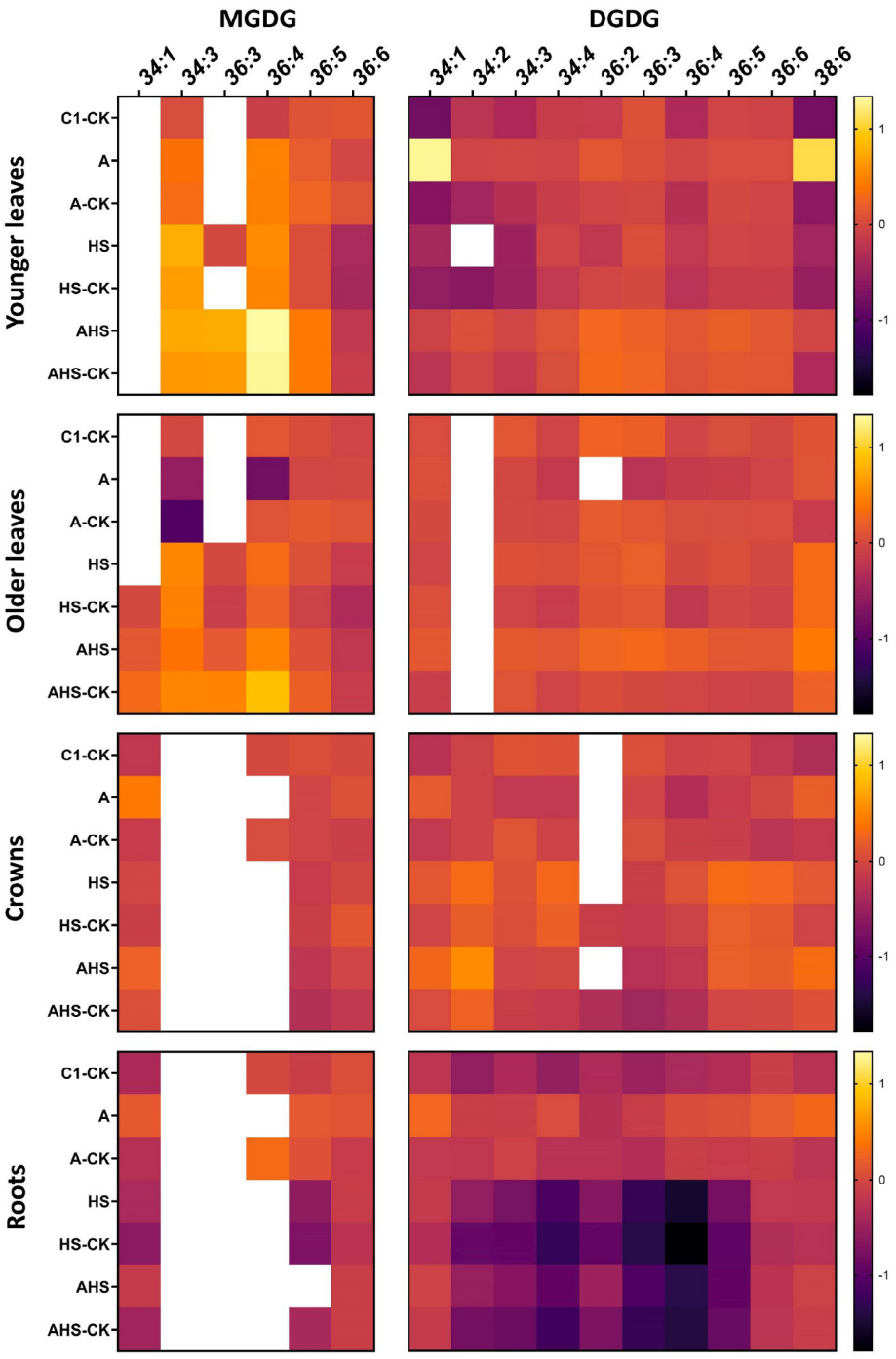

Fig. 4. Changes in lipid content. Heatmap showing composition of monogalactosyl diacylglycerols (MGDG) and digalactosyl diacylglycerols (DGDG) in younger and older leaves, crowns and roots of rice plants subjected to acclimation (A), heat stress (HS) or combined acclimation with heat stress (AHS), with and without exogenous application of the cytokinin mT9THP (CK) 24 h before heat stress. Samples were collected before and heat stress (with corresponding controls designated C1 and C2, respectively). The composition of acyl groups is indicated in number of carbons: number of double bonds format. At least four independent biological samples per treatment were evaluated. Data in the heatmap are normalized against control levels separately for each tissue and time-point (C1 or C2). The mean values are presented in $\log_{10}$ color scale. Missing lipid species are indicated in white. Means ± SD and other statistics are shown in Table S4.

we did not detect an increase in isoprene emissions in our rice plants after acclimation. At least a twofold decrease in emission was observed for N,N-dimethyl-2-butanamine, hexanal, 3,6-heptanedione, the terpenoids *cis*-calamenene and *δ*-cadinene. The down-regulation of hexanal may be related to enhanced phospholipase D activity and stress signaling as hexanal inhibits this enzyme (El Kayal et al., 2017; Prerostova and Vankova, 2023).

3.3. *Effects of exogenous cytokinin during acclimation*

The CK application followed by acclimation combined advantages of each treatment. The negative effects of the CK application on ABA and SA levels observed under control conditions disappeared due to acclimation (Fig. 2A, S4). The expression of the ABA biosynthetic genes *NCED3* and *NCED5* even increased in roots, and the expression of the ABA-response gene *ABI5* elevated in younger leaves (Fig. 3). Acclimation also maintained elevated tZ levels in roots (Fig. 2E). In contrast, exogenous CK reversed the negative effect of acclimation on JA and JA-Ile in crowns, increasing their levels, which indicates the importance of these hormones in crown protection (Fig. 2B, S3).

Many small changes in gene expression caused by either acclimation or CK application were enhanced by a synergistic combination of both factors. This combination resulted in a strong induction of genes encoding proteins involved in plant protection: *HSFA2d*, *HSP90.3*, as well as mitochondrial (*MSD1*) and plastid (*FSD1*) dismutases, particularly in roots (Fig. 3). Interestingly, the combined treatment stimulated

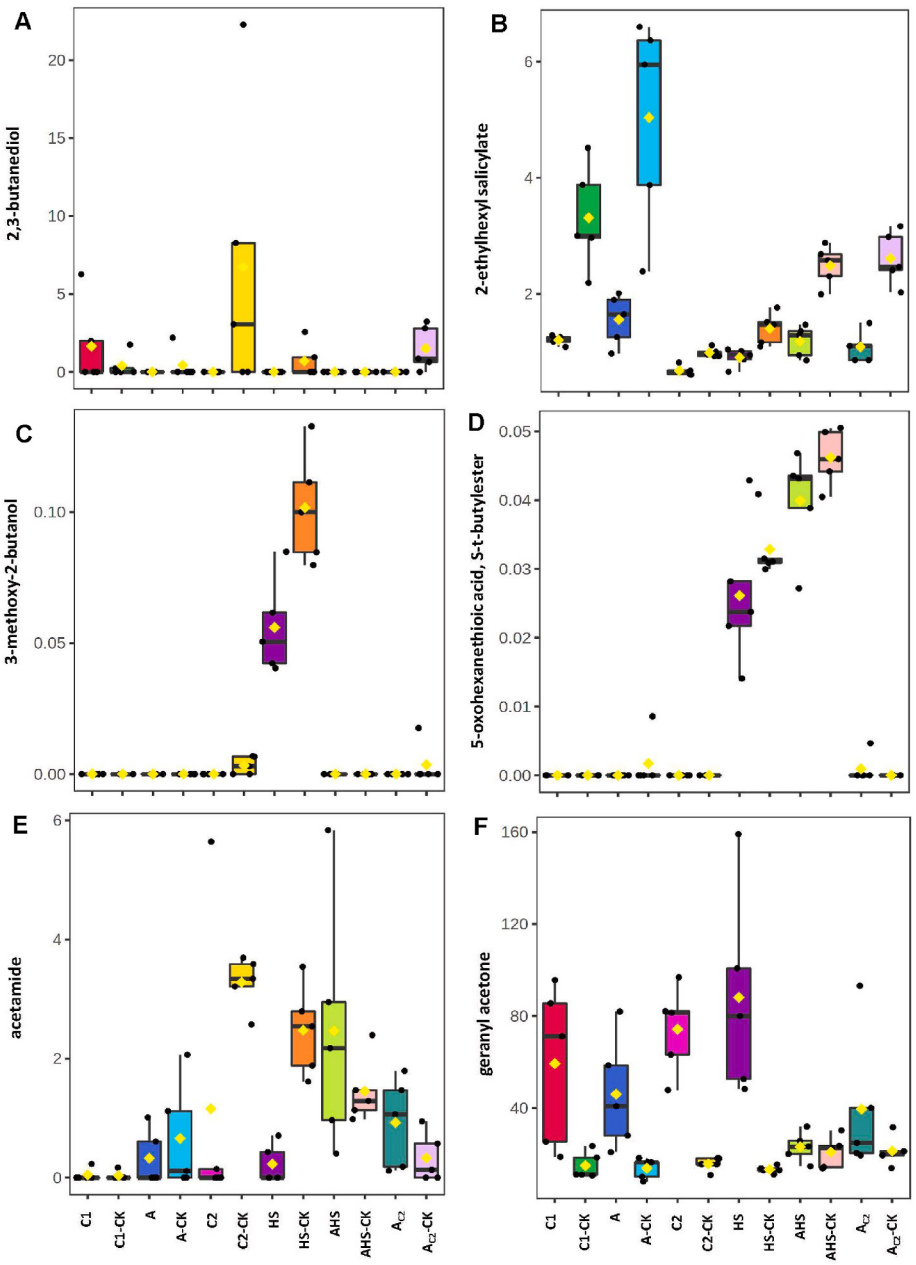

Fig. 5. Contents of selected volatiles released from rice seedlings subjected to acclimation (A), heat stress (HS) or combined acclimation with heat stress (AHS), with and without exogenous application of the cytokinin mT9THP (CK) 24 h before heat stress: (A) 2,3-butanediol; (B) 2-ethylhexyl salicylate; (C) 3-methoxy-2-butanol; (D) 5-oxohexanethioic acid, *S*-*t*-butyl ester; (E) acetamide; and (F) geranyl acetone. Volatile abundance is expressed in relative units corresponding to peak areas normalized to median by MetaboAnalyst 5.0. Samples were collected before and after heat stress (with corresponding controls designated C1 and C2, respectively). Values are shown in Table S5.

the expression of the type-B response regulator *RR26* in roots and younger leaves (Fig. 3) and thus CK signal transduction, suggesting that the CK signaling pathway is beneficial for thermotolerance (Prerostova and Vankova, 2023). As for the lipid composition of thylakoid membranes, the effects on MGDG and DGDG contents were similar to those of CK alone, indicating the dominant role of CKs in regulation of membrane composition as well as in maintenance of photosynthesis (Fig. 4; Dlouhý et al., 2020).

CK in combination with acclimation had a moderate effect on the volatile emissions by increasing the number of detected emitted volatiles, especially the SA derivative 2-ethylhexyl salicylate, which was also elevated under control condition after CK treatment (Fig. 5B), heterocyclic compounds furane and furanone derivatives, N,N-dimethyl-2-butanamine, lilial, patchoulane, coumarin and seven other compounds. Another 10 VOCs displayed more than twofold reduction (Table S5). Furanones function as signaling molecules, some have antioxidant, and/or antimicrobial or antifungal activities, while others are associated with fruit aromas (Slaughter, 1999). Lilial [3-(4-*t*-butylphenyl)-2-methylpropanal] was previously recognized as an insect repellent that increases the attraction of a parasitoid wasp to plants infested with leaf-chewing insects (Zeng et al., 2018). The tricyclic sesquiterpene patchoulane has antioxidant properties (Saleem et al., 2020) and coumarin (which is generated by the phenylpropanoid pathway) has antimicrobial, antiviral, and antioxidant functions and can improve iron nutrition (Stringlis et al., 2019). Interestingly, coumarin may be triggered by JA (Lourenco et al., 2016), which was upregulated by CK during acclimation (Fig. 2B).

In conclusion, despite the fact that acclimation was decisive for thermotolerance, the combination of acclimation with the CK treatment enhanced the acclimation effect on defense stimulation, improving both leaf and root protection.

3.4. Direct heat stress

Compared with acclimation, high temperature stress (45 °C, 6 h) resulted in greater changes in the rice metabolism. The greatest increase

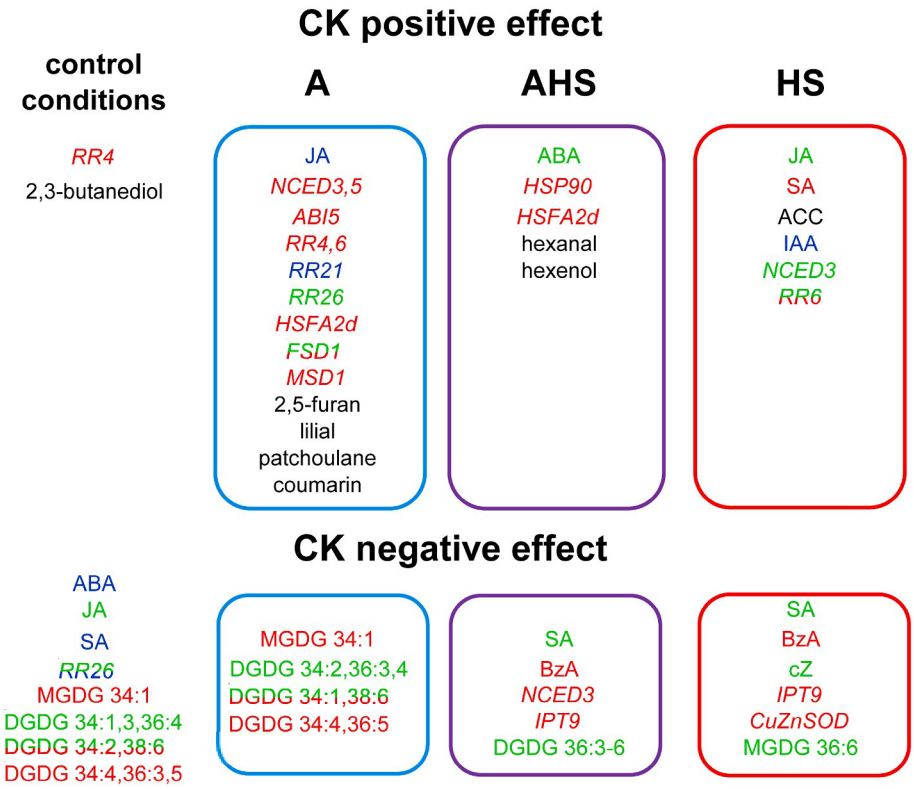

Fig. 6. Schematic diagram of the key positive and negative changes caused by exogenous CK in rice seedlings under control conditions, acclimation (A), heat stress (HS) and their combination (AHS). Up- or down-regulation of phytohormones, gene expression, lipids and volatile compounds relative to untreated controls are shown. The colors indicate changes in the whole plant (black), leaves (green), crowns (blue) and roots (red). For comparison, the impact of HS and AHS on plants is shown in Fig. S5.

was observed in the expression of heat shock factor *HSFA2d*, cytosolic heat-shock proteins *HSP90.2* and *HSP90.3*, and mitochondrial *HSP26.2* (Fig. 3), indicating intense protection of mitochondria (Prerostova and Vankova, 2023). Together with chloroplasts, these organelles were efficiently protected from oxidative stress by superoxide dismutases (mitochondrial *MSD1*, plastid *FSD1* and cytoplasmic *CuZnSOD;* Fig. 3).

Alternative oxidases play an important role in mitochondrial defense and plant thermotolerance (Borovik and Grabelnych, 2018). Accordingly, the expression of *AOX1a* and *AOX1c* was promoted by HS (Fig. 3).

HS treatment also affected almost all phytohormones measured. ABA is a key regulator of plant abiotic stress responses, involved in the regulation of water relations and production of protective compounds (Islam et al., 2018). ABA was up-regulated in younger leaves under HS, whereas it was reduced in roots, the major site of ABA biosynthesis (Zhang and Davies, 1987), which, together with up-regulated *NCED3* and *NCED5* expression in crowns and roots, indicates enhanced transport of ABA to shoots (Fig. 2A and 3), as described in Prerostova et al. (2022). HS resulted in a strong elevation of ACC content in both leaves and roots (Fig. 2C), consistent with the positive role of ethylene in thermotolerance reported by Larkindale and Knight (2002).

Despite the positive role of JA in plant thermotolerance demonstrated by Clarke et al. (2009), we found that direct HS triggered a reduction in JA content in roots (Fig. 2B), consistent with the results of a previous study (Prerostova et al., 2022). It is possible that the LOX pathway in roots was redirected to the production of volatiles and/or lipid signaling molecules (Niu and Xiang, 2018).

The content of the growth-promoting hormone auxin IAA increased in leaves but decreased in roots, suggesting a possible inhibition of IAA polar transport from shoots under stress conditions, as reported by Bielach et al. (2017). The elevation of IAA content (as well as the high accumulation of its metabolite IAA-aspartate; Fig. 2D, Table S2) in leaves is consistent with the stimulation of the expression of *YUCCA* auxin biosynthetic genes under HS (Feraru et al., 2019) and with the role of auxin in thermomorphogenesis, including shoot elongation and leaf hyponasty (Li et al., 2021).

Regarding the roles of CKs in the HS responses, HS greatly reduced the levels of tZ, the most physiologically active CK in stimulation of cell division (Hwang et al., 2012), its precursor tZRMP and isopentenyladenine-type CKs (Fig. 2E, Table S2). Simultaneously, the weakly active isomer of tZ *cis*-zeatin (cZ), and its precursor, *cis*-zeatin riboside monophosphate (cZRMP), were strongly up-regulated in all organs tested (Fig. 2F7, Table S2), consistent with the increase of cZ under various stress conditions (Prerostova and Vankova, 2023). These findings indicate cessation of cell division in the plants, whereas the other important CK functions (e.g., stabilization of the photosynthetic apparatus) were maintained. CK negative response regulators (*RR4*, *RR6*) were down-regulated in leaves, while positive regulators (*RR21, RR26*) were up-regulated in roots, which may reflect support of cZ functions.

One of the indicators of photosynthesis protection in leaves is stable expression of sigma factor *SIG5* (Fig. 3), the transcriptional regulator of the D2 protein in chloroplasts (Nagashima et al., 2004). *SIG5* expression is reportedly responsive to light quantity and quality, low temperature and abiotic stresses (Paajanen et al., 2021). Its expression was not significantly altered in leaves but was up-regulated by HS in non-green organs (crowns and roots; Fig. 3), which may be related to the protection of other type of plastids (Fu et al., 2021).

HS caused specific changes that mainly affected MGDG content in leaves and DGDG content in roots (Fig. 4). A direct role of MGDG in the photosynthetic machinery was indicated by the specific presence of 34:3, 36:3, and 36:4 species in leaves. Most MGDG species, with the exception of 36:6, were more abundant in leaves under all HS treatments. This membrane remodeling due to altered MGDG species composition caused by down-regulation of MGDG 36:6, accompanied by elevation of MGDG species with fewer double bonds (34:3, 36:3 and 36:4), is consistent with previously described reductions in the unsaturation of membrane lipids in response to HS, which contribute to the maintenance of optimal membrane fluidity and integrity (Murakami et al., 2000; Almeida et al., 2021). Interestingly, relatively small changes were detected in the lipid composition of the crowns, indicating the

stability of their membranes. In contrast, the levels of 34:1 and 36:5 MGDG forms were reduced in roots. Direct HS strongly down-regulated almost all detected forms of DGDG in roots (34:1, 34:2, 34:3, 34:4, 36:2, 36:3, 36:4, 36:5, 36:6 and 38:6).

High temperature has direct stimulatory effects on volatile emissions (Loreto and Schnitzler, 2010). The predominant volatiles emitted after HS were alcohol and acid derivatives of butane: 3-methoxy-2-butanol (Fig. 5C), *S-t*-butyl ester of 5-oxohexanethioic acid (Fig. 5D), 3-methyl- and 2-methylbutanoic acids (Table S5). Their production has been described in bacteria after anaerobic glycolysis and their occurrence in plants is unknown (Lan and Liao, 2012). Nevertheless, 3-methylbutanoic acid, known as isovaleric acid, has been found to inhibit plant growth (Murata et al., 2022). Emissions of another 16 detected VOCs were elevated at least twofold (compared with controls), including some furanone derivatives (γ-valerolactone, *cis*-whiskey lactone, γ-propyl-γ-butyrolactone, and γ-heptyl-γ-butyrolactone) (Table S5), which are signaling molecules with antioxidant properties (Slaughter, 1999). Apart from the LOX-mediated volatiles mentioned above, HS reduced the emission of 1-penten-3-ol, (*Z*)-2-penten-1-ol, and three heterocyclic VOCs by more than twofold. In contrast to acclimation, benzoic acid accumulated under HS like a solid compound rather than being emitted as VOC (Tables S2 and S5), suggesting that the shikimate pathway was mainly redirected to the production of secondary metabolites (Qin et al., 2005).

3.5. Acclimation with heat stress

Exposure of plants to moderately elevated temperature (acclimation, priming) increased substantially, their tolerance of subsequent severe temperature stresses and enabled faster recovery (Sharma et al., 2020). Hormone responses to AHS, including ABA dynamics, showed significant similarities to HS responses (Fig. 2). In addition, AHS led to an increase in JA content in leaves and ACC content in roots (Fig. 2B and C). The IAA content was elevated in crowns (Fig. 2D), whereas tZ content was significantly down-regulated in leaves and crowns. The lower content of tZ was compensated by enhanced CK signaling (up-regulated *RR21*, *RR26*; Fig. 3), which could help the plant to maintain its functions during stress and renew growth during subsequent recovery (Prerostova et al., 2020). In roots, tZ was moderately enhanced compared with HS (comparably to control values), which could indicate better plant fitness (Fig. 2E). Content of low active cZ did not increase as much as after direct HS, especially in younger leaves, possibly reflecting lower stress strength (Fig. 2F; Prerostova et al., 2020).

Acclimation before HS strongly enhanced the expression of many monitored genes in roots during heat treatment, including heat shock protein genes, genes related to hormones, photosynthesis, or reactive oxygen species (*HSFA2d*, *HSP90.3*, *HSP26.2*, *NCED3*, *NCED5*, *RR4*, *RR6*, *SIG5*, *FSD1, MSD1*, *AOX1a*; Fig. 3). These changes highlight the important role of roots in stress responses and stress tolerance. In crowns, preceding acclimation slightly reduced the expression of these genes compared with HS.

AHS had very specific effects on MGDG composition in leaves (Fig. 4). Pre-acclimation attenuated the reduction in MGDG 36:6 content in leaves accompanied by an increase in 36:3, 36:4, and 36:5, close to control values. Moreover, the most profound changes were observed in roots, where the contents of DGDG species with less than six double bonds were diminished.

AHS responses were associated with much stronger volatile emissions than HS, e.g. of 5-oxohexanethioic acid *S-t*-butyl ester (Fig. 5D). AHS promoted emissions of acetic acid butyl ester, 5-oxo-2-pyrrolidinecarbonitrile, 4-methyl-5H-furan-2-one (Table S5). Acetamide concentrations were not as much reduced as in the case of HS (Fig. 5E). The enhanced emissions could be partly given by a higher rate of photosynthesis during AHS, since the production of volatiles is energy-demanding. According to Sharkey and Yeh (2001), emissions of isoprene, the most abundant volatile in some species, can be as high as 5–10% of photosynthetically assimilated carbon. Thus, under HS-induced suppression of photosynthetic activity (see Prerostova et al., 2022), production of volatiles may be limited (Loreto and Schnitzler, 2010). However, some pathways normally used for the intensive synthesis of photosynthesis-related compounds (such as the methylerythritol 4-phosphate pathway in plastids) can be diverted to VOC production (Fineschi and Loreto, 2012). In addition, the greater emission of volatiles under AHS could contribute to the promotion of plant tolerance. Nevertheless, similarly to CK treatments, AHS led to down-regulation of geranyl acetone, the isoprenoid-based volatile compound (Fig. 5F), indicating more complex regulatory mechanisms in VOC production.

AHS also downregulated the production of some butane derivatives such as 3-methoxy-2-butanol (Fig. 5C) or the growth inhibitor 3-methylbutanoic acid (Murata et al., 2022), which were up-regulated by HS (Table S5), indicating more effective growth regulation due to acclimation.

3.6. Effects of exogenous cytokinin during direct heat stress

Exogenous CK application 24 h before HS elicited some changes similar to those of the pre-acclimation (AHS), mainly in leaves. These changes included increases in JA and JA-Ile levels in older leaves and IAA levels in crowns (Fig. 2B and D, S3). Expression of *NCED3* also increased in leaves, similarly as in AHS response, despite the fact that ABA levels were not significantly affected by CK treatment (Fig. 2A and 3). Another change shared with acclimation was a smaller increase in cZ in younger leaves, and in methylthio-derivatives in younger leaves and crowns (Fig. 2F, Table S2).

Under HS, mT9THP stimulated the biosynthesis of endogenous CKs in younger leaves and roots, as indicated by elevation of CK nucleotides, and promoted *IPT9* expression (Fig. 3, Table S2). Simultaneously, expression of the positive CK response regulator *RR26* was stimulated in crowns and younger leaves, resembling the CK treatment combined with acclimation (Fig. 3). This could indicate reduced stress severity or stress sensing, as enhanced CK levels reportedly delayed sensing of drought stress in tobacco (Havlova et al., 2008). It could also be the reason for better protection of younger leaves from senescence (Fig. 1B). Nevertheless, the contents of the ethylene precursor ACC (Fig. 2C) were elevated in all organs by HS-CK treatment, which is in agreement with our previous study on *Arabidopsis* plants treated with an inhibitor of the CK deactivation enzyme CKX (Prerostova et al., 2020). Thus, enhancing CK contents appears to increase ACC contents during HS in both monocots and dicots. Exogenous CK also specifically diminished SA content in younger leaves (Fig. S4).

CK application before HS affected DGDG composition in roots intensifying the decrease of all DGDG species caused by HS (Fig. 4). Considering that the absolute numbers of DGDG in roots were not decreased as much as those of MGDG (due to photosynthesis in leaves), a possible role of DGDG lipids in plastids besides photosynthetic function arises. However, data on this topic are sparse. DGDG have been found to replace plasma membrane glycerophospholipids in the roots under Pi deficiency (Andersson et al., 2003) or reduce activity of membrane-bound-proteins and the membrane permeability due to a decrease in DGDG content (Chaffai et al., 2005).

Application of exogenous CK before HS resulted in increased release of isatin, similarly to CK treatment under control conditions, and of 4-methyl-5H-furan-2-one, whereas the releases of some other furanone derivatives were diminished (Table S5). Despite enhanced JA production, CK also strongly stimulated the emission of LOX-pathway volatiles, particularly hexanal, which may indicate a tendency to suppress strong stress signaling through phospholipase D (El Kayal et al., 2017). Addition of CK before HS enhanced similar emissions of the volatile compounds (5-oxo-2-pyrrolidinecarbonitrile and acetamide) as AHS (Fig. 5E, Table S5).

The ability of CKs to mimic acclimation is consistent with Černý et al.

(2014), who suggested that some protein-level responses to CK are similar to HS treatment. Nevertheless, our results indicate that this correlation between the CK and HS responses relates to lower HS temperatures (such as the acclimation temperature).

3.7. Effects of exogenous cytokinin during acclimation followed by heat stress

The combination of exogenous CK and acclimation was the treatment that most strongly promoted rice thermotolerance (Fig. 1B). Due to the relative similarity of changes observed in AHS and CK-treated HS plants, differences between AHS with and without CK application were small. For example, CK strongly enhanced ACC in leaves and roots, similarly to AHS (Fig. 2C). This suggests that the positive impacts of CK on HS tolerance partially mimic those of acclimation.

Combining the CK treatment with acclimation enhanced the up-regulation of *HSFA2d* and *HSP90* expression in roots induced by AHS (Fig. 3). This treatment also resulted in the highest levels of ABA in younger leaves, although *NCED* transcript levels were similar to those recorded after AHS treatment (Fig. 2A and 3). Levels of ABA metabolites in leaves were also slightly higher, suggesting preceding ABA elevation and a more rapid stress response (Table S2). Both *HSP* expression and ABA content indicate that the treatment enhanced synthesis of protective compounds, as suggested by the expression profile of the ABA-signaling gene *ABI5* in roots (Fig. 3). Exogenous CK in combination with AHS increased transcription of *SIG5* in crowns, indicating that it also affects non-green and/or developing plastids (Fu et al., 2021).

As under HS-CK, the levels of JA (and especially JA-Ile) and SA were slightly reduced in younger leaves by AHS-CK compared with AHS (Fig. 2B, S3, S4). This might be related to the production of volatile compounds. Volatile emissions were strongest after combination of AHS with CK, resulting in an increase in LOX-volatiles and SA derivatives, including (*Z*)-3-hexen-1-ol, hexanal, 2-ethylhexyl salicylate, and N,N-dimethyl-2-butanamine (Fig. 5, Table S5). Both CK and AHS diminished emissions of geranyl acetone (Fig. 5F). Compared to HS-CK, the AHS-CK treatment resulted in enhanced emissions of heptyl ester of cyclobutanecarboxylic acid, 1-decen, propylene carbonate, 1,1′-oxybis-2-propanol, 2,2′-oxybis-1-propanol, and 3-ethylhexane (Table S5). These results indicate that intensive cross-talk among phytohormones during HS responses is also tightly connected with the production of VOCs.

CK application before AHS had little additional effects on lipid composition compared with AHS treatment. It again affected mainly DGDG species, namely 36:3, 36:4, and 36:5 in older leaves and 34:2 in roots (Fig. 4), which indicates that CKs also regulate the response in older leaves.

4. Conclusion

Evaluation of the role of CK in HS responses showed that CK treatment can partially substitute for heat acclimation (the CK effects are summarized in Fig. 6, and the HS and AHS effects are summarized in Fig. S5). The combined CK and acclimation application significantly improved plant thermotolerance, including changes in the lipid composition of the thylakoid membrane (increasing membrane fluidity given by reduction of lipid unsaturation), and, in particular, the production of protective compounds and volatiles. CKs were found to stimulate the emission of 2,3-butanediol, a noncanonical linear isoprenoid volatile, and the SA derivative 2-ethylhexyl salicylate already under control conditions. During acclimation, CKs further enhanced the emission of the SA derivative 2-ethylhexyl salicylate, furane and furanone derivatives, while during AHS CKs promoted the emission of JA-related LOX-volatiles (hexenol and hexanal). Our results reveal a novel CK function – stimulation of emission of specific volatiles, which seems to contribute significantly to CK positive effects in HS responses. Comparison of responses of different organs disclose important functions of roots in stress responses. Overall, our data clearly indicate an efficient strategy to promote tolerance to HS in plants.

Author contributions

Conceptualization: R.V., S.P., K.D.; Resources: J.K., K.D., L.P.; Validation, Formal analysis: S.P., J.J., J.R., P.M., J.L., P.D.; Investigation: S. P., J.R., J.J., P.M., A.G., V.K.; Writing - Original Draft: R.V, J.J., J.R., P. M., S.P., J.K., K.D., L.P.; Writing - Review & Editing: R.V, J.J., J.R., A.G., P.M., S.P., J.K., K.D., L.P.; Visualization: S.P., J.J., J.R., V.K., A.G.; Supervision: R.V.; Funding acquisition: R.V., J.K., T.V.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2023.107683.

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