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Characterization of the role of cytokinins and abscisic acid during abiotic stress response

Charakterizace úlohy cytokininů a kyseliny abscisové při abiotickém stresu

Doctoral thesis

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Prague, 2018

DECLARATIONS

I hereby declare that this Ph.D. thesis documents my own work and I wrote it independently. This work or any substantial part of the text is not a subject of any other defending procedure. I declare that all used sources were cited and acknowledged properly.

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Signature

In the name of other co-authors, I declare that Sylva Přerostová has substantially contributed to all selected publications and I agree with the fact that these articles are presented as an integral part of this Ph.D. thesis. The publications were created by collectives of authors and the participation of the author of this thesis is specified below.

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ABSTRACT

Abiotic stresses significantly reduce crop yield, causing serious problems in agriculture. Understanding the mechanisms of plant stress responses could contribute to the improvement of their stress tolerance. Phytohormones play an important role in plant stress defence as well as in regulation of growth and development. This thesis summarizes the results published in four articles focused on the evaluation of the effects of phytohormones during abiotic stresses, namely salinity, drought, ZnO nanoparticle treatment and cold stress. The main emphasis is put on abscisic acid as the key regulator of water status and stress defence, and on cytokinins, which regulate plant growth and stabilize photosynthetic machinery. Cytokinins act antagonistically to abscisic acid.

Our results showed that abscisic acid is a general abiotic stress response regulator. Stress-tolerant plants (halophyte *Thellungiella salsuginea* or winter line of einkorn wheat *Triticum monococcum*) had a higher basal level of this hormone, especially in shoot meristematic tissues (apices, crowns), than stress-sensitive plants. Stress-tolerant plants reacted faster and in a more flexible way to stress.

Active cytokinins were negatively affected by stress, which was associated with growth suppression. The drought stress study showed that higher level of cytokinins improves stress tolerance, but discriminates plants during long-term stress. The rate of recovery after stress was dependent on cytokinin elevation. Up-regulation of the low active cytokinin *cis*-zeatin was characteristic during severe stress conditions, mainly in roots.

This thesis compares cytokinin/abscisic acid ratio dynamics during different kinds of stresses in relation to the response phases, as well as highlights specificity of the reactions of individual organs, where the meristematic tissue was the most affected by all stresses.

Key words: abscisic acid, *cis*-zeatin, cold stress, cytokinins, drought stress, phytohormones, salt stress, ZnO nanoparticles

ABSTRAKT

Abiotické stresy výrazně snižují výnosy plodin, čímž způsobují vážné problémy v zemědělství. Pochopení mechanizmů stresových odpovědí u rostlin by mohlo pomoci zvýšit jejich odolnost vůči stresu. Fytohormony hrají důležitou roli v obranných reakcích rostlin, jakož i v růstu a vývoji. Tato práce shrnuje výsledky čtyř publikací zaměřených na působení fytohormonů v průběhu abiotických stresů, konkrétně zasolení, sucha, kontaminace ZnO nanočásticemi a chladu. Hlavní důraz je kladen na kyselinu abscisovou, jako na klíčový regulátor obsahu vody v rostlině a stresových odpovědí, a na cytokininy, které regulují růst rostlin a stabilizují fotosyntetický aparát. Cytokininy působí antagonisticky vůči kyselině abscisové.

Naše výsledky ukázaly, že kyselina abscisová je univerzální regulátor odpovědí na abiotický stres. Rostliny odolné vůči stresu (halofyt *Thellungiella salsuginea* nebo ozimá linie jednozrnné pšenice *Triticum monococcum*) měly vyšší bazální hladiny tohoto hormonu, zejména ve meristematickém pletivu prýtu (vzrostné vrcholy, odnožovací uzly), než rostliny citlivé. Odolné rostliny reagovaly na stres rychleji a pružněji.

Aktivní cytokininy byly stresem negativně ovlivněny, což souviselo s potlačením růstu. Studie zaměřená na stres suchem ukázala, že vyšší hladiny cytokininů vedou ke zlepšení odolnosti rostlin, ale znevýhodňují je v případě dlouhodobého stresu. Rychlost zotavení po stresu závisela na hladinách cytokininů. Stimulace syntézy *cis*-zeatinu, málo aktivního cytokininu, byla charakteristická pro silný stres, převážně v kořenech.

Tato práce porovnává průběh změny poměru cytokininy/kyselina abscisová během jednotlivých stresových odpovědí v relaci k fázi odezvy a ukazuje specifickou reakci jednotlivých orgánů, přičemž nejcitlivější byla meristematická pletiva.

Klíčová slova: *cis*-zeatin, cytokininy, fytohormony, chlad, kyselina abscisová, sucho, zasolení, ZnO nanočástice

ABBREVIATIONS

35S:CKX	transformant with *CKX1* gene under constitutive *35S* promoter
ABA	abscisic acid
ABAR	abscisic acid receptor
ABCG	ATP-binding cassette type G
ABF	abscisic acid responsive factor
ABI	abscisic acid insensitive
AIT	abscisic acid importing transporter
AMP/ADP/ATP	adenosine mono/di/triphosphate
CK	cytokinin
CKX	cytokinin oxidase/dehydrogenase
DEX:CKX	transformant with *HvCKX2* gene (from *Hordeum vulgare*) under dexamethasone-inducible *pOp/LhGR* promoter
DEX:IPT	transformant with *ipt* gene (from *Agrobacterium tumefaciens*) under dexamethasone-inducible *pOp/LhGR* promoter
DTX	detoxification efflux carrier
DV92	spring line of *Triticum monococcum*
ENT	equilibrative nucleoside transporter
G3116	winter line of *Triticum monococcum*
HK	histidine kinase
HP	histidine phosphotransfer protein
HPLC-MS/MS	high-pressure liquid chromatography with tandem mass spectrometry
IPT	isopentenyl transferase
LOG	lonely guy
NCED	9-*cis*-epoxycarotenoid dioxygenase
PUP	purine permease
PYR/PYL/RCAR	pyrabactin resistance/PYR-like/regulatory components of abscisic acid receptors
RR	response regulator
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SAG	senescence-associated gene
SAG:IPT	transformant with *ipt* gene (from *Agrobacterium tumefaciens*) under senescence-inducible *SAG12* promoter
SARK	senescence-associated receptor-like kinase
SnRK	SNF1-related protein kinase
tRNA	transfer ribonucleic acid
WRKY	transcription factor with domain W-R-K-Y (Trp-Arg-Lys-Tyr)
WT	wild-type

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1 INTRODUCTION

Plants as sessile organisms had to develop mechanisms to cope with stresses caused by many different stressors, like unfavourable environmental conditions, pathogen infection or grazing by herbivores. Under stress conditions, plants need to modify their growth and development in order to reallocate energy sources to defence (Cramer et al., 2011). Suppression of biomass production is usually associated with a reduction of crop yield, which may lead to serious problems in agriculture.

Understanding the mechanisms underlying plant responses to stresses could contribute to the improvement of their stress tolerance and adaptability. The elucidation of complex physiological interactions under stress conditions represents the real challenge to modern science.

1.1 Abiotic Stressors

Plant stressors can be sorted into two groups – abiotic and biotic. The main topic of this thesis is the evaluation of hormonal functions in plant responses to abiotic stresses (for more information about biotic stresses, see the reviews Tenenboim and Brotman, 2016; Verma et al., 2016). Abiotic stresses involve the wide range of environmental disturbances (Cramer et al., 2011), like extreme changes of temperature, non-optimal light irradiance, nutrient deficiency and excess, or water shortage and overabundance. Many stresses are overlapped in their manifestation. For example, hot weather may be associated with high temperature, high irradiance and drought, which can result in osmotic stress, problems with nutrient uptake, increased concentration of toxic ions and other harmful compounds in soil. Plant protective mechanisms can be specific to individual stresses or common for more stresses. It follows that plants tolerant to one kind of stress may effectively resist other stresses (for example the halophyte *Thellungiella salsuginea* is capable to resist salt stress, drought, as well as cold stress; Wong et al., 2005).

This thesis is focused on characterization of plant responses to abiotic stresses, namely salinity, drought, ZnO nanoparticle contamination and cold stress (each stress is the main topic of one of the four attached manuscripts). Salinity (the topic of the manuscript Prerostova et al., 2017) is the serious problem of maritime lands, soils suffering by drought or draining as well as over-fertilized agriculture soils (Munns and Tester, 2008). Apart from the toxicity of Na^+ and Cl^- ions, salt stress causes osmotic unbalance, which complicates uptake of water

and other nutrients (Munns and Tester, 2008). The similar obstacle can be caused by metal nanoparticles (like ZnO nanoparticles used in the study Vankova et al., 2017). Zinc ions are toxic for plants in higher concentrations (Sofo et al., 2013; Landa et al., 2015) and the attempt to suppress their transport to the plants is accompanied by difficulties in water and nutrient uptake. A characteristic feature of drought and cold stresses is water deprivation (Vankova et al., 2012). Moreover, cold stress causes a slowdown of metabolism and of other physiological processes (Cramer et al., 2011; Kosova et al., 2012).

1.2 Phytohormones during Abiotic Stresses

Phytohormones act as the crucial regulators of plant stress responses, as well as of growth and development (Cramer et al., 2011). Cross-talk among phytohormones enables to fine-tune stress responses. This thesis is focused on the elucidation of the functions of abscisic acid (ABA) and cytokinins (CKs). For that reason, other phytohormones are not mentioned in detail, although they play important roles in abiotic stress responses. The dynamics of the other hormones (namely auxins, gibberellins, salicylic acid, jasmonic acid and ethylene) during stress responses are discussed in attached articles.

1.2.1 Abscisic Acid (ABA)

ABA belongs to isoprenoid hormones (see the review Nambara and Marion-Poll, 2005). It is synthesized in plastids from C_{40} carotenoids, which are cleaved by the enzyme 9-*cis*-epoxycarotenoid dioxygenase (NCED) to form xanthoxin, the linear precursor of ABA. The synthesis of xanthoxin by the enzyme NCED is the limiting step of the ABA synthesis. Xanthoxin is transported to the cytosol, where its subsequent modifications and cleavage give rise to ABA.

ABA can be inactivated by glucosylation creating the storage form ABA-glucosyl ester (capable to be hydrolysed back into the active form) or by irreversible degradation (hydroxylation) to phaseic acid, neophaseic acid or 7′-hydroxy-ABA (Nambara and Marion-Poll, 2005).

ABA is transported through symplast or apoplast as a free molecule or in the glucosylated form (Hartung et al., 2002; Merilo et al., 2015). ABA is predominantly synthesized in roots (Wolf et al. 1990). The main flow across the whole plant is mediated by xylem from roots to

shoots (Wolf et al. 1990; Hartung et al., 2002). The transport of ABA into or out of the cells is facilitated by ABCG transporters or AIT1 and DTX50 proteins (Merilo et al., 2015; Do et al., 2018).

The signalling pathway of ABA starts with the receptors from PYR/PYL/RCAR family or the ABA receptors ABAR localized in plastids (Hauser et al., 2011; Rushton et al., 2012). The receptor with bound ABA inhibits the repressor protein phosphatase 2C (e.g. ABI), which blocks the SnRK2 kinase. The released kinase phosphorylates transcription factors ABFs (ABA-responsive factors) and ABI5 protein, which activate transcriptions of many ABA-responsive genes directly or through WRKY factors.

The link between ABA and stress responses was observed in many studies (for example Arbona et al., 2010; Kosova et al., 2012; Syu et al., 2014). The fast reaction of plants to the ABA signal includes stomata closure (Munemasa et al., 2015; Albert et al., 2017). ABA stimulates the production of reactive oxygen species and vice versa, especially during the stomata closing (Mittler and Blumwald, 2015). Closed stomata lead to down-regulation of photosynthesis, decrease of growth and subsequently lowered nutrition demand (Munemasa et al., 2015). Water loss via the stomata is diminished. Simultaneously, synthesis of protective compounds, like dehydrins and proline, is up-regulated, which helps plants to maintain water potential (Lugan et al, 2010). The synthesis of cuticular waxes is stimulated, too (Teusink et al., 2002; Mackova et al., 2013b). ABA induces accumulation of antioxidants, e.g. anthocyanins, carotenoids, ascorbic acid, etc. (Lacampagne et al., 2009; Gururani et al., 2015). ABA is apparently able to protect photosynthetic apparatus, although the activity of photosynthesis is diminished (probably because of lowered gas exchange caused by closed stomata; Gururani et al., 2015).

Many works showed that ABA is a good marker of stress responses (e.g. Kosova et al., 2012; Mackova et al., 2013a). For that reason, the level of ABA was measured in the attached articles to assess the stress intensity. ABA was used for the evaluation of mild stress (in the case of salinity) and the existence of stress itself (as was used in the evaluation of the impact of ZnO nanoparticle treatment). The level of ABA was also taken into consideration when stress-sensitive and stress-tolerant plants were compared in salt stress (*Arabidopsis thaliana* x *Thellungiella salsuginea*), or cold stress (winter x spring lines of *Triticum monococcum*), as well as in drought (comparison of the responses of plants with modified CK levels).

1.2.2 Cytokinins (CKs)

CKs are adenine derivatives with isoprenoid or aromatic side chain (Zurcher and Muller, 2016). Due to the very limited abundance, the aromatic CKs have not been included in this thesis, so the whole text deals with the isoprenoid type CKs. In plants, four main active CKs occur: *trans*-zeatin, *cis*-zeatin, dihydrozeatin, and isopentenyladenine. CK biosynthesis (except *cis*-zeatin) takes place in plastids and includes the binding of dimethylallyl diphosphate to AMP/ADP/ATP with the assistance of the enzyme isopentenyl transferase (IPT). The side chain can be modified by enzymes of the cytochrome P450 type forming ribotides. Ribotides containing monophosphate are the direct precursors of active forms (conversion by the enzyme LOG) or they can be modified to active forms via dephosphorylation to ribosides and subsequent hydrolysis to bases. The synthesis of *cis*-zeatin takes place in the cytosol and the reaction of dimethylallyl diphosphate is realized by tRNA-IPTs (Schafer et al., 2015). After hydroxylation of the side chain to *cis*-form, tRNA is cleaved off and the other steps of synthesis are similar as in the case of the other CKs. The possibility of conversion among the four types of CKs was proved *in vivo* only for isopentenyladenine to *trans*-zeatin, but other conversions have not been described or they were observed only *in vitro* (Hluska et al., 2016). Neither the *trans*-*cis* isomerisation of zeatin nor the occurrence of the *cis*-*trans* isomerase was detected (e.g. Gajdosova et al., 2011; Hluska et al., 2016).

The synthesis of CKs takes place mostly in roots (Zurcher and Muller, 2016). Active forms, as well as ribosides, can be transported to long distance (Kang et al., 2017). ABCG transporters were found to export CKs from cells to xylem; and some of the PUP and ENT transporters import CKs across the plasma membrane (Kang et al., 2017).

CK receptors have been found on plasma membrane or endoplasmic reticulum (Zurcher and Muller, 2016). The histidine kinase receptors (HKs) are dimers composed of the CK binding site and the histidine kinase domain, which transfer the CK signal by phosphorylation to histidine phosphotransfer proteins (HPs). HPs proteins are mobile and they are transported to the nucleus, where they activate response factors (RRs). RRs of type B are transcription factors, which activate the transcription of CK-related genes and also of the transcription of RRs of type A. RRs of type A work as the negative regulators of CK signalling and they block the activation of RRs of type B.

CKs can be deactivated by ribosylation or by O-glucosylation (Zurcher and Muller, 2016). Degradation of CKs consists in N^7- or N^9-glucosylation or cleavage of the side chain by the enzyme cytokinin oxidase/dehydrogenase (CKX) to adenine and the aldehyde originated from the side chain.

CKs are very important growth regulators participating in stress reactions (summarized in O'Brien and Benkova, 2013). Their content during abiotic stresses was observed to be down-regulated (Kosova et al., 2012; Liu et al., 2012; Mackova et al., 2013a). CKs are the main coordinators of cell division (Laureys et al., 1998). Down-regulation of CKs during stress progression is associated with the growth suppression. CKs were also found to promote stomata opening, antagonizing ABA (Munemasa et al., 2015; Nguyen et al., 2016). The low level of CKs during stress leads to the inhibition of buds and shoot meristematic tissue development (Zurcher and Muller, 2016); to inhibition of photosynthesis (Gururani et al., 2015); and to changes in the source-sink relation (Kosova et al., 2012). CKs were also shown to be the inhibitor of senescence (for all observed CK functions see the book chapter Zurcher and Muller, 2016). The stress responses are not only regulated by the changes in phytohormone content, but also by regulation of the signalling pathway (Mason et al., 2010; Jeon and Kim, 2013; Nguyen et al., 2016).

The evaluation of the impact of CKs to stress tolerance is not straightforward, as both the up-regulation of CKs levels as well as their down-regulation were reported to enhance plant tolerance, e.g. to salinity (Tran et al., 2007; Mason et al., 2010; Liu et al., 2012) and drought stress (Rivero et al., 2010; Werner et al., 2010; Nishiyama et al., 2011; Merewitz et al., 2012). The detail characterisation of the role of CKs during drought stress progression has become one of the main goals of this thesis.

The second main issue has been the role of *cis*-zeatin during stress responses (Kaminek, 2015). This CK is often associated with stress (growth limiting) conditions, as was observed in the studies Vyroubalova et al. (2009), Gajdosova et al. (2011) or Kosova et al. (2012). The amount of relevant studies is rather low, as many reports did not distinguish *trans*-zeatin and *cis*-zeatin. This might cause problems in the interpretation, as the levels of *trans*-zeatin are usually down-regulated under stress conditions, but the levels of *cis*-zeatin can be increased.

1.3 Subject of the Study

The main aim of this study was to elucidate the roles of ABA and CKs during abiotic stress responses. This topic was addressed by determination of the content of phytohormones (including *cis*-zeatin) by HPLC-MS/MS in combination with analyses of gene transcription (RT-qPCR), in relation to changes of physiological parameters (plant growth, plant water content, membrane stability, ion content, frost tolerance etc.). The used methods enabled to characterize plant responses to different stresses, including the hormonal dynamics during the stress progression. The stress responses in roots, leaves and shoot meristematic tissues were evaluated separately. The possibility to utilize the achieved knowledge of ABA or CK functions in their exogenous application, gene manipulation or in breeding in order to increase plant stress tolerance was studied using stress-resistant and stress-sensitive plants or transformants with modified hormone contents.

The four attached manuscripts summarize the results from the studies of plant responses to salt stress, drought, ZnO nanoparticle contamination and cold stress. This thesis is focused on the evaluation of common and specific features of plant stress responses, which are highlighted in the Discussion.

2 AIMS

The aims of this doctoral thesis are comprised of the following points:

1) To characterize the impact of abiotic stresses on the level of ABA
    a) Impact of salt stress
    b) Impact of drought stress
    c) Impact of ZnO nanoparticles
    d) Impact of cold stress
2) To characterize the impact of abiotic stresses on the level of CKs
    a) Impact of salt stress
    b) Impact of drought stress
    c) Impact of ZnO nanoparticles
    d) Impact of cold stress
3) To discuss the mutual relationship between ABA and CKs during abiotic stresses

3 SUMMARY OF PUBLISHED RESULTS

This thesis summarizes results presented in four attached publications (out of 9 published original papers), where I am the first author (2) or co-author (2). All selected articles are focused on phytohormones in the relation to plant abiotic stress responses. Results achieved in these studies enabled to fulfil the aims of PhD study.

This chapter briefly summarizes data presented in the chosen publications. Evaluation of ABA and CK functions are discussed later.

3.1 1st Article – Salt Stress

The article Prerostova et al. (2017) published in *Plant Science* (IF 3.437) is focused on salt stress responses of two model plants: salt-sensitive *Arabidopsis thaliana* and salt-tolerant *Thellungiella salsuginea*. Plants were exposed 1 week to different NaCl concentrations in the growth medium (2–150 mM NaCl in the case of *Arabidopsis*; 150–350 mM NaCl in the case of *Thellungiella*). The highest concentrations were also used for investigation of the short-term dynamics of hormonal responses (15 min to 24 h). Apart from hormonal analysis, transcription levels of selected genes, Na^+ and K^+ contents, relative water content and membrane stability were measured in shoot apical meristem (apices), leaves and roots.

Our results showed that salt stress tolerance of the halophyte *Thellungiella* is associated with higher basal levels of ABA, jasmonic acid and *cis*-zeatin in shoot apices. Stress responses of both species can be separated in relation to stress strength. Very mild stress (2–25 mM NaCl in the case of *Arabidopsis*) affected the transcription of genes involved in CK biosynthesis (*AtIPTs*) or degradation (*AtCKXs*). Mild stress (50 mM NaCl in the case of *Arabidopsis*, and 150 mM in the case of *Thellungiella*) led to stress responses only in glycophyte shoot apices, while in all tested organs of the halophyte. Severe stress caused serious metabolic changes in both, *Arabidopsis* (above 75 mM NaCl) and *Thellungiella* (above 225 mM NaCl). Although *Arabidopsis* showed with increasing salt concentrations progressive damages of shoot apices leading to plant death in the case of 150 mM NaCl (lethal stress), *Thellungiella* was able to protect the apex in severe stress at the expense of older leaves. The analysis of the early stress responses (15 min to 24 h) revealed that the dynamics of the responses were similar in both plants but the halophyte reacted faster and stronger than *Arabidopsis*.

3.2 2nd Article – Drought Stress

The article Prerostova et al. (2018) published in *Frontiers in Plant Science* (IF 4.291) elucidates the role of CKs during drought stress and subsequent re-watering. The study was performed using high throughput phenotyping system combined with hormonal and transcriptomic analyses. Responses of *Arabidopsis thaliana* wild-type (WT) plants were compared with those of transformants with modified CK biosynthesis or degradation. Down-regulation of endogenous CK content was achieved by overexpression of the gene for CK deactivating enzyme *CKX* either constitutively using 35S promoter (*35S:CKX*), or induced at the stress onset using dexamethasone-inducible one (*DEX:CKX*). CK levels were increased by stimulation of the expression of CK biosynthetic gene *IPT* at the stress onset (*DEX:IPT*), under senescence-inducible SAG12 promoter (*SAG:IPT*) or by application of exogenous CK *meta*-topolin.

Constitutive CK down-regulation slowed down ontogenesis and led to elevated expression of stress-related genes. These plants showed higher drought stress tolerance than WT, but their recover after re-watering was slow. Plants with higher CK levels resisted drought at the beginning of stress period. With prolonged drought, they were losing water to a higher extent than WT, probably due to the promotion of stomata opening by elevated CK content. Only *SAG:IPT* plants showed better drought tolerance than WT. Stimulation of CK synthesis during the stress progression did not interfere with activation of defence mechanisms at the beginning of stress period, but effectively diminished negative drought effects on photosynthetic activity. Higher levels of CKs promoted faster recovery after re-watering. In general, drought responses were associated with elevated levels of ABA, jasmonic acid and salicylic acid and decreased levels of auxins and (with the exception of *ipt* overexpressers) of CKs.

3.3 3rd Article – ZnO Nanoparticles

The article Vankova et al. (2017) published in the journal *Science of the Total Environment* (IF 4.900) describes the effect of ZnO nanoparticles on *Arabidopsis* plants. The diameter of ZnO nanoparticles was 30 nm and concentration range 0.16–100 mg L^{-1} was used.

Increasing concentration of ZnO nanoparticles suppressed synthesis of CKs and auxins in shoot apices. The level of the low active CK *cis*-zeatin was highly elevated in the presence of the contaminant. The levels of ABA raised in the presence of the nanoparticles in shoots,

while those of salicylic acid in leaves and roots. Our results showed that ZnO nanoparticles in higher concentrations activate stress responses in plants.

3.4 4th Article – Cold Stress

The article Vankova et al. (2014) published in *Environmental and Experimental Botany* (IF 3.003) is focused on the comparison of cold stress responses of the einkorn wheat *Triticum monococcum* cultivars DV92 (spring line) and G3116 (winter line). Plants were treated by cold (4 °C) 1–42 days. Water saturation deficit, osmotic potential, days to heading, acquired frost tolerance (lethal temperature for 50% plants), contents of dehydrins, phytohormones and phenolic acids levels were measured in crowns, leaves, and roots.

The first reaction to cold stress (alarm phase; 1 day) was characteristic by water deficit associated with the increase of the level of ABA. Other hormones, namely jasmonic acid and salicylic acid as well as gibberellins and CKs were down-regulated, to a higher extent in winter line. During the acclimation phase (3–7 days), plants accumulated dehydrins and increased their frost tolerance. Hormones promoting growth (gibberellins, auxins and CKs) were stimulated, faster in the spring line. The ABA level decreased and the content of phenolic acids increased probably to protect plants against reactive oxygen species. After 21 days, the spring line passed into the generative stage, which was indicated by the maximum of active CKs. The winter line fulfilled its vernalisation requirement after 42 days, which was indicated by similar hormonal profiles as observed earlier in the spring line, as well as by the decrease of frost tolerance and dehydrin levels. The results were compared with the responses of common wheat *T. aestivum*.

4 DISCUSSION

4.1 The Role of ABA in Abiotic Stress Responses

4.1.1 ABA under Salt Stress Conditions

Our salt stress study (Prerostova et al., 2017) revealed that the level of ABA increases in plants with rising salt concentration (see Fig. 4A in the Attachment). In the case of salt-sensitive *Arabidopsis thaliana*, the increase was detected mainly in shoot apical meristem (apices), under severe stress also in leaves. The elevation of ABA content was fast (within 15 min in roots, within 1 h in apices and leaves; Fig 4C). ABA content in roots decreased after 24 h to the control level. Interestingly, the transcription of ABA biosynthetic gene *NCED3* was strongly up-regulated in the same organ (Fig. 3C). Simultaneously, high elevation of ABA degradation metabolites (phaseic acid, neophaseic acid) was found (Supplementary Fig. A.4).

The halophyte *Thellungiella salsuginea* had a higher basal level of ABA in apices than *Arabidopsis*. The ABA content in the halophyte exposed to salt stress rose in apices and leaves but decreased in roots. The transcription of *ThNCED3* gene was stimulated strongly in roots (Fig. 3B), which shows fast transport of ABA from roots to shoots (Wolf et al., 1990). The response of the halophyte was slightly faster in apices than in the case of *Arabidopsis*. The response of the halophyte was stronger than in *Arabidopsis*. *Thellungiella* preferentially stored ABA into the reversible conjugate ABA-glucosyl ester (Supplementary Fig. A.4). The possibility of fast hydrolysis to active hormone under stress conditions is obvious (Xu et al., 2014).

Our results together with other comparative studies (Taji et al., 2004; Gong et al., 2005) showed that salt-tolerant *Thellungiella* has similar transcriptomic, metabolomic and hormonal equipment as salt-sensitive *Arabidopsis*. Nevertheless, *Thellungiella* constitutively expresses many compounds that are stress-inducible in *Arabidopsis*, including those associated with the ABA biosynthetic pathway. For example, we found a higher level of ABA in shoot apices under control conditions than in *Arabidopsis*. *Thellungiella* also prefers to down-regulate ABA content by its glucosylation into the storage form (ABA-glucosyl ester), avoiding the formation of irreversible degradation forms such as phaseic acid and neophaseic acid, which are characteristic for *Arabidopsis*. This indicates the energy-saving strategy of the halophyte.

The higher level of ABA seems to be crucial for plant responses under salt stress conditions (Tester and Davenport, 2003; Arbona et al., 2010). Faster and stronger up-regulation of ABA

in the whole plant facilitates effective stress reaction. However, the higher ABA levels, even though only in the actively growing shoot apical meristematic tissue, lead to slower growth, smaller stomata aperture and more leaf wax content (Teusink et al., 2002; Mackova et al., 2013b). The closing of stomata enables plants to stabilize water potential in leaves; however, it simultaneously leads to decrease of the photosynthesis rate, and subsequently of growth, which is connected with lower nutrient demand (Munemasa et al., 2015).

Elevation of the ABA content is noticeable also under lower salt concentrations, which correspond to salinization of field soil (Cramer et al., 2011). This explains why crops grown on moderately salted lands (e.g. 40 mM NaCl) show suppressed growth.

Our results showed that the response of the shoot apical meristem is crucial during salt stress, but the responses of roots and older leaves are also necessary for plant resistance.

4.1.2 ABA under Drought Stress Conditions

Our phenotyping experiment (Prerostova et al., 2018), which compared different transformants with modulated CK content, confirmed the important role of ABA during drought stress (from other articles see e.g. Lugan et al., 2010; Mackova et al., 2013b; Gururani et al., 2015). ABA activates stomata closure and stimulates expression of stress-related genes (O'Brien and Benkova, 2013; Leng et al., 2014; Verma et al., 216).

The Figure 11 from the paper Prerostova et al. (2018) demonstrates that slow growth rate and higher stress tolerance correlate with the higher ABA level. A typical example is a transformant constitutively overexpressing CKX gene, 35S:CKX, which showed better stress tolerance but slower recovery ability. This is related to the fact that the CKX overexpressing genotypes maintains a higher level of ABA also during recovery, while the other genotypes down-regulated ABA content quickly upon rehydration (Fig. 5). In general, the elevated ABA levels (at least in the long-term stress duration) are required for the efficient drought tolerance of plants, while active recovery process is associated with ABA suppression.

4.1.3 ABA under ZnO Nanoparticle Influence

The effects of the stress caused by nanoparticles depend on multiple factors, namely nanoparticle composition, charge, size, shape, concentration, time of exposure, used plant species and substrate (Nair and Chung, 2014; Arruda et al., 2015).

Transcriptomic studies showed that nanoparticles up-regulate the expression of genes related to stress responses, including ABA biosynthetic genes (Kaveh et al., 2013; Syu et al., 2014; Thiruvengadam et al., 2015). Similar results were obtained also in our transcriptomic study Landa et al. (2015) and hormonal study Vankova et al. (2017), which were focused on the effect of ZnO nanoparticles.

The results summarized in Vankova et al. (2017; Fig. 5) indicate the elevation of ABA levels in roots of plants exposed already to 4 mg L^{-1} ZnO nanoparticles. In this case, plant roots were directly exposed to nanoparticles in a hydroponic system. Higher nanoparticle concentrations led to increasing of ABA content also in shoot apices and leaves. These data are in accordance with Sofo et al. (2013) and Syu et al. (2014). Interestingly, ABA content in roots was diminished after exposition to very high concentration (100 mg L^{-1}). The regulation of ABA levels was connected with an elevation of ABA metabolites arising from ABA degradation (Table 4). The higher content of ABA is most likely associated with the higher content of reactive oxygen species, anthocyanins and heat shock proteins (Nair and Chung, 2014; Thiruvengadam et al., 2015; Zhang et al., 2015).

Lower levels of ABA in shoot apices of plants exposed to very low ZnO nanoparticles concentrations (0.16 and 0.8 mg L^{-1}) could reflect the fact that lower concentrations slightly suppress stress responses, as was described in Nyitrai et al. (2009) or Prasad et al. (2012).

However, it is necessary to take into consideration the effect of the nanoparticle size as well as the toxicity of the ion. It is obvious that the main negative effect of ZnO nanoparticles is given by Zn^{2+} ions (Landa et al., 2015). The same results were obtained also in our experiments with CuO nanoparticles (Landa et al., 2017). In that study, we found the negative impact caused by Cu^{2+}. Nevertheless, the nanoparticles were more soluble than bulk particles and they could enter the cells more easily (Arruda et al., 2015; Zhang et al., 2015; Landa et al., 2017). The nanoparticles exhibited also higher adhesion to root surface, which could help plants to keep toxic ions outside, and in this way reduce the uptake into cells (Landa et al., 2017).

ABA is an important regulator of stress responses to heavy metal stress (Sofo et al., 2013; Shukla et al., 2014; Syu et al., 2014), but it seems that it is also involved in the reaction to nanoparticles *per se*.

4.1.4 ABA under Cold Stress Conditions

ABA is known for a long time to be associated with early cold stress responses (Galiba et al., 1993; Janowiak et al., 2002; Kosova et al., 2012). The first phase (ca 1 day) of cold stress response is associated with fast decrease of root hydraulic conductivity, which results in the drop of plant water content, and subsequently in the increase of ABA levels regulating stomata aperture and synthesis of protective compounds (Vankova et al., 2014; Figs. 1, 4). During acclimation, ABA levels decrease and its role as a stress response regulator is replaced by salicylic acid, ethylene and jasmonic acid.

As demonstrated in our study Vankova et al. (2014), plants grown in soil evidenced ABA elevation in leaves and crowns exposed to cool air, but roots hidden in soil did not show any reaction. Interestingly, the spring genotype reduced the ABA levels over a longer period than the winter line, which exhibited also higher basal level of ABA in actively developing crowns.

4.1.5 ABA under Stress Conditions – Summary

ABA is the key phytohormone involved in the response to abiotic stresses associated with dehydration, such as salt stress associated with ion misbalance, drought stress characteristic by water deficiency, and cold stress connected with diminished water transport during alarm phase. The universal function of ABA in many stress responses suggests that plants with elevated content of this hormone could exhibit resistance to more stresses (e.g. *Thellungiella salsuginea*, which resists salinity, drought and cold; Wong et al., 2005). The higher basal content of ABA in meristematic tissues (apices, crowns) seems to be a good prerequisite for better tolerance of the stress (as was shown in the case of salinity and cold stress); however, slower growth is the potential risk of this strategy. The fast and flexible modulation of ABA signalling and ABA content under stress conditions (by stimulation of synthesis or by activation of storage forms) may improve the ability of plants to withstand the stress.

4.2 The Role of CKs in Abiotic Stress Responses

4.2.1 CKs under Salt Stress Conditions

Our experiments on salt stress (Prerostova et al., 2017; see Figs. 3, 4, A.6, A.7) showed that low concentrations of NaCl do not change the levels of active CKs (*trans*-zeatin, dihydrozeatin, isopentenyladenine and *cis*-zeatin), but they modify the expression of genes associated with CKs. Specifically, up-regulation of CK biosynthesis (*IPT3* expression) and down-regulation of CK degradation (expression of several *CKX*) were observed throughout the plants. Severe stress increased the level of *cis*-zeatin at the expense of *trans*-zeatin content. This was observed in *Arabidopsis* apices but in all organs of *Thellungiella*. A similar response was described in the case of *Arabidopsis* (Gajdosova et al., 2011) and maize (Vyroubalova et al., 2009). The increased content of *cis*-zeatin was accompanied by much higher elevation of the level of *cis*-zeatin riboside, the direct precursor and the transport form of *cis*-zeatin. The decrease of *trans*-zeatin was connected with lower synthesis (*IPT* expression) and diminished signalling (*ARR* type B expression) in the whole plant (the role of response regulators was examined e.g. in Vyroubalova et al., 2009; Mason et al., 2010). The lethal stress was characteristic by hormonal imbalance with the huge content of low-active CKs (dihydrozeatin, isopentenyladenine and *cis*-zeatin), probably as a part of the trigger of cell death (Mlejnek and Prochazka, 2002).

The early plant responses to salt stress were associated with the increase of expression of several *IPTs*. However, within 2 h, the expression of CKXs was up-regulated and the expression of IPTs was diminished. The CK signalling was also inhibited. Within 24 h, the content of active CKs decreased in all studied organs.

Despite the fact that some studies showed better salt stress tolerance of plants with up-regulated CK synthesis (Liu et al., 2012) or after exogenous CK application (Iqbal and Ashraf, 2005), other articles (Tran et al., 2007; Mason et al., 2010; Nishiyama et al., 2011) described the negative role of CKs during salt stress. Our results (Prerostova et al., 2017) on hormonal analysis of the salt-tolerant *Thellungiella* tend to agree with the latter authors. The halophyte *Thellungiella* had lower basal levels of active CKs and their metabolites (around half of those in *Arabidopsis*), and an order of magnitude lower content of their precursors in the whole plant. Only the level of the low-active *cis*-zeatin was higher in *Thellungiella*. Also, the expression profile of the receptor *TsHK3* related to the signalling pathway of *cis*-zeatin (Spichal et al., 2004) was different from the *AHK3* in *Arabidopsis*. The low level of *trans*-

zeatin, which is highly active in cell division (Laureys et al., 1998) and stomata opening (Munemasa et al., 2015), clarifies the slow growth rate of *Thellungiella*.

4.2.2 CKs under Drought Stress Conditions

The article Prerostova et al. (2018) contributed to the elucidation of the role of CKs during drought. Drought, in general, leads to down-regulation of CK content, which is connected with growth suppression. From active CKs, mainly *trans*-zeatin was diminished by drought; by contrast, the low active *cis*-zeatin was up-regulated (this effect was observed also in roots in studies Havlova et al., 2008; and Mackova et al., 2013a). Re-watering after the stress was connected with growth acceleration associated with the elevation of the *trans*-zeatin level.

Previous studies differed in their conclusions about positive or negative impacts of CKs on drought resistance of plants. Some studies showed that the lower level of CKs supports plant tolerance (e.g. Werner et al., 2010; Nishiyama et al., 2011). The other articles brought the finding that the higher levels of CKs or the application of an exogenous CK stimulate drought resistance (e.g. Rulcova and Pospisilova, 2001; Rivero et al., 2010; Merewitz et al., 2012).

Our experiment focused on the wide range of *Arabidopsis* transformants with the modified endogenous CK content together with the WT exposed to exogenous *meta*-topolin showed the following results. At the beginning of the drought stress, CKs supported the growth probably by blocking the stomatal closure and diminishing the negative stress effects on the photosynthesis and cell division. Nevertheless, the lower level of CKs was an advantage in the long-term stress (1 week and more). The growth rate of the plants with low content of active CKs was suppressed by the stress less than that of WT plants and their water loss was smaller. It is possible to say that plants with the lower CK levels were better “prepared” for the potential stress.

Re-watering was associated with vigorous growth. The growth acceleration was the highest in plants containing the higher levels of CKs; by contrast, plants overexpressing the CK degradation enzyme CKX maintained the stress regime. It follows that CKs are necessary for the fast recovery after drought stress and inhibition of the stress mechanisms.

Our results illustrate that the advantages of different strategies comprising CK modulation depend significantly on the stress duration, severity and speed of dehydration. For example, the best variant applicable for the long-term stress conditions seems to be the usage of plants

overexpressing the enzyme for the CK biosynthesis IPT under the stress-inducible promoter (like *SAG12* or *SARK*; similar results were achieved by Rivero et al., 2010; Merewitz et al., 2012). These plants do not actually suffer from enhanced water loss (as was observed in the case of dexamethasone-inducible *IPT* gene). They activate the expression of *IPT* gene during the stress progression, which partially suppresses stress responses and renews the growth close to the time of re-watering. The growth of plants is not affected before the beginning of the senescence. However, the duration of the drought cannot be too long and the maturing of fruits of these plants might be affected (Chang et al., 2003; Calderini et al., 2007). The beneficial effect of the *SAG12* promoter was demonstrated also on tobacco plants exposed to severe drought stress (Havlova et al., 2008; Vankova et al., 2012).

4.2.3 CKs under ZnO Nanoparticle Influence

The negative effect of ZnO nanoparticles (Vankova et al., 2017; Figs. 1, 2, Table 1) on *Arabidopsis* plants positively correlated with increasing concentration of the contaminant in the medium. Suppression of growth was accompanied by the decrease of the CKs content. The strongest stress influence was detected in the shoot apical meristem, the tissue specific by intensive growth and development. These results are in agreement with the role of CKs in cell division and growth (Laureys et al., 1998). The highest concentrations (4, 20 and 100 mg L^{-1}) led to significant increase of the low active CK *cis*-zeatin in roots. Low sensitivity of roots on ZnO nanoparticles suggests high stress resistance of this organ.

A slight hormonal disturbance was observed in the case of O- and N-glucosyl forms of CKs also at mild stress (0.8 and 4 mg L^{-1}, respectively; see Table 2 in the publication). The elevation of these deactivation forms indicates the tendency to maintain CK homeostasis under stress progression.

The negative influence of ZnO nanoparticles on plants was evident. The decrease of CKs might by caused probably by the metal toxicity, as described also in the case of arsenic (Srivastava et al., 2009) or cadmium (Lomaglio et al., 2015). The tissue most vulnerable to the toxic compounds seems to be the shoot apex. This fact should be taken into the consideration. Thus, the stress strength evaluation should include characterization of the response of this meristematic tissue.

4.2.4 CKs under Cold Stress Conditions

The effect of cold stress on the level of CKs in einkorn wheat *Triticum monococcum* is described in our article Vankova et al. (2014; see Fig. 6). The characteristic feature of the alarm phase response was a decrease of CKs content in leaves and crowns, which was faster in the winter line of einkorn wheat and more profound in the case of winter common wheat *T. aestivum* (Kosova et al., 2012). The CK signalling was also suppressed by cold (Jeon and Kim, 2013). The acclimation was connected with up-regulation of CK levels in both wheat species and partial resumption of growth, which was associated with the inhibition of the activity of the CK degradation enzymes CKXs (Kosova et al., 2012; Vankova et al., 2014). During the phase of stress resistance, winter line enhanced its frost tolerance, which was associated with a further decrease of CKs (mainly in the fast-growing crowns). The vegetative-generative transition was then preceded by a transient elevation of *cis*-zeatin and isopentenyladenosine (Corbesier et al., 2003; Tarkowska et al., 2012; Vankova et al., 2014).

Comparing *T. monococcum* and *T. aestivum*, we can draw the conclusion that cold stress decreases the levels of active CKs, but their levels increase again after acclimation.

4.2.5 CKs under Stress Conditions – Summary

All studied abiotic stresses (salinity, drought, cold and metal nanoparticle pollutants) could be described as the stresses affecting water usage in plants. The described stresses caused inhibition of growth rate associated with down-regulation of the levels of active CKs and of CK signal transduction. Especially the alarm phase (in the case of fast stress application – salinity, low temperature) or severe stress (in the case of drought progression) can be characterized by *trans*-zeatin suppression. This decrease was also connected with the regulation of stomatal aperture and other stress mechanisms, as described by Nguyen et al. (2016). CKs can stabilize the photosynthetic apparatus, which means that down-regulation of CKs during stress coincides with photosynthesis suppression and growth repression (Gururani et al., 2015). Inhibition of growth rate might be associated with the reallocation of resources to defence (Werner et al., 2010; Vankova et al., 2012; O'Brien and Benkova, 2013).

The drought stress experiment (Prerostova et al., 2018) revealed that higher level of active CKs helps plants to tolerate the stress, but discriminates these plants in the respect to the resistance to long-term stress. CKs are also important for the recovery after stress and for growth renewal.

All our studies showed the importance of *cis*-zeatin during stress progression. Its content increases during stress, especially in roots. Its synthesis is localized in the cytosol, in contrast to *trans*-zeatin, which is synthesized in plastids. This CK is low active and might compete with *trans*-zeatin for the binding site of receptors (Gajdosova et al., 2011; Schafer et al., 2015).

4.3 Relationship between ABA and CKs under Abiotic Stresses

The interaction between ABA and CKs might be observed in many processes. Firstly, both hormones are involved in regulation of stomata aperture. CKs promote the opening of stomata and the exchange of gases, while ABA gives the signal to close stomata (Albert et al., 2017; Munemasa et al., 2015). This regulation is important for water management (Munemasa et al., 2015) and fundamentally affects the photosynthesis rate (Gururani et al., 2015). In the majority of cases, ABA inhibits growth, induces developmental stages connected with desiccation (like seed maturation and pollen dormancy; Cramer et al., 2011) as well as anthocyanin and carotenoid synthesis (e.g. during fruit ripening; Lacampagne et al., 2010; Leng et al., 2014). By contrast, CKs support growth and development and they stimulate cell division (O'Brien and Benkova, 2013; Zurcher and Muller, 2016). Both hormones are able to regulate the synthesis and signalling pathway of one another (O'Brien and Benkova, 2013). Defence mechanisms might be also regulated by ABA as well as by CKs.

The antagonism between ABA and CKs in stress responses seems to be a good indicator of the plant status. The ratio between ABA and total active CKs (*trans*-zeatin, *cis*-zeatin, isopentenyladenine and dihydrozeatin) measured in the four presented studies is shown on Fig. 1. The lower the ratio was, the higher influence of ABA was detected. The low ratios in leaves after short-term stress impact (cold stress, salt stress) or after the long-term duration of severe stresses connected with impaired water uptake (high concentrations of ZnO nanoparticles, NaCl or drought) were mainly associated with regulation of stomata aperture. In the case of CKX transformants exposed to drought, it is obvious that the higher level of ABA established during the drought was maintained also after re-watering.

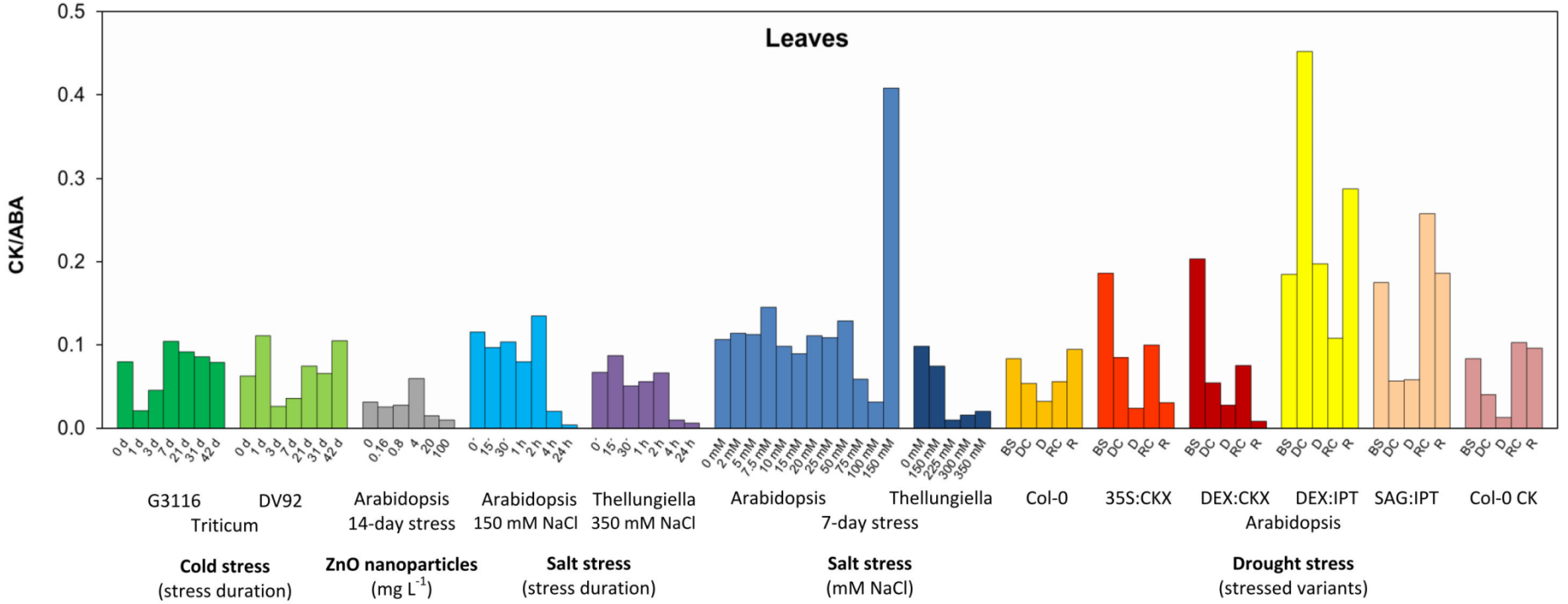
The stress-tolerant plants (winter line G3116 of *Triticum monococcum* and halophyte *Thellungiella salsuginea*) had much lower basal CK/ABA ratio in the fast-growing tissues (apices or crowns) than their sensitive relatives. In the stress-tolerant plants, the decrease of CK/ABA ratio during stress progression was faster than in the stress-sensitive ones. High

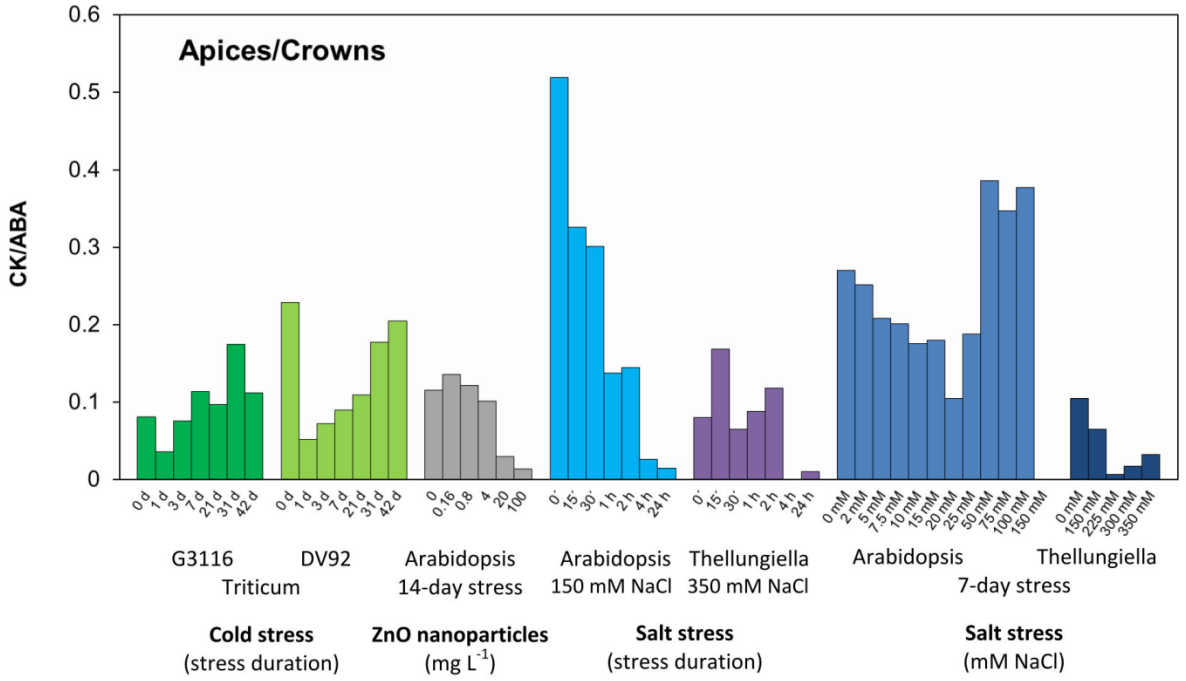
CK/ABA ratio in plants exposed to severe stress (mostly in roots) is caused by high levels of low active CKs, especially of *cis*-zeatin. On the example of *Arabidopsis* WT plants grown in hydroponics, it is nicely visible the effect of plant age on the content of CKs and ABA. Plants exposed to short-term salt stress (time scale) were 28 days old, plants after 7-day application of NaCl were 35 days old, and plants treated by nanoparticles were 42 days old. The descendent trend of the CK/ABA ratio with plant age clearly demonstrates the inhibition of growth, or more precisely the decrease of active CKs content in the shoot apical meristem with ageing.

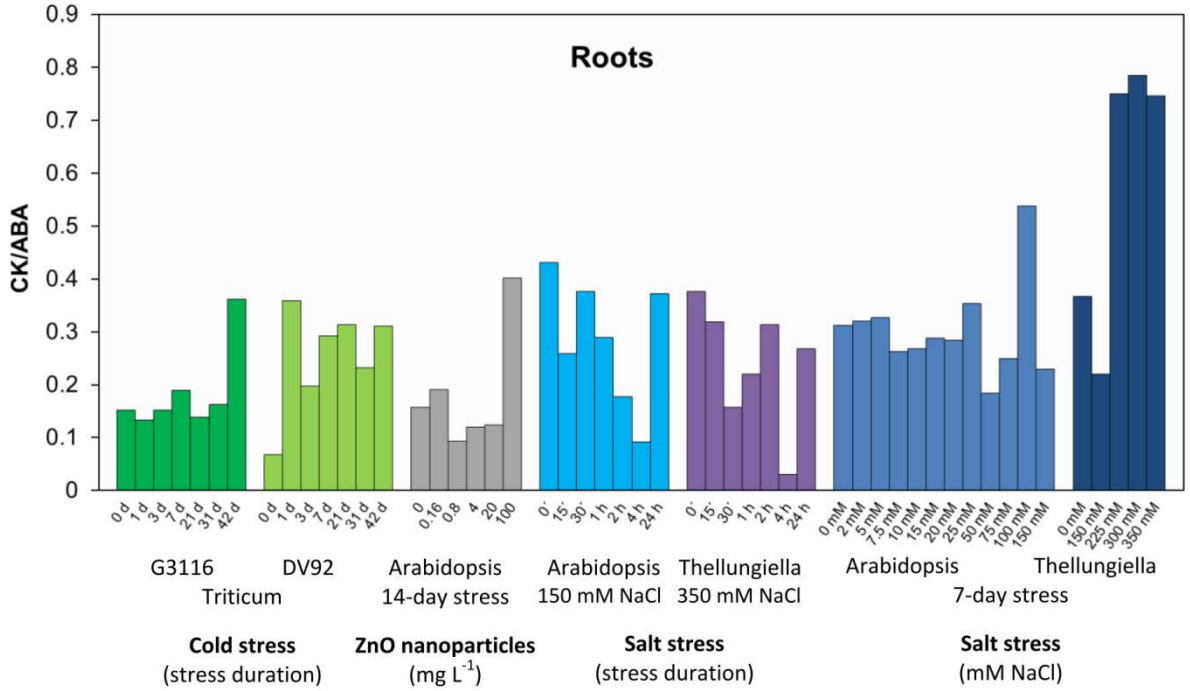
4.4 Conclusion

In conclusion, the role of ABA in responses to studied abiotic stresses connected with water deficiency seems to be universal. The antagonistic effect of active CKs and the tight relationship between both hormones was also demonstrated. The results show the importance of considering complex hormonal cross-talk in order to characterise the stress responses. The involvement of the low active CK *cis*-zeatin in the responses to severe stress (especially in roots) was evident and the specific role of this CK in stressed plants should be further investigated. All manuscripts included in this thesis show the importance of separate evaluation of leaves, roots and fast-growing meristematic tissues.

Figure 1: Ratio of the levels of active CKs (*trans*-zeatin, *cis*-zeatin, dihydrozeatin and isopentenyladenine) to the content of ABA in leaves, fast-growing shoot apices or crowns (*Triticum monococcum*) and roots of all tested variants mentioned in the four selected manuscripts. All variants are described in detail in the respective articles. Cold stress: *Triticum monococcum* winter line G3116 and spring line DV92 exposed to cold stress (4 °C) 1–42 days. ZnO nanoparticles: *Arabidopsis thaliana* exposed 14 days to different concentrations of ZnO nanoparticles (mg L^{-1}). Salt stress: *Arabidopsis thaliana* or *Thellungiella salsuginea* exposed from 30 min to 24 h to high concentrations of NaCl, and plants exposed to different concentrations of NaCl during 1 week. Drought stress: Arabidopsis thaliana before stress (BS), after 13 days of drought (D; the respective control variant – DC), and after 6 days of recovery (R; the respective control variant – RC).




Apices/Crowns
CK/ABA
0.6
0.5
0.4
0.3
0.2
0.1
0
0 d 1 d 3 d 7 d 21 d 31 d 42 d
0 d 1 d 3 d 7 d 21 d 31 d 42 d
0 0.16 0.8 4 20 100
0' 15' 30' 1 h 2 h 4 h 24 h
0' 15' 30' 1 h 2 h 4 h 24 h
0 mM 2 mM 5 mM 7.5 mM 10 mM 15 mM 20 mM 25 mM 50 mM 75 mM 100 mM 150 mM
0 mM 150 mM 225 mM 300 mM 350 mM
G3116 DV92
Triticum
Arabidopsis
14-day stress
Arabidopsis
150 mM NaCl
Thellungiella
350 mM NaCl
Arabidopsis Thellungiella
7-day stress
Cold stress
(stress duration)
ZnO nanoparticles
(mg L-1)
Salt stress
(stress duration)
Salt stress
(mM NaCl)


Roots
CK/ABA
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 d 1 d 3 d 7 d 21 d 31 d 42 d
0 d 1 d 3 d 7 d 21 d 31 d 42 d
0 0.16 0.8 4 20 100
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0 mM 150 mM 225 mM 300 mM 350 mM
G3116 DV92
Triticum
Arabidopsis
14-day stress
Arabidopsis
150 mM NaCl
Thellungiella
350 mM NaCl
Arabidopsis Thellungiella
7-day stress
Cold stress
(stress duration)
ZnO nanoparticles
(mg L-1)
Salt stress
(stress duration)
Salt stress
(mM NaCl)

5 SUMMARY OF THE RESULTS

This doctoral thesis summarized the results from four studies focused on dynamics of phytohormone levels during stress responses. The roles of ABA and CKs during responses to salinity (Prerostova et al., 2017), drought (Prerostova et al., 2018), ZnO nanoparticle treatment (Vankova et al., 2017) and cold stress (Vankova et al., 2014) were discussed in detail.

The achieved results showed that ABA is the key hormone involved in the responses to all studied stresses. Stress-tolerant plants (halophyte *Thellungiella salsuginea* or the winter line G3116 of *Triticum monococcum*) had higher basal levels of ABA in shoot meristematic tissues (apices, crowns) than stress-sensitive plants. The meristematic tissue was also the most affected tissue by all stresses. Our results showed that stress-tolerant plants can faster and in more flexible way modulate ABA signalling and ABA content under stress conditions (by stimulation of synthesis or by activation of storage forms), which may enhance the ability of plants to withstand the stress.

The studied stresses caused a decrease of the active CK (mainly *trans*-zeatin) content, especially in the alarm phase or under severe stress conditions. CK down-regulation was associated with growth suppression. The drought experiment (Prerostova et al., 2018) revealed that higher level of active CKs helps plants to tolerate the stress, but discriminates these plants in the respect to the resistance to long-term stress. A higher level of CKs was shown to be very important for the recovery after stress release and for growth renewal. Up-regulation of the low active CK *cis*-zeatin during stress progression was detected in all studied stresses. The level of *cis*-zeatin was elevated mainly in roots. This hormone is synthesized in the cytosol (in contrast to *trans*-zeatin, which is synthesized in plastids), which means that plants prefer the CK synthesis through cytosolic pathway than the plastid pathway under stress conditions.

Both hormones play an important role in the regulation of stomata aperture, stabilization of photosynthetic apparatus, control of water relations, cell division and activation of defence mechanisms. ABA and CKs effects are antagonistic and the CK/ABA ratio seems to be a good indicator of stress duration.

This thesis showed the important roles of ABA and CKs during abiotic stresses connected with water deficiency. Based on the specific stress responses of leaves, roots and meristematic tissues, the importance of separate evaluation of these tissues was highlighted.

6 ZÁVĚRY PRÁCE

V rámci této doktorské práce byly shrnuty výsledky čtyř publikací zaměřených na dynamiku hladin fytohormonů během odpovědí na stresové podmínky. Role kyseliny abscisové a cytokininů v odpovědi na zasolení (Prerostova et al., 2017), sucho (Prerostova et al., 2018), kontaminaci ZnO nanočásticemi (Vankova et al., 2017) a chlad (Vankova et al., 2014) byly podrobně diskutovány.

Dosažené výsledky ukázaly, že kyselina abscisová je klíčovým hormonem, který je zapojen do odpovědí na všechny sledované stresy. Rostliny odolné vůči stresu (halofyt *Thellungiella salsuginea* nebo ozimý kultivar G3116 pšenice *Triticum monococcum*) měly v porovnání s citlivými odrůdami a druhy vyšší bazální hladinu kyseliny abscisové v meristématickém pletivu prýtu (vzrostné vrcholy, odnožovací uzly). Meristematické pletivo bylo také nejvíce zasaženo všemi zkoumanými druhy stresu. Naše výsledky ukázaly, že odolné rostliny umí v průběhu stresu rychleji a flexibilněji regulovat signální dráhu kyseliny abscisové a obsah kyseliny abscisové (podpořením syntézy nebo aktivací zásobních forem), což může zlepšit schopnost rostlin odolávat stresu.

Sledované stresy způsobily pokles obsahu aktivních cytokininů (zejména *trans*-zeatinu), a to hlavně během rané fáze stresu nebo v případě působení intenzivního stresu. Pokles obsahu cytokininů byl doprovázen potlačením růstu. Pokus zaměřený na sucho (Prerostova et al., 2018) ukázal, že vyšší obsah aktivních cytokininů pomáhá rostlinám odolávat stresu, ale znevýhodňuje je v případě dlouhotrvajícího stresu. Vyšší hladiny cytokininů se ukázaly být důležité pro rychlejší zotavení po stresu a pro obnovení růstu. V rámci všech studií bylo zjištěno navýšení hladiny *cis*-zeatinu, cytokininu s nízkou fyziologickou aktivitou, v průběhu stresu. Hladiny *cis*-zeatinu se zvedaly hlavně v kořenech. Syntéza tohoto hormonu probíhá v cytosolu (narozdíl od *trans*-zeatinu, který je tvořen v plastidech), z čehož plyne, že rostliny během stresu upřednostňují syntézu cytokininů cytosolickou dráhou spíše než plastidovou.

Oba hormony jsou důležité pro regulaci otevřenosti průduchů, stabilizaci fotosyntetického aparátu, pro kontrolu vodního režimu, buněčného dělení a pro aktivaci obranných mechanizmů. Kyselina abscisová a cytokininy působí antagonisticky a jejich poměr (CK/ABA) se zdá být dobrým indikátorem síly stresu.

Tato práce ukázala důležitou roli kyseliny abscisové a cytokininů v průběhu abiotických stresů spojených s nedostatkem vody. Vzhledem k sledovaným specifickým stresovým odpovědím v kořenech, listech a meristematických pletivech byla v rámci disertační práce také zdůrazněna důležitost vyhodnocovat tato pletiva jednotlivě.

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8 ATTACHMENTS

Published articles summarized in this Ph.D. thesis are attached to this part of the document. Four articles have been selected.

1st Article

Prerostova S., Dobrev P. I., Gaudinova A., Hosek P., Soudek P., Knirsch V., Vankova R. (2017): Hormonal dynamics during salt stress responses of salt-sensitive *Arabidopsis thaliana* and salt-tolerant *Thellungiella salsuginea*. *Plant Science*, 264, 188-198. (IF 3.437)

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This article was published in 2017 and I am the first author. I substantially participated in the preparation of hydroponics, the sampling of material, in the preparation of samples for hormonal analysis, in the measurement of RNA content by RT-qPCR, evaluation of the results and I prepared the manuscript.

Supplementary Figures are attached. Supplementary Table A.1 is available on-line.

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Hormonal dynamics during salt stress responses of salt-sensitive *Arabidopsis thaliana* and salt-tolerant *Thellungiella salsuginea*

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ABSTRACT

Salt stress responses in salt-sensitive *Arabidopsis thaliana* (2–150 mM NaCl) and the closely related salt-tolerant *Thellungiella salsuginea* (*Eutrema halophila,* 150–350 mM NaCl) were compared to identify hormonal and transcriptomic changes associated with enhanced stress tolerance. Phytohormone levels, expression of selected genes, membrane stability, and Na^+ and K^+ concentrations were measured in shoot apices, leaves, and roots. *Thellungiella* exhibited higher salt stress tolerance associated with elevated basal levels of abscisic acid and jasmonic acid, and lower levels of active cytokinins (excluding *cis*-zeatin) in shoot apices. Analysis of the dynamics of the early salt stress response (15 min to 24 h) revealed that the halophyte response was faster and stronger. Very mild stress, in our hydropony arrangement 2–25 mM NaCl, affected the transcription of genes involved in cytokinin metabolism (*AtIPTs*, *AtCKXs*). Mild stress induced in *Arabidopsis* (50 mM) stress responses only in shoot apices, while in *Thellungiella* (150 mM) across the whole plant. *Arabidopsis* exhibited in hydropony evidence of severe stress above 75 mM NaCl and died in 150 mM, whereas the halophyte only became severely stressed above 225 mM. The responses of individual phytohormones (cytokinins, auxin, abscisic acid, jasmonic acid, salicylic acid and their metabolites) to salinity are discussed.

1. Introduction

Salinization is an increasingly serious problem in modern agriculture due to irrigation with briny water and over-fertilization of high yield cultivars [1,2]. Approximately 30% of the irrigated land and 6% of the total land area are affected by soil salinity [3]. Salt-tolerant plants can grow and thrive in saline soils, helping to restore the productivity of salt-contaminated land. Consequently, there is great interest in understanding their salt tolerance mechanisms.

Considerable effort has been invested into characterization of the salt stress response of *Arabidopsis thaliana* (e.g. [4]). In addition, to identify characteristics associated with effective salt tolerance, comparative studies have been performed using the salt-sensitive *Arabidopsis thaliana* and its highly salt-tolerant relative *Thellungiella salsuginea* (*Eutrema halophila*) (e.g. [5,6]). These two species exhibit high nucleotide sequence identity (on the order of 92–95% across their genes) [5,7,8], enabling the use of a single microarray platform in such comparative analyses. By comparing transcriptome changes in the two species following salt stress, Gong et al. [9] identified a number of shared responses (ca. 40% transcripts) mainly in regulation of ribosomal functions, photosynthesis, cell growth, osmolyte accumulation and abscisic acid (ABA)-dependent pathway. Unique responses observed in *Thellungiella* involved stimulation of genes whose functions relate to protein folding, post-translational modifications and protein redistribution, which suggests the activation of energy saving response mechanisms. In contrast, *Arabidopsis* exhibited a general and rather energy-intensive defence response that required bulk protein synthesis.

Metabolite analysis of *Arabidopsis* and *Thellungiella* plants revealed that the halophyte contained higher levels of sugars, organic acids, and amino acids (especially proline) [9]. This was interpreted to mean that

Abbreviations: ABA, abscisic acid; AHK, *Arabidopsis* histidine kinase; ARR, *Arabidopsis* response regulator; CK, cytokinin; CKX, cytokinin oxidase/dehydrogenase; COR47, cold-regulated 47; cZ, *cis*-zeatin; DZ, dihydrozeatin; HKT, high-affinity K^+ transporter; IAA, indole-3-acetic acid; iP, isopentenyladenine; IPT, isopentenyl transferase; JA, jasmonic acid; MSI, membrane stability index; NCED3, 9-*cis*-epoxycarotenoid dioxygenase 3; OxIAA, 2-oxindole-3-acetic acid; OxIAA-GE, 2-oxindole-3-acetate glucosyl ester; PA, phaseic acid; PDF2, protein phosphatase 2A subunit A3; PSBO, photosystem II subunit O; RD26, responsive to desiccation 26; RD29B, responsive to desiccation 29B; RH, relative humidity; RWC, relative water content; SA, salicylic acid; TsHK, *Thellungiella salsuginea* histidine kinase; TsRR, *Thellungiella salsuginea response regulator*; tZ, *trans*-zeatin; UBQ10, ubiquitin 10

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Thellungiella exhibits better "stress-anticipatory preparedness". Proline, as one of the most common osmolytes, seems to play an important role in salinity responses because osmotic stress is one of the two major components of salt stress [10].

The other component of salinization is ion toxicity, which occurs when Na^+ or Cl^- accumulate in cytosol or chloroplasts. High Na^+ concentrations can displace Ca^{2+} from the plasma membrane or inhibit acquisition of K^+ [11]. Both *Arabidopsis* and *Thellungiella* accumulate Na^+ when exposed to salt stress [12]. However, enhanced stress tolerance in the halophytes is associated with comparatively low Na^+ levels (at a given environmental salt concentration) and higher K^+ levels. These responses are tissue-specific [13].

Phytohormones play central roles in modulating physiological responses and stimulating transcriptome changes in responses to challenging environmental conditions. The key hormone in the salinity response is ABA. The cross-talk between ABA, jasmonic acid (JA), and ethylene in *Thellungiella* has been studied recently [12,13]. In addition, the roles of growth-promoting hormones, i.e. auxins and cytokinins (CKs), in salt stress responses have been studied in *Arabidopsis* [14–16] and tomato plants [17]. Surprisingly, plant stress tolerance is reportedly enhanced by both increased CK levels resulting from over-expression of the biosynthetic gene *isopentenyl transferase* [18], and decreased CK levels caused by over-expression of the CK degradation gene *cytokinin oxidase/dehydrogenase* [14].

This work presents a comprehensive analysis of phytohormonal dynamics in the salt-sensitive *Arabidopsis thaliana* and salt-tolerant *Thellungiella salsuginea* over a wide range of salt concentrations, and a description of the hormonal changes occurring in both species during the early stages of severe salt stress. The results obtained are supported by RT-qPCR expression analyses of the key phytohormone-related genes. The identified hormonal changes were correlated with changes in the plant physiological state. In addition, because of the crucial role of shoot meristematic tissue in stress responses, responses in the shoot apex were studied separately to complement measurements on leaves and roots.

2. Materials and methods

2.1. Plant material and stress application

Arabidopsis thaliana (Col-0) and *Thellungiella salsuginea* (Shandong) were grown hydroponically using modified Hoagland medium [19]. *Arabidopsis* plants were cultivated in climate chambers (SANYO MLR 350H) at 20 °C, 75% RH, 8/16 h light/dark, under an optimal light intensity of 150 μmol m^{-2} s^{-1}. *Thellungiella* plants were grown in a PGC20 FLEX chamber (Conviron), at 20 °C, 75% RH, 8/16 h light/dark. In accordance with previous reports [20,21], the slow-growing *Thellungiella* was cultivated at an optimal light intensity of 230 μmol m^{-2} s^{-1}. Short-day conditions were chosen to maintain the plants in the vegetative state and thereby avoid the hormonal changes associated with the onset of the vegetative-generative transition. To ensure that the species were compared at the same developmental stage, *Arabidopsis* and *Thellungiella* were exposed to salt stress 28 and 35 days after sowing, respectively, at which points both species had achieved similar rosette sizes. *Arabidopsis* was grown in salt concentrations of 0, 2, 5, 7.5, 10, 15, 20, 25, 50, 75, 100 and 150 mM NaCl, while *Thellungiella* was grown in salt concentrations of 0, 150, 225, 300 and 350 mM NaCl. Shoot apex, leaf (the 8th–12th leaves from ca. 20), and root samples were collected after 1 week. Time-course experiments were performed by sampling at 0, 15 min, 30 min, 1 h, 2 h, 4 h and 24 h of severe stress (corresponding to treatment with 150 mM NaCl for *Arabidopsis* or 350 mM NaCl for *Thellungiella*). Three independent biological experiments were performed, and at least three replicate samples were collected in each experiment. The samples were immediately frozen in liquid nitrogen. All samples were taken between 1 and 2 p.m. to avoid diurnal variation.

2.2. Relative water content (RWC)

Three middle leaves from each experiment were weighed (fresh weight–FW), saturated with water for 1 day (turgid weight–TW), and dried overnight at 70 °C (dry weight–DW). The relative water content (RWC) was then calculated as RWC (%) = [(FW − DW)/(TW − DW)] × 100.

2.3. Membrane stability index (MSI)

The membrane stability index (MSI) was estimated according to Tripathy et al. [22]. Three middle leaves from each experiment were incubated 24 h in double-distilled water at 4 °C in darkness, after which their electrical conductivity (C1) was measured using a conductivity meter 4520 (Jenway). The samples were then boiled in a water bath for 20 min and their electrical conductivity was measured again (C2). The MSI was calculated as MSI (%) = [1 − (C1/C2)] × 100.

2.4. Na^+ and K^+ determination

Shoot and root samples (ca 0.25 g DW) from two independent experiments were solubilized according to Soudek et al. [23] in HNO_3/$HClO_4$ solution (7:1) and mineralized by microwaves (Multi-wave PRO, Anthon Paar GmbH). The concentration of Na^+ and K^+ was measured in an air-acetylene flame using a SensAA atomic absorption spectrometer (GBC) with GBS Avanta software 2.02 [24]. Absorbance was measured at 589.0 and 404.3 nm, respectively. Na^+ and K^+ concentrations were calculated from calibration curves.

2.5. Phytohormone analyses

Samples were purified and analysed as described earlier [25,26]. Frozen samples (ca 20 mg) were homogenised and extracted with cold (−20 °C) methanol/water/formic acid (15/4/1 v/v/v). The following isotope-labelled internal standards (10 pmol/sample) were then added: $^{13}C_6$-IAA (Cambridge Isotope Laboratories); $^2H_4$-SA (Sigma-Aldrich); $^2H_3$-PA, $^2H_3$-DPA (NRC-PBI); $^2H_6$-ABA, $^2H_5$-JA, $^2H_5$-transZ, $^2H_5$-transZR, $^2H_5$-transZ7G, $^2H_5$-transZ9G, $^2H_5$-transZOG, $^2H_5$-transZROG, $^2H_5$-transZRMP, $^2H_3$-DHZ, $^2H_3$-DHZR, $^2H_3$-DHZ9G, $^2H_6$-iP, $^2H_6$-iPR, $^2H_6$-iP7G, $^2H_6$-iP9G, $^2H_6$-iPRMP (Olchemim). Two hormone fractions were extracted with a reverse phase–cation exchange SPE column (Oasis-MCX, Waters): the acid fraction was eluted with methanol (auxins, ABA, SA, JA), and the second fraction was eluted with 0.35 M NH_4OH in 60% methanol (CKs). Fractions were analysed using HPLC (Ultimate 3000, Dionex) coupled to a 3200 Q TRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems). Hormone quantification was performed by the isotope dilution method with multilevel calibration curves ($r^2 > 0.99$). Data processing was performed with the Analyst 1.5 software package (Applied Biosystems).

2.6. Quantitative RT-PCR

Frozen material (ca 100 mg FW) was pulverised in liquid nitrogen. Total RNA was extracted using the RNeasy Plant Kit (Qiagen) and treated with the Ambion DNA-free kit (Thermo Fisher Scientific Inc.). Reverse transcription was done with the M-MLV Reverse Transcriptase (RNase H Minus, Point Mutant, Promega), oligo-dT primers, and Protector RNase Inhibitor (Roche Applied Science). qPCR was performed using a LightCycler 480 DNA SYBR Green I Master or a LightCycler 480 Probes Master with UPL Probes (Roche Applied Science). Primer sequences and PCR conditions are shown in Table A.1. qPCR amplification was monitored using a Light Cycler 480 (Roche Applied Science).

Relative RNA levels were calculated as described by Hellemans et al. [27]. Transcript levels were normalised to *AtUBQ10* (*At4g05320*) and *AtPDF2* (*At1g13320*) in the case of *Arabidopsis*, and to *TsUBQ10*

(AK353089) and *TsPDF2 (XM_006417069)* in the case of *Thellungiella*. The invariant levels of *UBQ10* and *PDF2* transcripts among the tested tissues under salt stress conditions were selected from microarray data [28].

Gene sequences were obtained from TAIR [29] and the NCBI [30]. Primers were designed using Primer3Plus [31]. The quality of primer pairs was verified by AlleleID (PREMIER Biosoft; [32]) and the probability of folding secondary structures was predicted with mfold [33].

2.7. Statistical analysis

Normalized gene expression data and data from hormone analyses were standardised using the equation: $y_{ij} = (x_{ij} - \bar{x}_j)/s_j$. Here, y_{ij} is the standardized value of x_{ij}, $\bar{x}_j$ is the mean value of x_j, and s_j is the standard deviation of x_j. Standardized data or data from MSI, RWC and rosette measurements were evaluated by the Mann-Whitney *U* test with the Bonferroni correction in the program PAST 3.01 [34].

3. Results

3.1. Arabidopsis vs. Thellungiella – impact of salt stress on plant physiology

To identify characteristic features associated with salt stress tolerance, the response of the salt-sensitive *Arabidopsis thaliana* was compared to that of the halophyte *Thellungiella salsuginea*. The response of *Arabidopsis* to NaCl exposure was monitored over 7 days at salt concentrations of 2–150 mM. No significant changes in variables such as rosette growth were observed at concentrations up to 25 mM NaCl (Fig. A.1A). Above 75 mM NaCl, the plant leaves became dark green and their relative water content (RWC; Fig. A.2 A) fell. Early leaf senescence was observed after 6, 3, and 2 days at NaCl concentrations of 75, 100 and 150 mM, respectively. After 7 days in 150 mM NaCl, the shoot apex and young leaves of *Arabidopsis* were dead, and only older leaves remained viable (Fig. 1A). The RWC and membrane stability index (MSI) both dropped significantly in middle leaves after 7 days at 100 and 150 mM NaCl (Figs. A.2A, A.3A).

In contrast, *Thellungiella* plants exposed to 150 mM NaCl exhibited no detectable differences from controls. At NaCl concentrations of 225, 300 and 350 mM NaCl, *Thellungiella* exhibited dark leaves with violet shade and decreased RWC (Fig. A.2B). However, its rosette diameter did not decrease in parallel with the salt concentration (Fig. A.1B). At 350 mM NaCl, *Thellungiella* preferentially protected shoot apices and young leaves, which remained alive while the older leaves entered early senescence (Fig. 1B). There was also a small but statistically significant difference in MSI between stressed and control *Thellungiella* plants (Fig. A.3B).

The dynamics of the changes in RWC and MSI upon treatment with high salt concentrations (150 mM NaCl in the case of *Arabidopsis*, and 350 mM in the case of *Thellungiella*) were relatively similar in both species. The RWC fell to 70% (from the control level of 80%) after 4 h, then to ca. 55% after 24 h, and remained at the latter level over the following six days (Fig. A.2). *Arabidopsis* also exhibited a small reduction in MSI after 24 h (Fig. A.3A).

3.2. Na^+ and K^+ content

After one week, the levels of accumulated Na^+ in *Arabidopsis* (which were slightly higher in roots than in shoots) were proportional to the salt concentrations in the media up to 75 mM (Fig. 2A). Plants treated with 100 mM NaCl had 2.5 times more Na^+ in their shoots than in their roots. *Thellungiella* exposed to 150 mM NaCl had higher levels of Na^+ in their shoots than in their roots, but the total Na^+ content was half that in *Arabidopsis* treated with 100 mM NaCl (Fig. 2B). The Na^+ content of shoots grew linearly with the NaCl concentration in the media, but in

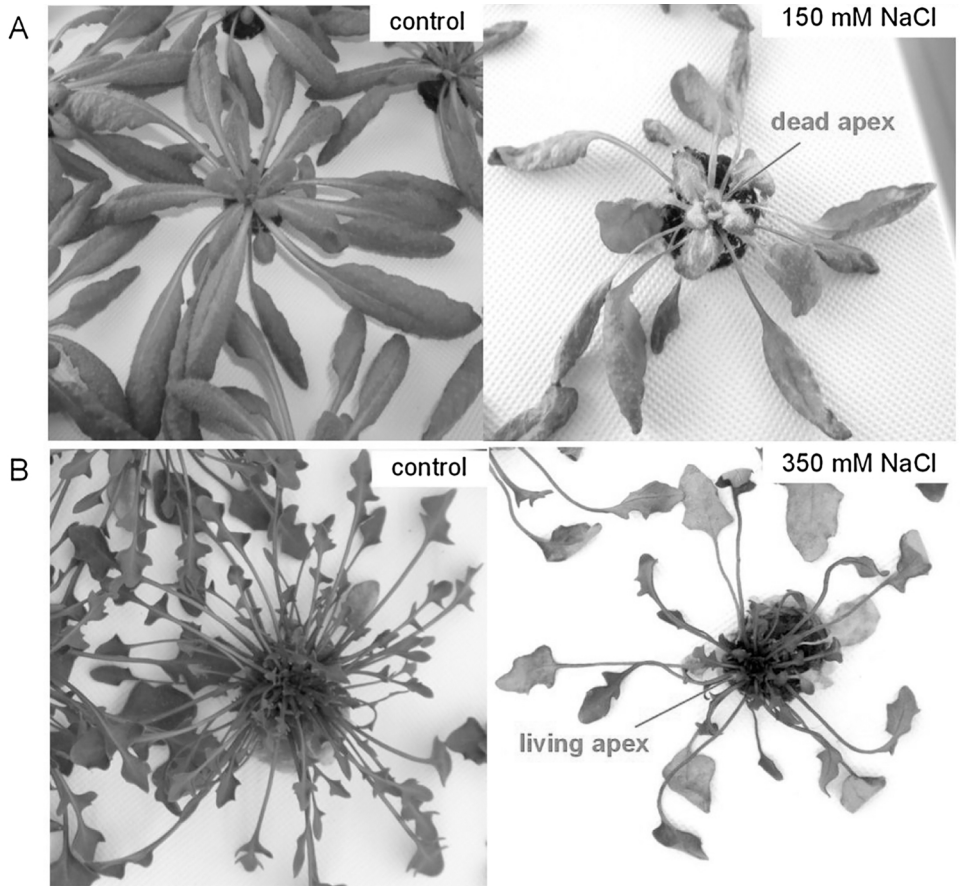

Fig. 1. The effect of one-week severe salt stress on (A) salt-sensitive *Arabidopsis thaliana* (150 mM NaCl) and (B) salt-tolerant *Thellungiella salsuginea* (350 mM NaCl).

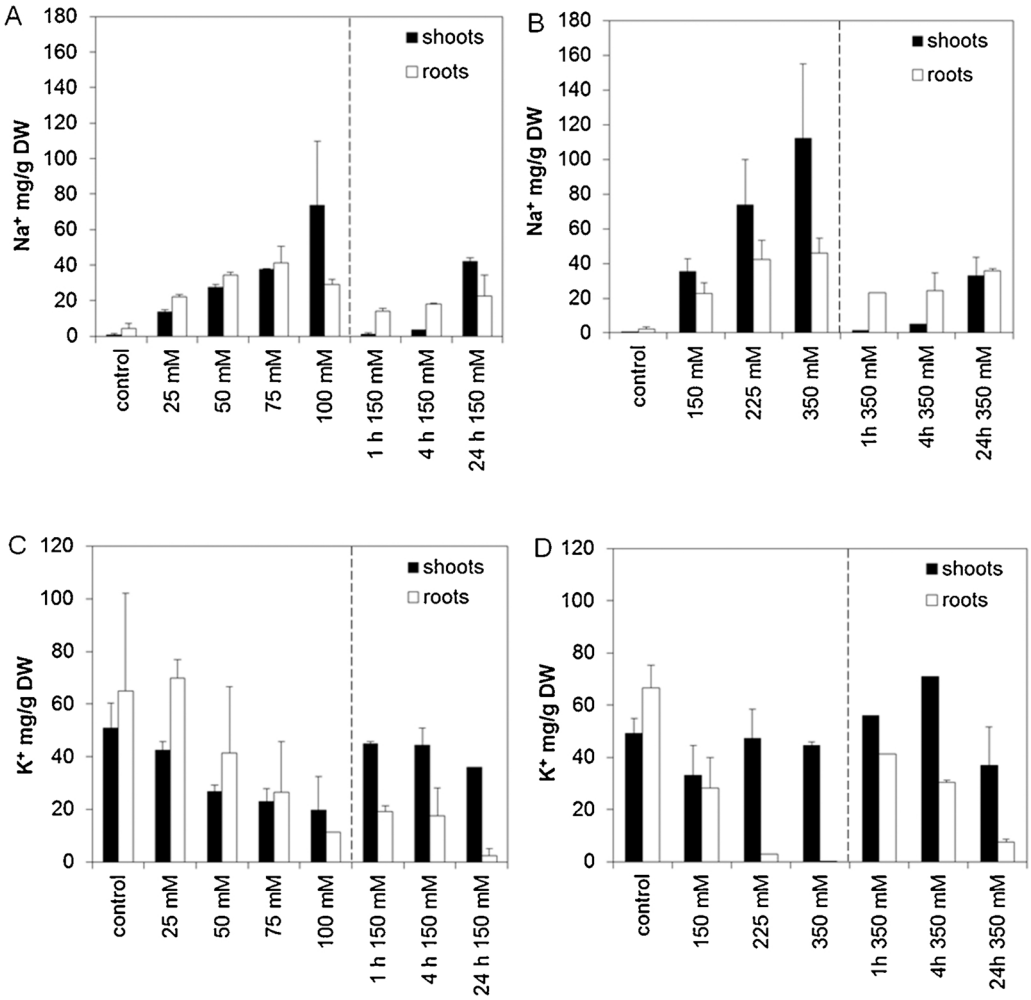

Fig. 2. Na^+ and K^+ content. Levels of Na^+ in shoots and roots of (A) *Arabidopsis* or (B) *Thellungiella*; and levels of K^+ in shoots and roots of (C) *Arabidopsis* or (D) *Thellungiella* exposed to different salt concentrations for 1 week or after exposure to high NaCl concentrations (150 or 350 mM) for 1, 4 and 24 h.

roots it stopped increasing at ca. 40–45 mg g^{-1} DW.

The basal K^+ concentration was similar in both species. One week of salt stress caused a gradual decline of the K^+ content in *Arabidopsis*, particularly in roots (Fig. 2C). *Thellungiella* plants had almost no K^+ in their roots when the external NaCl concentration was above 225 mM NaCl (Fig. 2D). However, the K^+ concentration in their shoots stabilised at 45 mg g^{-1} DW at higher NaCl concentrations.

The time course of Na^+ uptake over the first 24 h of severe salt stress was similar in both species. Within 24 h, the Na^+ concentration increased rapidly in shoots (Fig. 2A, B). The K^+ levels in roots started falling within 1 h (Fig. 2C, D). Interestingly, the Na^+ content rose rapidly in shoots, but the K^+ concentration decreased to much greater extent in roots.

3.3. Stress-related genes

Stress marker genes (*AtRD26*, *AtRD29B, AtCOR47*) were selected by comparing microarray data for *Arabidopsis* and *Thellungiella* [5,9,35,36]. The gene that exhibited the strongest transcriptional response to salt stress in *Arabidopsis* was *AtRD26,* which was highly upregulated in shoot apices at NaCl concentrations above 50 mM (Fig. 3A). Similarly, *AtRD29 B* was upregulated in apices above 10 mM NaCl. Both genes were strongly upregulated in leaves exposed to 100–150 mM NaCl. No stimulation of *AtCOR47* was observed.

The early response of *Arabidopsis* to 150 mM NaCl involved a transient increase in *AtRD26* expression in leaves that began within 15 min of treatment and peaked at 30 min. In addition, a weak increase in *AtRD29B* transcription was detected after 1 h. *AtRD26* and *AtRD29B* transcript levels increased in shoots after 4 and 24 h, and *AtRD26* was also up-regulated in roots between 15 min and 24 h (Fig. 3C).

The salt stress response of *Thellungiella* was much stronger than that in *Arabidopsis*. *TsRD29B* expression was strongly upregulated throughout the plant at external NaCl concentrations above 225 mM, and at concentrations above 150 mM in roots (Fig. 3B). *TsCOR47* also responded strongly in shoots, as did *TsRD26* in roots. Up-regulation of *TsRD26* was detected within 15 min of the start of the salt treatment in shoot apices and roots (Fig. 3D); *TsRD29 B* expression increased after 30 min in roots and after 4 h in shoots. Similar, but less profound, profile was exhibited by *TsCOR47*.

The *High-affinity K^+ transporter 1;1* (*AtHKT1;1*) had high basal level of expression in *Arabidopsis* roots. Its transcription was moderately stimulated in leaves by 2–20 mM NaCl, but it was suppressed in apices exposed to salinities of 50 mM and above (Fig. 3A). Treatment with 150 mM NaCl caused *AtHKT1;1* transcription to decrease throughout the plant by a factor of around 20 between 4 h and 24 h after the start of treatment (Fig. 3C).

In *Thellungiella*, *TsHKT1;2* was the most salt-sensitive transporter [37]. It was most strongly transcribed in apices. Salt stress (225–350 mM NaCl) caused up-regulation of *TsHKT1;2* transcription in shoots (Fig. 3B). During the early response, *TsHKT1;2* expression decreased in the apices and increased transiently in leaves and roots.

3.4. Hormone responses

3.4.1. Abscisic acid

Seven-day exposure of *Arabidopsis* to 7.5–50 mM NaCl more than

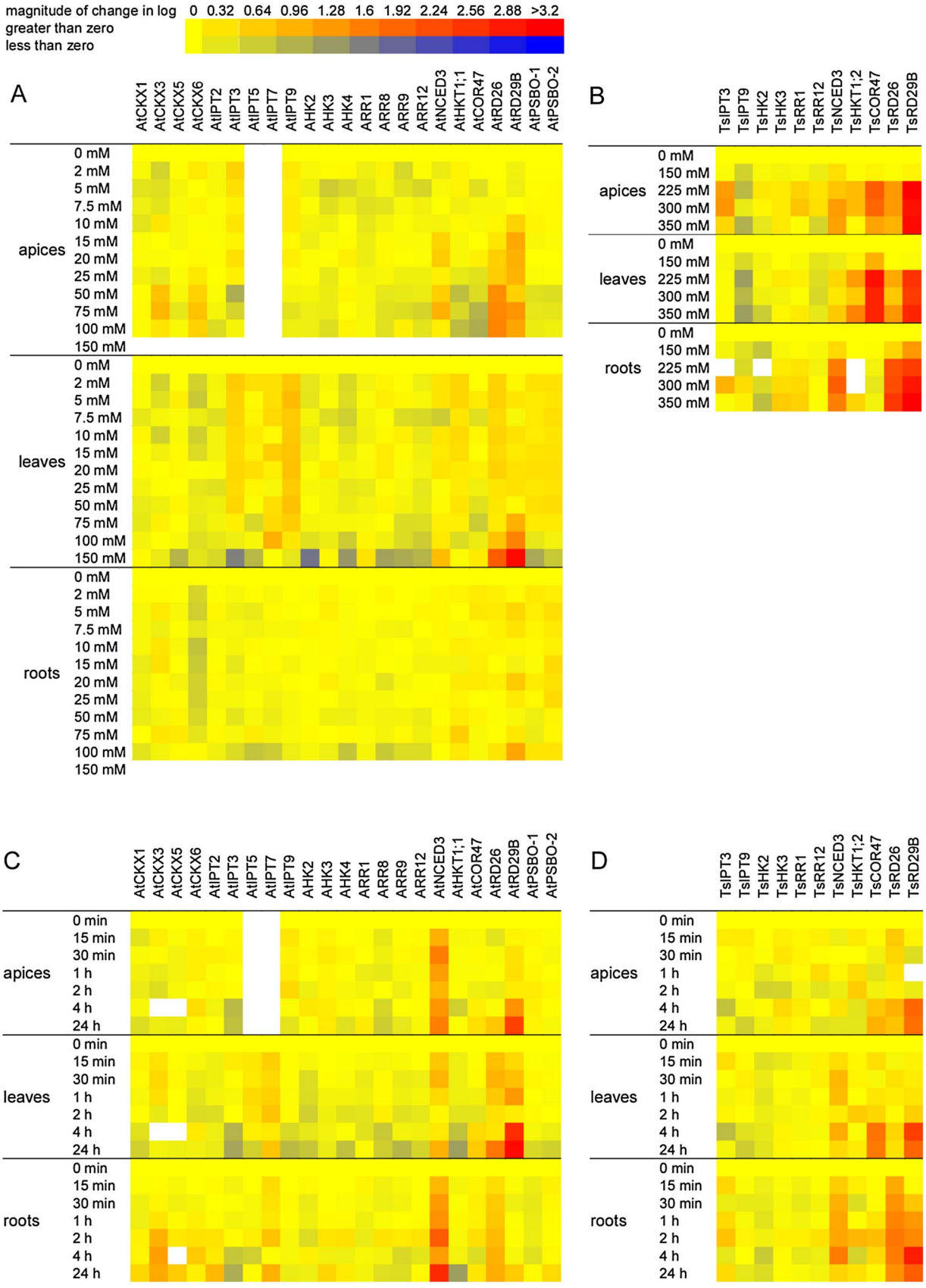

Fig. 3. Heatmaps showing the transcription levels of selected genes in apices, leaves and roots of (A) *Arabidopsis* and (B) *Thellungiella* exposed to different salt concentrations for 1 week; and (C) *Arabidopsis* or (D) *Thellungiella* exposed to high NaCl concentrations (150 or 350 mM, respectively) for 24 h. Relative values from RT-qPCR experiments were normalised separately for each plant tissue against results for control conditions. Logarithmic values were converted into heatmaps using the BAR HeatMapper Plus Tool (http://bar.utoronto.ca).

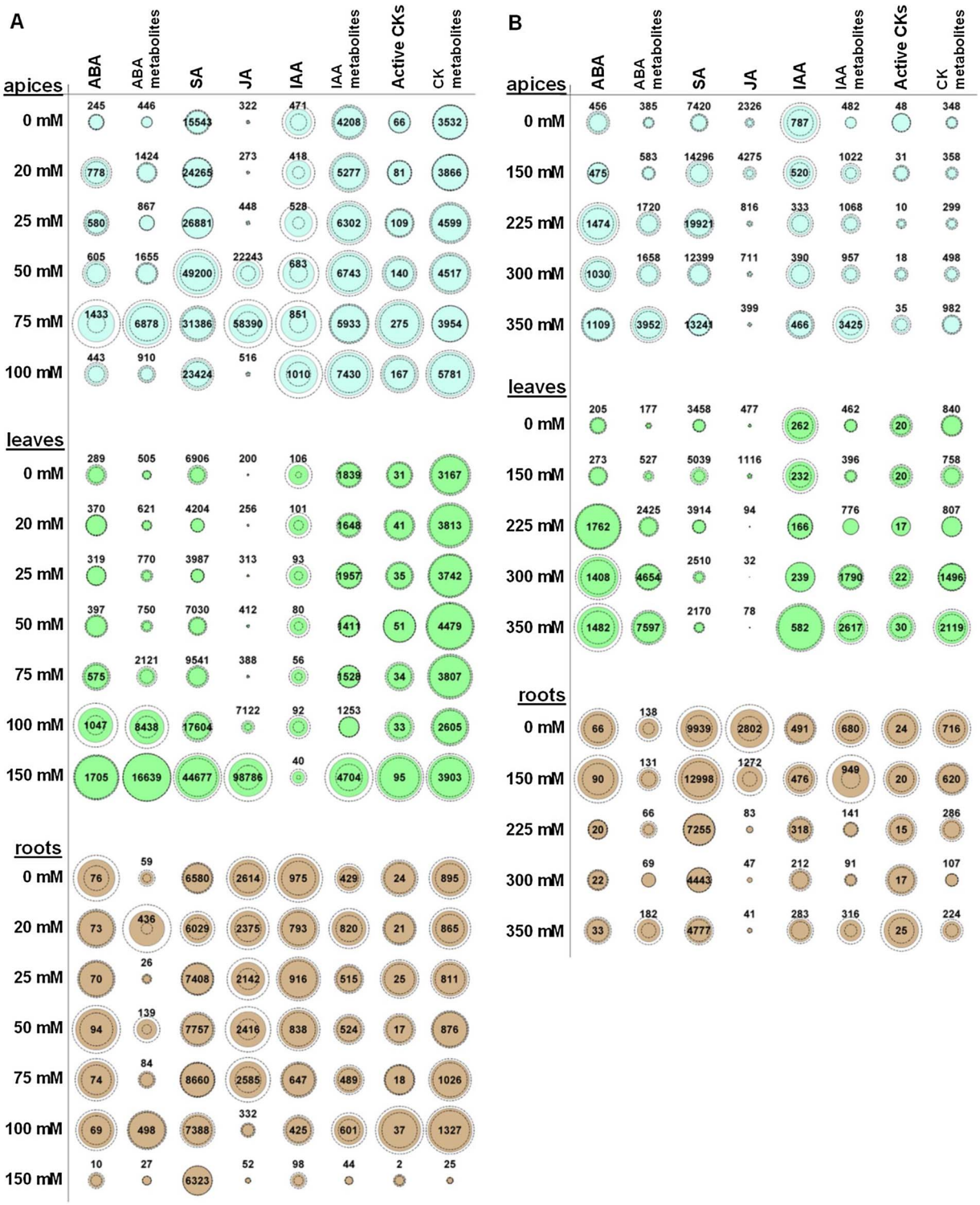

Fig. 4. Contents of phytohormones in apices, leaves and roots of (A) *Arabidopsis* and (B) *Thellungiella* exposed to different salt concentrations for 1 week; and (C) *Arabidopsis* or (D) *Thellungiella* exposed to high NaCl concentrations (150 or 350 mM, respectively) for 24 h. The plots represent mean phytohormone concentrations (pmol g^{-1} DW) as circles, with larger circles corresponding to higher concentrations. Dashed lines indicate standard deviations. Circle areas are normalised for each hormone-tissue combination together in both species. Independent normalization was performed within the concentration-scale (A, B) or time-scale (C, D). The plot was created in MATLAB 8.0 (The MathWorks Inc.). ABA: abscisic acid; ABA metabolites: dihydrophaseic acid, phaseic acid, neophaseic acid, abscisic acid glucosyl ester, 9′-hydroxyabscisic acid; IAA: indole-3-acetic acid; IAA metabolites: indole-3-acetyl aspartate, 2-oxindole-3-acetic acid, 2-oxindole-3-acetic acid glucosyl ester, indole-3-acetyl-1-glucosyl ester; JA: jasmonic acid; SA: salicylic acid; Active CKs: *trans*-zeatin, dihydrozeatin, isopentenyladenine, *cis*-zeatin; CK metabolites: CK ribosides, CK phosphates, CK O- and N-glucosides (described in detail in Supplementary Figures A.7–A.10).

doubled the levels of ABA in shoot apices, with further profound increase in 75 mM NaCl (Fig. 4A). Substantial increase in ABA levels was also detected in leaves of plants treated with 75, 100, and 150 mM NaCl (2, 3.5, and 5.8-times more than controls, respectively). The ABA levels in roots remained low throughout. The levels of ABA metabolites – mainly neophaseic acid and phaseic acid (PA) – increased strongly in apices of plants treated with 75 mM NaCl and leaves treated with 100 and 150 mM NaCl (Fig. A.4).

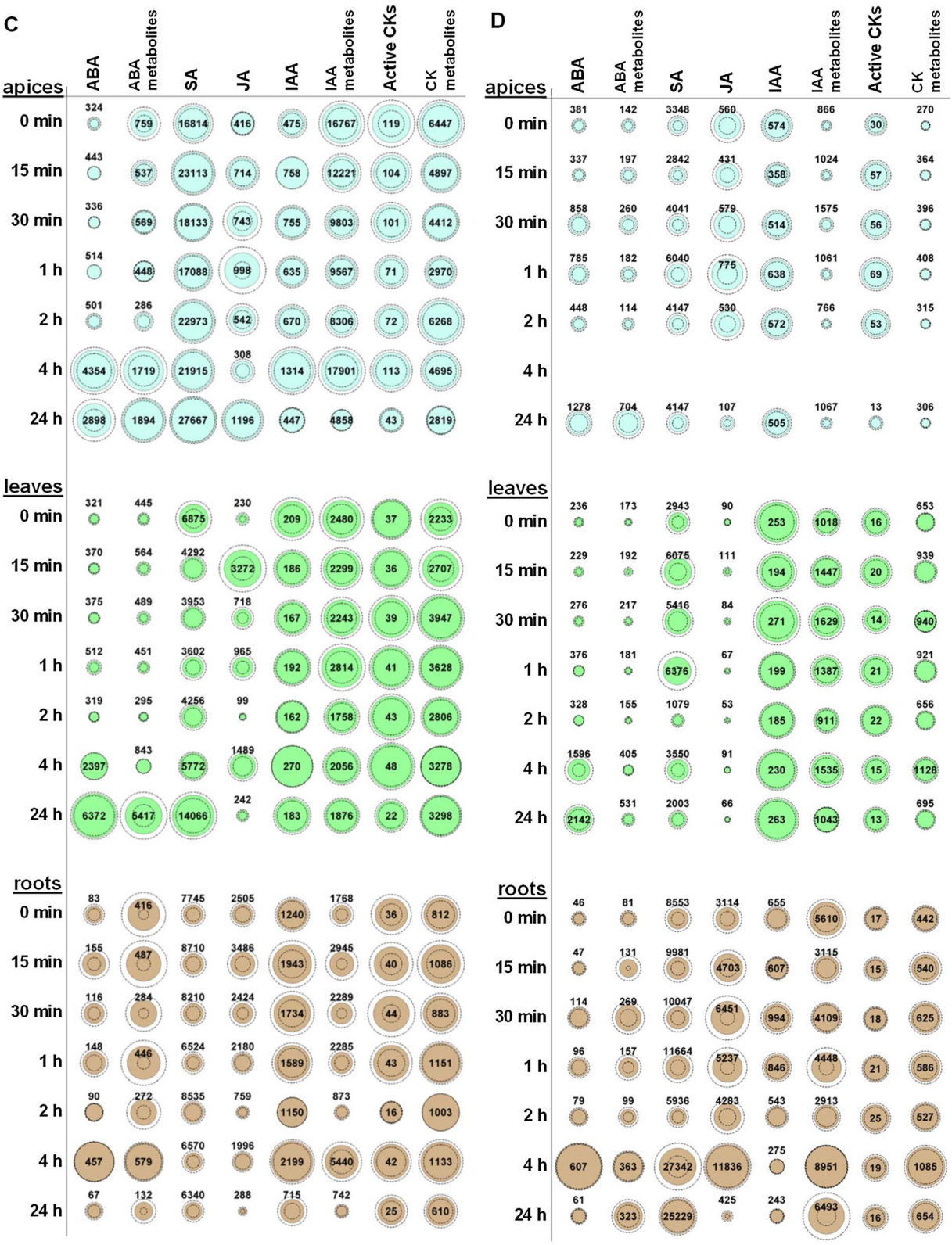

Fig. 4. (*continued*)

Severe salt stress (150 mM NaCl) caused the ABA levels to increase moderately within 15 min in *Arabidopsis* roots, and within 1 h in leaves and apices (Fig. 4C). This was followed by more pronounced increase across the aboveground parts of the plant that began after 4 h and continued for 24 h in apices and leaves (leading to 7-fold and 20-fold increase in concentration relative to the basal levels, respectively). In roots, ABA fell to the control level. The transcription of the rate limiting gene of ABA biosynthesis *AtNCED3* was upregulated within 15 min, with transient maxima after 2 and 24 h in roots, and after 30 min and 24 h in apices (Fig. 3C). Its upregulation in leaves was much less pronounced. ABA metabolites (mainly PA) were elevated in apices after 4–24 h and strongly in leaves after 24 h (Fig. A.4).

In *Thellungiella*, the levels of ABA in shoot apices were higher than those in *Arabidopsis*. Week-long saline treatments (225–350 mM NaCl) caused ABA levels to rise in apices (2.5-fold) and leaves (7-fold), but to fall significantly in roots (Fig. 4B). ABA metabolites followed the same trend as the active hormone (Fig. A.4). *TsNCED3* transcription was stimulated at salt concentrations of 225 mM and above – strongly in roots (10 times more than control), less so in apices, and only slightly in leaves (Fig. 3D).

Severe salt stress (350 mM NaCl) caused ABA levels to rise after 30–60 min in roots and apices (Fig. 4B), peaking after 4 h in roots and 24 h in apices (Fig. 4D). In leaves, a moderate increase was detected after 1–2 h and the increase continued gradually until 24 h. ABA metabolites (especially PA and ABA-glucosyl ester) increased after 4–24 h. *TsNCED3* expression was stimulated after 15 min of stress, predominantly in roots and somewhat less in leaves (Fig. 3D).

3.4.2. Salicylic acid and jasmonic acid

Arabidopsis plants exposed to NaCl concentrations above 50 mM for 7 days exhibited elevated levels of SA in shoots and JA in apices (Fig. 4A). Salt concentrations above 100 mM caused JA levels to increase sharply in leaves and decline in roots.

Severe salt stress (150 mM NaCl) caused SA levels to increase in *Arabidopsis* shoots after 24 h (Fig. 4C). JA was transiently upregulated in shoots between 15 and 60 min, and after 4 h in leaves. After 24 h, JA levels fell sharply in leaves and roots (Fig. 4C). The dynamics of JA and of its active conjugate, jasmonate-isoleucine were similar (Fig. A.11).

In *Thellungiella,* salt exposure increased SA levels in apices. In roots and leaves, levels of this hormone were higher than in controls at a salt concentration of 150 mM NaCl (Fig. 4B). Similarly, the content of JA in shoots was greater than in controls at a salt concentration of 150 mM NaCl, while JA levels decreased gradually in roots. Severe salt stress (350 mM NaCl) caused a transient increase in SA levels in *Thellungiella* leaves between 15 and 60 min (Fig. 4D), while the levels of SA and JA in the roots increased after 4 h. In the case of SA, these elevated levels remained 24 h after the start of the treatment.

3.4.3. Auxins

Week-long salt stress caused a progressive decrease in the levels of indole-3-acetic acid (IAA) in *Arabidopsis* leaves and roots treated with up to 50 mM NaCl (Fig. 4A). Higher levels of salt stress caused a non-significant increase in IAA levels in shoot apices. Major auxin metabolites detected in the salt-stressed plants were 2-oxindole-3-acetate glucosyl ester (OxIAA-GE) and 2-oxindole-3-acetic acid (OxIAA), which were especially abundant in shoot apices (Fig. A.5). The inactive conjugate indole-3-acetyl aspartate was also detected in apices at NaCl concentrations above 50 mM. Severe salt stress (150 mM NaCl) caused the IAA content in *Arabidopsis* apices and roots to fall over 24 h, but IAA levels in leaves were unaffected (Fig. 4C). Similar trends were observed for IAA degradation metabolites, primarily OxIAA and OxIAA-GE (Fig. A.5).

The basal IAA level in *Thellungiella* apices was slightly higher than in *Arabidopsis*, but that in roots was lower. Week-long treatment with 225–350 mM NaCl caused IAA levels to fall in apices and roots of *Thellungiella* (Fig. 4B), and severe stress (350 mM NaCl) caused IAA levels in leaves to increase moderately (Fig. 4B). The main IAA metabolites detected in *Thellungiella* were indole-3-acetyl aspartate and OxIAA (Fig. A.5). Levels of IAA metabolites in the leaves increased at 300 and 350 mM NaCl, and in apices at the latter concentration. Severe salt stress (350 mM NaCl) caused IAA levels to decrease slightly between 4 and 24 h; this change was accompanied by an increase in the levels of IAA metabolites (Fig. 4D).

3.4.4. Cytokinins

Because there have been contradictory reports on the role of CKs in salt stress responses (e.g. [18]), we combined CK metabolite analysis with determination of the expression of selected CK-related genes to clarify their functions. In *Arabidopsis*, active CKs [*trans*-zeatin (tZ), *cis*-zeatin (cZ), dihydrozeatin (DZ) and isopentenytladenine (iP)] and their ribosides were primarily detected in apices (Figs. 4 A, A.6, A.7). No significant changes in active CK levels were detected at low salt concentrations (2–75 mM NaCl) even though the CK biosynthetic gene *AtIPT3* was slightly stimulated in leaves and the CK-degrading cytokinin oxidase/dehydrogenases (*AtCKX1, AtCKX3* and *AtCKX6*) were slightly down-regulated throughout the plants. Week-long treatment with NaCl concentrations above 75 mM significantly reduced tZ levels in apices. The levels of active CKs in leaves only changed significantly relative to controls at salt concentrations of 100 mM or above, while the levels of active CKs and CK ribosides in roots fell significantly at NaCl concentration 150 mM. The content of cZ (and its riboside) increased strongly in apices at 50 mM, and especially at 75 mM NaCl; in leaves, a similar increase was observed only at 150 mM NaCl. Levels of CK precursors (CK phosphates) and CK O-glucosides fell at NaCl concentrations of 75 mM and above, and were most pronounced at 150 mM NaCl (Figs. A.8, A.9). Levels of CK N-glucosides in apices increased significantly at 100 mM NaCl (Fig. A.10). High salt concentrations (100–150 mM) suppressed the expression of *AtIPT3* in leaves and roots, and that of *AtIPT7* in roots. Transcription of *ARR12,* the CK type-B response regulator, was inhibited throughout the plant at NaCl concentrations of 75 mM and above. The type-A response regulators *ARR8* and *ARR9* were inhibited in roots above 100 mM and in leaves at 150 mM NaCl.

Severe salt stress (150 mM) reduced the levels of active CKs throughout *Arabidopsis* plants over 24 h (Figs. 4C, A.6), as well as those of CK ribosides and phosphates (Figs. A.7, A.8). The early response (15 min to 2 h) involved upregulation of *AtIPT9* transcription in apices and *AtIPT7* in leaves and roots. After 2 h, *AtCKX3* and *AtCKX5* were upregulated in roots, along with *AtCKX6* after 4 h (Fig. 3C). Between 4 and 24 h, *AtIPT3* was strongly suppressed throughout the plants. Transcription of the CK receptors *AHK2* and *AHK4* was diminished in leaves after 24 h. *ARR1* transcription was downregulated between 30 min and 4 h in shoots. Transcription of *ARR8* and *ARR9* was transiently up-regulated in roots after 1 h, that of *ARR8* also in leaves. After 4 h, their transcription was diminished throughout the plants.

The basal levels of active CKs in *Thellungiella* shoots were around half those in *Arabidopsis* (Figs. 4 B, A.6). In addition, the relatively low CK N-glucoside levels in *Thellungiella* apices seemed to be related to their low levels of active CKs (Fig. A.10). Whole-plant levels of CK precursors in *Thellungiella* were around an order of magnitude lower than in *Arabidopsis* (Fig. A.8). Simultaneously, the expression of *TsIPT3* in roots was around half that of *AtIPT3*. Interestingly, however, *Thellungiella* had a higher level of cZ than *Arabidopsis* (Fig. A.6). Week-long heavy salt stress (225–350 mM NaCl) reduced the levels of tZ, its riboside, tZ phosphate, and CK O-glucosides, while increasing those of cZ, its riboside and isopentenyladenosine phosphate throughout the plant (Figs. A.6–A.9). Simultaneously, transcription of *TsIPT3* increased strongly in apices (Fig. 3B) while that of *TsIPT9* decreased in shoots. Treatment with 350 mM NaCl for one week also reduced the transcription of the CK receptor *TsHK2* across the whole plant. *TsHK3* and *TsRR1* transcript levels increased gradually in roots at NaCl concentrations of 225 mM and above. Interestingly, *TsHK3* expression was almost identical in all tissues, whereas *AHK3* was expressed most strongly in leaves, around 50% lower in apices, and around 33% lower in leaves.

Severe salt stress (350 mM NaCl) caused a transient increase in the levels of active CKs in *Thellungiella* apices and roots between 1 and 2 h after the start of treatment (Figs. 4 D, A.6), which was preceded by transient up-regulation of *TsIPT3* expression in roots (after 15 min − 2 h). *TsIPT3* and *TsIPT9* expression were downregulated in shoots after 4 h and 24 h, respectively (Fig. 3D). Down-regulation of *TsHK2* started within 15 min of stress treatment (and persisted for 24 h) in leaves and roots, but was only observed between 1 and 2 h after the start of treatment in apices.

4. Discussion

4.1. Differences between Arabidopsis and Thellungiella under control conditions

A comparative analysis of the *Arabidopsis* and *Thellungiella* transcriptomes showed that they share most of their salt tolerance genes but differ strongly in their expression patterns [5]. *Thellungiella* constitutively overexpresses many genes that are stress-inducible in *Arabidopsis* [5,9], including those associated with the ABA biosynthetic pathway. In accordance with the transcriptome analyses, under control conditions we observed moderately elevated (relative to *Arabidopsis*) levels of ABA and JA in *Thellungiella* shoot apices, the meristematic tissue crucial for plant survival. This is consistent with the results of Taji et al. [5], who found mildly enhanced ABA levels in *Thellungiella*. Other studies reported that the basal levels of ABA (and JA) in *Thellungiella* leaves and roots are similar to those in *Arabidopsis* [13]. This is consistent with our analysis, which revealed comparable or even slightly lower levels of ABA in the halophyte leaves and roots. The basal level of JA was moderately elevated in *Thellungiella* leaves, and also in roots (where the basal level of SA was also slightly higher than in *Arabidopsis*). *Thellungiella* shoots had significantly higher auxin (IAA) levels than their *Arabidopsis* counterparts, which is reflected in their enhanced leaf serration [38]. However, levels of CKs were lower, especially in shoots. The most physiologically active CK in terms of stimulating cell division, tZ, was less abundant in *Thellungiella*, while the less-active cZ was more abundant. Consequently, *Thellungiella* plants grew more slowly while maintaining higher basal levels of protective compounds and the corresponding transcripts (e.g. *TsCOR47*).

4.2. Salt concentration range dependence

The effects of week-long salt exposure depended on the NaCl concentration in the hydroponic system. Four distinct effect classes were defined: 1) no visible or significant metabolic changes (*Arabidopsis* in 2–25 mM NaCl); 2) moderate changes in growth, hormone levels, or stress-marker gene expression (*Arabidopsis* in 50 mM NaCl, *Thellungiella* in 150 mM NaCl); 3) severe stress (*Arabidopsis* in 75–100 mM NaCl, *Thellungiella* in 225–350 mM NaCl); and 4) lethal stress (*Arabidopsis* in 150 mM NaCl).

4.2.1. Group 1–very mild stress

Very mild stress (2–25 mM NaCl) did not cause distinct phenotypic changes in *Arabidopsis* plants, but affected the expression of some CK-related genes in leaves. However, due to natural variability, these changes were not statistically significant. The CK biosynthetic genes *AtIPT3* and *AtIPT9* were upregulated, while *AtCKX3* and *AtCKX6* were downregulated, indicating stimulation of CK production, which might be necessary to compensate for the increased ABA levels in regulation of stomatal behavior. A similar response was observed in shoot apices, but only in 2–10 mM NaCl. The stimulatory effect of very mild stresses seems to be a general phenomenon − it has also been observed, e.g., after heavy metal treatment (ZnO; [39]).

4.2.2. Group 2–mild stress

Week-long exposure of *Arabidopsis* to 50 mM NaCl or *Thellungiella* to 150 mM NaCl caused considerable changes in transcript and hormone levels but did not significantly affect RWC or MSI. Both species had similar concentrations Na^+ and K^+ in their shoots and roots (ca 30 mg g^{-1} DW). *Arabidopsis* exposed to 50 mM NaCl exhibited higher levels of *AtRD26* and *AtRD29B* transcripts and of ABA, JA, and SA in apices. The levels of the stress-associated CK cZ [40] also increased in these tissues. Photosynthesis markers (*AtPSBO1, AtPSBO2*) were downregulated only in apices. Only minor hormonal changes occurred in leaves and roots. The results indicate that shoot apex with new leaf primordia started to react to the stress, activating defence mechanisms, but other tissues showed no significant changes.

The response of *Thellungiella* to 150 mM NaCl was also most pronounced in shoot apices: levels of SA and JA rose, while those of auxins and active CKs (predominantly tZ) fell. These effects were linked to faster ABA turn-over resulting from the upregulation of *TsNCED3* transcription and the promotion of ABA deactivation. Mild increase in ABA levels and substantial increase in those of SA and JA occurred in leaves, while levels of SA and ABA increased in roots. The low intensity of the stress can be deduced from the moderate increase in the transcription of *TsRD29B* in roots and *TsCOR47* in leaves. Previous publications have also reported that treatment with 150 mM NaCl has only minor effects on *Thellungiella* growth and transcriptomic activity (e.g. [41]). Our results show that while these responses are modest, they occur across the plant.

4.2.3. Group 3–severe stress

In our experimental setup, exposure of *Arabidopsis* to 75–100 mM NaCl or *Thellungiella* to 225–350 mM NaCl caused severe stress, characterised by smaller rosettes consisting of dark green leaves and reduction in both RWC and MSI. The more severe the salt stress, the sooner senescence started in older leaves.

In *Arabidopsis*, transcription of the stress-related genes expressed most strongly in apices and leaves were *AtRD26* and *AtRD29B*, respectively. Surprisingly, *AtCOR47* expression decreased in apices. In *Thellungiella*, *TsRD26* and *TsRD29B* were strongly upregulated across the plant, and *TsCOR47* was strongly upregulated in shoots. The difference in *COR47* expression between the tested species might be given by the fact that *Thellungiella* tolerates a wider range of abiotic stresses [42] and so its stress response may be broader. *COR47*, as a primarily cold-regulated gene, need not be activated in *Arabidopsis* during salinity responses.

In 75 mM NaCl, *Arabidopsis* apices exhibited high levels of ABA, JA and SA. Exposure to 100 mM NaCl seemed to reduce the apex ability to withstand stress because levels of ABA and JA were much lower than in 75 mM NaCl. Levels of both these hormones increased in the leaves, but only SA increased in roots. A stronger increase was detected in 100 mM NaCl. It thus appears that *Arabidopsis* was able to protect shoot apices in 75 mM NaCl, and to lesser extent in 100 mM NaCl.

Thellungiella exposed to 225–350 mM NaCl exhibited very high ABA levels in shoots. High ABA levels seem to be important for salt tolerance because they allow stomatal conductance to be kept low, stabilizing the plant water potential. Simultaneously, growth inhibition reduced plant nutrient demands [43]. ABA levels in roots were low even though *TsNCED3* expression was highest in roots and relatively low in leaves. These results are consistent with the finding that ABA is primarily synthesised in roots and transported upwards through the xylem [44]. Salt stress-induced increase in ABA levels has previously been reported in leaves and roots of *Thellungiella* [12,13]. Interestingly, large quantities of ABA in *Thellungiella* were glucosylated to the storage form, i.e. the ABA glucosyl ester. This represents an energy-saving strategy because it means that ABA levels can be increased by glucosyl ester hydrolysis in the event of stress, without any need for *de novo* synthesis.

At NaCl concentrations of 225–350 mM, *Thellungiella* apices exhibited elevated SA levels (albeit to a lesser degree at higher salt concentrations) but gradually diminished JA levels. In addition, the levels of SA and JA in leaves declined. This contradicts the results of Ellouzi et al. [13], who reported increased JA levels. However, they focused on the early response (within 72 h). Given the dynamic nature of the regulation of hormone levels, it is possible that JA levels were already downregulated after seven days of stress exposure.

The IAA content in leaves was reduced at lower salt concentrations, but increased in *Arabidopsis* and *Thellungiella* in 100 mM and 350 mM NaCl, respectively. Increased levels of IAA and its catabolites might suggest that abscission of old leaves was delayed until their nutrient content could be translocated to younger leaves [45]. In 100 mM NaCl, IAA levels in *Arabidopsis* roots decreased progressively.

Arabidopsis exposed to 75 mM NaCl exhibited high levels of IAA and active CKs (including tZ and the less active forms cZ, iP and DZ) in apices. Severe stress suppressed CK signalling in both leaves and roots, as indicated by the down-regulation of the CK receptor genes *AHK2* and *AHK4*, and the type-B response regulator *ARR12*. These results are consistent with those of Mason et al. [15], who reported that CKs negatively affect salt stress tolerance by down-regulating *AtHKT1;1* expression via ARR1 and ARR12. Also analyses of CK-deficient plants, which exhibit a strong stress-tolerant phenotype associated with ABA hypersensitivity, showed the negative role of CKs in salt stress signalling [14].

In 225–350 mM NaCl, apices of *Thellungiella* exhibited lower levels of IAA and active CKs (with the exception of cZ in 350 mM salt). However, the activity of the CK signaling pathway seems to be increased, as indicated by the up-regulation of *TsIPT3*, *TsRR1* and *TsHK3*. This feature, together with high ABA and SA levels, might be associated with preferential protection of apices in this species.

The studied species differed in their expression of the K^+ transporter *HKT*. *AtHKT1;1* was expressed mainly in roots, while *TsHKT1;2* was predominantly localised in apices. This is in accordance with the results of Wu et al. [46], who detected a higher basal level of *TsHKT1;2* in shoots. In severe stress, the expression of *HKTs* increased in shoots of *Thellungiella,* but was unchanged in *Arabidopsis*, which is in agreement with other studies [37,46].

4.2.4. Group 4–lethal stress

Seven-day exposure to 150 mM NaCl was lethal for *Arabidopsis* plants in hydroponics; shoot apices died first. Highly stressed leaves exhibited significant membrane damage and decreased RWC. Strong up-regulation of *AtRD29B* and *AtRD26* transcripts and increase in levels of ABA (which correlated well with *AtNCED3* transcription), as well as of JA and SA, were accompanied by reduced auxin levels. Even though all the CK signaling-related genes were downregulated, the levels of CKs (mainly cZ-, iP- and DZ-types) were high. One potential explanation for this is that the increased CK levels were due to enhanced degradation of specific tRNAs. In fact, CKs, especially cZ, are bound close to the codon to facilitate codon-anticodon interaction and to enhance the efficiency of specific tRNAs in translation [47]. This possibility was suggested in the study on salinity responses in maize [48]. Alternatively, the increase in CK levels could be linked to the fact that extreme CK concentrations may induce programmed cell death [49].

4.2.5. Time course of the early response to severe stress

The early transcriptomic and hormonal responses (15 min–24 h) to severe salt stress (150 mM NaCl in *Arabidopsis* and 350 mM in *Thellungiella*) were similar, although there were some differences in their extent and timing.

The lower salt tolerance of *Arabidopsis* was reflected by significantly decreased MSI and higher Na^+ accumulation in leaves. Stimulation of stress marker genes started in both species after 15 min in roots and after 4 h in apices and leaves, much more strongly in halophyte. Another early up-regulated gene (after 15 min) was the ABA biosynthetic gene *NCED3*, whose expression increased gradually over the first 24 h in roots. Hormonal responses were also very fast, especially in directly exposed roots. Increased levels of ABA, SA and JA in roots of both species were detected after 15–60 min. The halophyte exhibited a much stronger response, especially in terms of the increase in SA and JA levels in roots and the upregulation of the stress-related genes *RD26*, *RD29B* and *COR47*.

Transient peaks in JA levels may indicate that this hormone is a trigger of stress responses. JA maxima occurred in roots and leaves after 15 min and 4 h, and in apices after 1 h. The halophyte exhibited an early transient SA maximum in leaves (between 15 and 60 min), whereas this hormone maximum in *Arabidopsis* occurred after 24 h. The ABA response in apices was observed after 30 min in *Thellungiella* and after 1 h in *Arabidopsis*. Strongly up-regulated ABA levels were found in shoots after 4–24 h. The ABA dynamics in leaves and roots were consistent with the results reported by Ellouzi et al. [13]. Generally, the glycophyte showed a stronger hormonal response in shoots than the halophyte, which primarily reacted to salinity in roots. These data indicate differences in the targeting of defence stimulation between the species.

IAA levels exhibited a minor transient decrease after 15 min in shoots of both plants. Profound downregulation was detected in roots of the halophyte after 2–24 h; in *Arabidopsis*, this response occurred after 24 h. Active CK levels were maintained in *Arabidopsis* apices for 30 min, whereas in *Thellungiella* apices they were transiently mildly elevated between 15 and 60 min. After 24 h, CK levels decreased substantially in shoots of both species, and also in *Arabidopsis* roots. *Thellungiella* consistently had lower levels of CKs (including precursors and metabolites) than the glycophyte. Down-regulation of CK signaling-related genes (*AHK2*, *AHK4*, and *ARR1*) started after 30 min in *Arabidopsis* shoots. Transient stimulation of the expression of the type-A response regulators *ARR8* and *ARR9*, which switch off CK signal transduction, was observed after 2 h in roots. These data on severe stress response are in accordance with the results of Nguyen et al. [50], who demonstrated that repression of CK response, and thus CK signaling, is one of the strategies plants use to cope with abiotic stresses associated with water deficit. *Thellungiella* also exhibited reduced *TsIPT3* expression in shoots after 4 h, the downregulation of *TsHK2* transcription started earlier than in *Arabidopsis* (after 15 min) across the whole plant. It seems that the early response to salt stress (the “alarm phase”) is characterised by decrease in auxin and CK levels as well as down-regulation of CK signal transduction to suppress growth and allow the reallocation of resources to defence. This reaction seems to be similar to the responses to other abiotic stresses, e.g. cold [51].

In conclusion, the enhanced salt stress tolerance of *Thellungiella* is associated with higher basal levels of ABA and JA in apices. *Thellungiella* also responds to stress more rapidly than *Arabidopsis* by increasing the levels of ABA in its apices and reducing those of auxins and CKs, with the exception of *cis*-zeatin, the CK associated with low growth rates. The halophyte *Thellungiella* is able to protect the shoot apex, even under severe salt stress. Finally, the definition of different salt stress intensity classes based on characteristic plant stress responses could be useful for selecting specific stress markers.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2017.07.020.

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Hormonal dynamics during salt stress responses of salt-sensitive *Arabidopsis thaliana* and salt-tolerant *Thellungiella salsuginea*

Sylva Prerostova, Petre I. Dobrev, Alena Gaudinova, Petr Hosek, Petr Soudek, Vojtech Knirsch, Radomira Vankova

Fig. A.1. The diameter of (A) *Arabidopsis* or (B) *Thellungiella* rosettes after 7 days in medium with selected salt concentration. Error bars represent standard deviations; asterisks mark statistical significance in comparison with control (Mann-Whitney U test; * $p < 0.05$, ** $p < 0.01$).

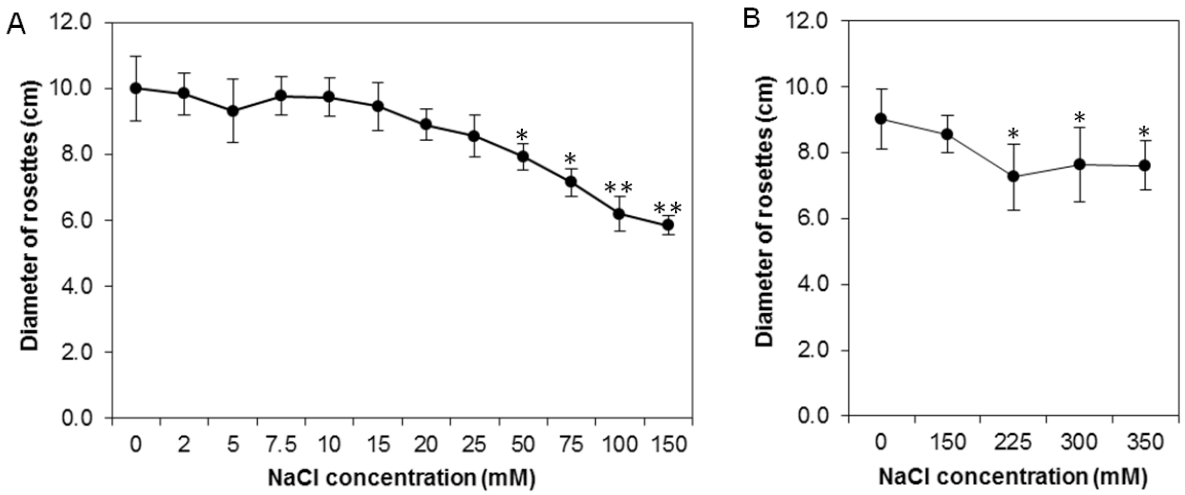


Fig. A.2. Relative water content (RWC) in the leaves of (A) *Arabidopsis* or (B) *Thellungiella* exposed to different salt concentrations for 1 week or exposed to high NaCl concentration (150 or 350 mM) 4 h and 24 h. Error bars represent standard deviations; asterisks mark statistical significance in comparison with control (Mann-Whitney U test; * $p < 0.05$, ** $p < 0.01$).

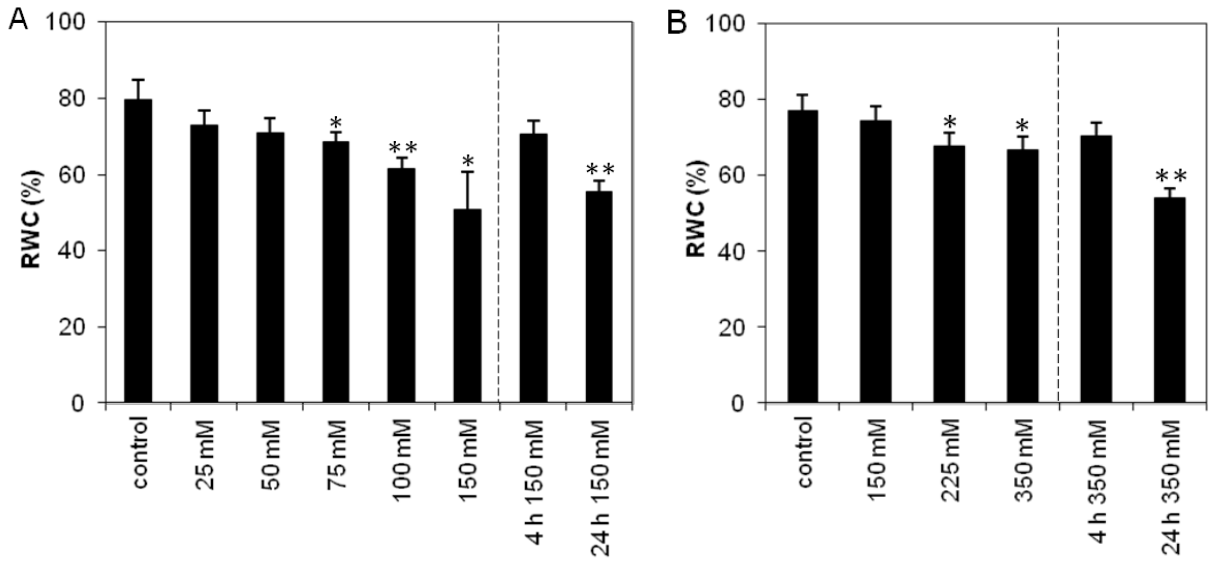


Fig. A.3. Membrane stability index (MSI) of the leaves of (A) *Arabidopsis* or (B) *Thellungiella* exposed to different salt concentrations for 1 week or exposed to high NaCl concentration (150 or 350 mM) 4 h and 24 h. Error bars represent standard deviations; asterisks mark statistical significance in comparison with control (Mann-Whitney U test; * $p < 0.05$, ** $p < 0.01$).

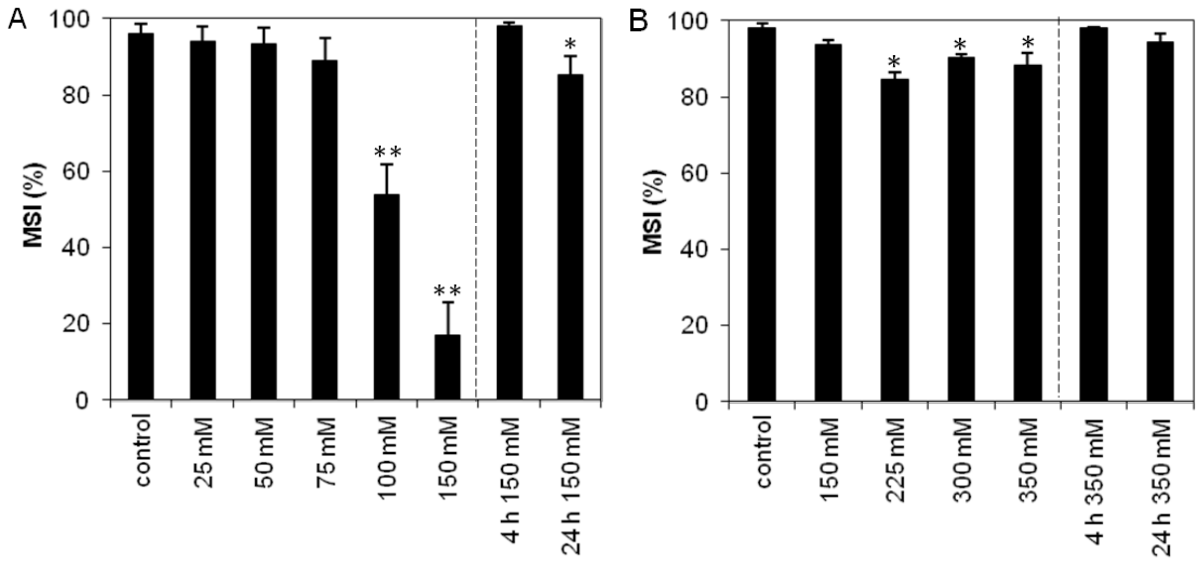


Fig. A.4. Levels of abscisic acid (ABA) metabolites in (A) *Arabidopsis* and (B) *Thellungiella* exposed to different salt concentrations for 1 week; and (C) *Arabidopsis* or (D) *Thellungiella* exposed to high NaCl concentration (150 or 350 mM, respectively) for short time period (from 15 min to 24 h). DPA: dihydrophaseic acid; PA: phaseic acid; NPA: neophaseic acid; ABA-GE: abscisic acid glucosyl ester; 9'-OH-ABA: 9'-hydroxyabscisic acid. A – apices; L – leaves; R – roots.

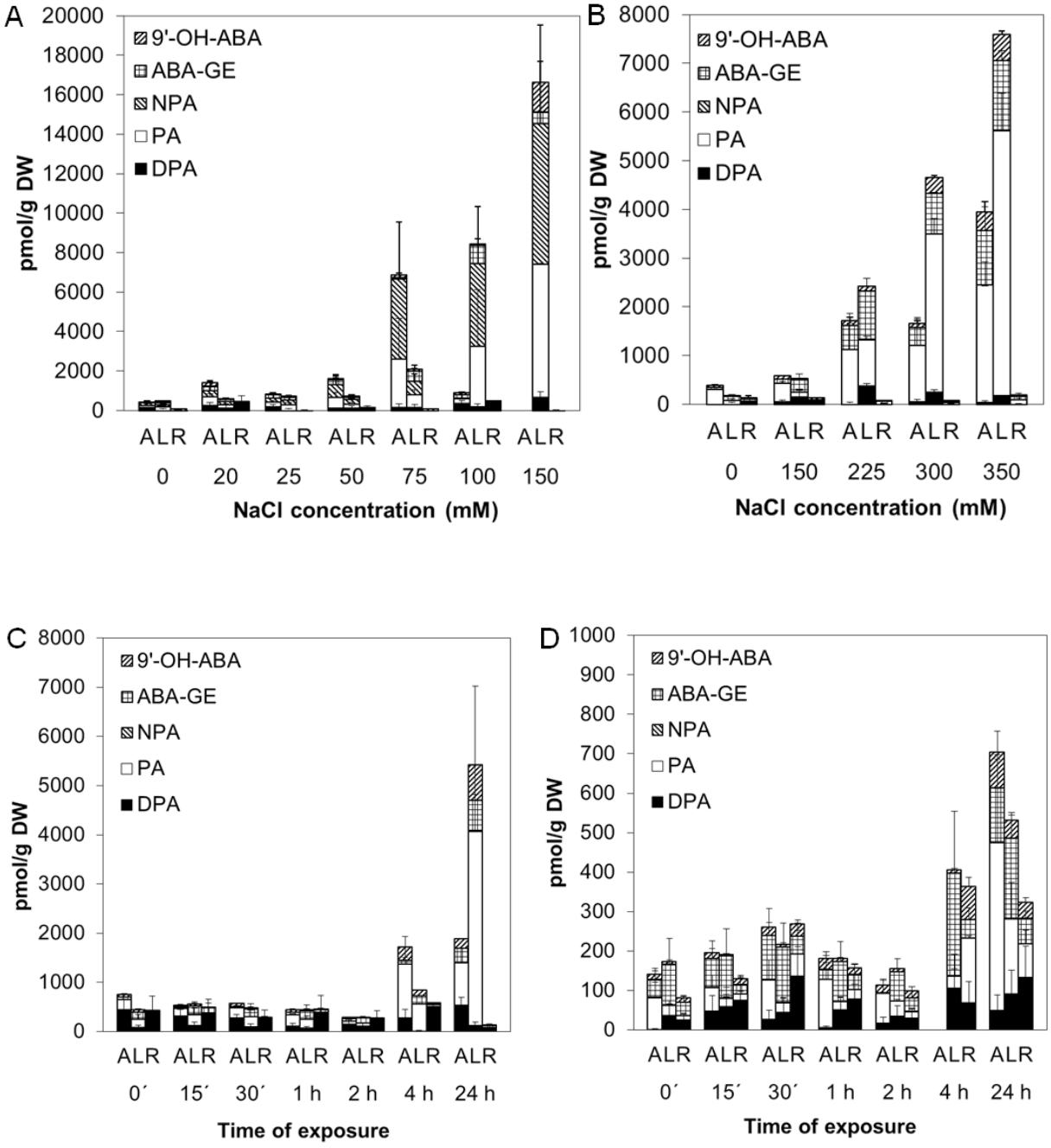


Fig. A.5. Levels of the auxin indole-3-acetic acid (IAA) metabolites in (A) *Arabidopsis* and (B) *Thellungiella* exposed to different salt concentrations for 1 week; and (C) *Arabidopsis* or (D) *Thellungiella* exposed to high NaCl concentration (150 or 350 mM, respectively) for short time period (from 15 min to 24 h). IAA-Asp: indole-3-acetyl aspartate; OxIAA: 2-oxindole-3-acetic acid; OxIAA-GE: 2-oxindole-3-acetic acid glucosyl ester; IAA-GE: indole-3-acetyl-1-glucosyl ester. A – apices; L – leaves; R – roots.

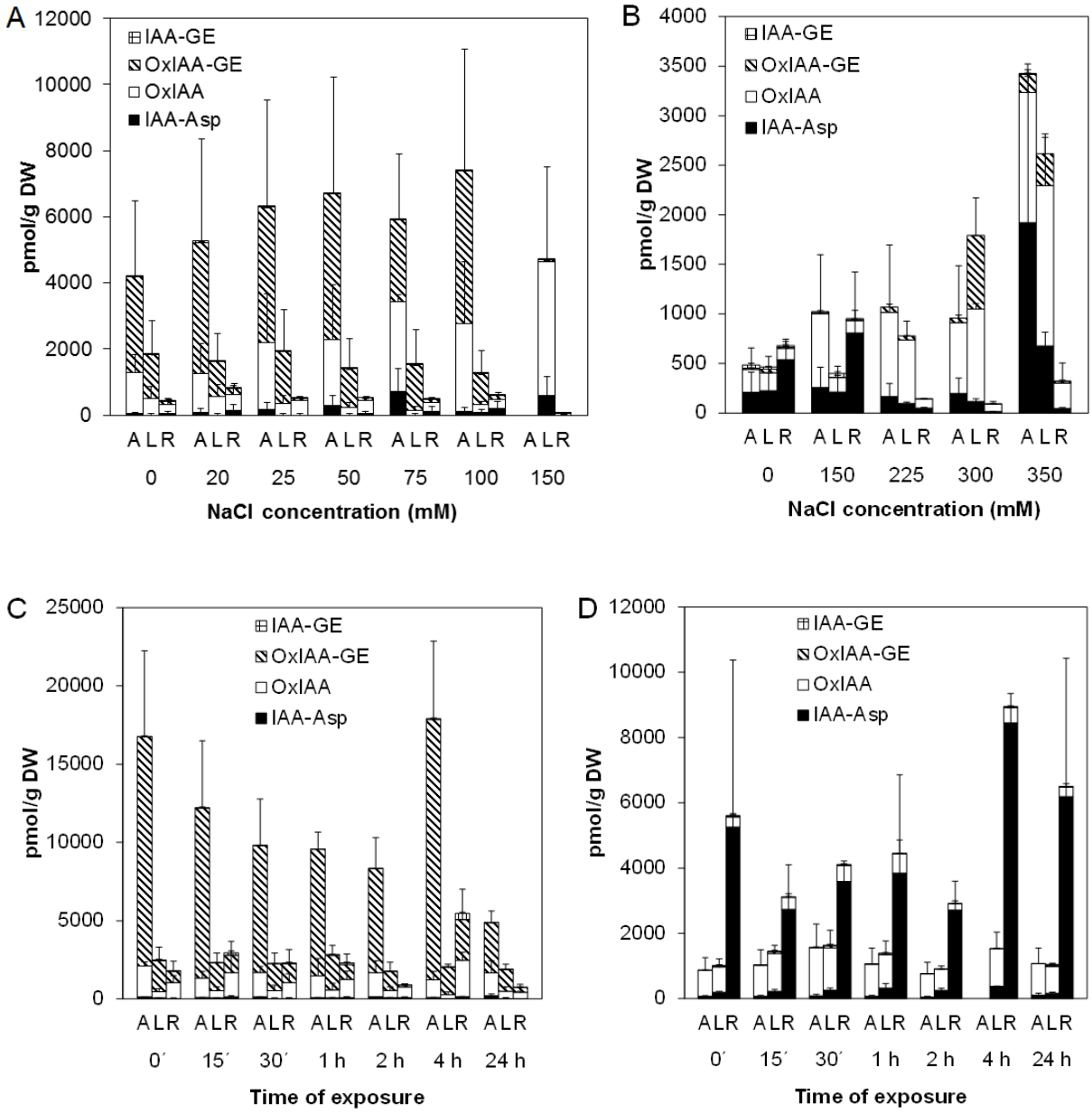


Fig. A.6. Levels of active cytokinins (CKs) in (A) *Arabidopsis* and (B) *Thellungiella* exposed to different salt concentrations for 1 week; and (C) *Arabidopsis* or (D) *Thellungiella* exposed to high NaCl concentration (150 or 350 mM, respectively) for short time period (from 15 min to 24 h). tZ: *trans*-zeatin; DZ: dihydrozeatin; iP: isopentenyladenine; cZ: *cis*-zeatin. A – apices; L – leaves; R – roots.

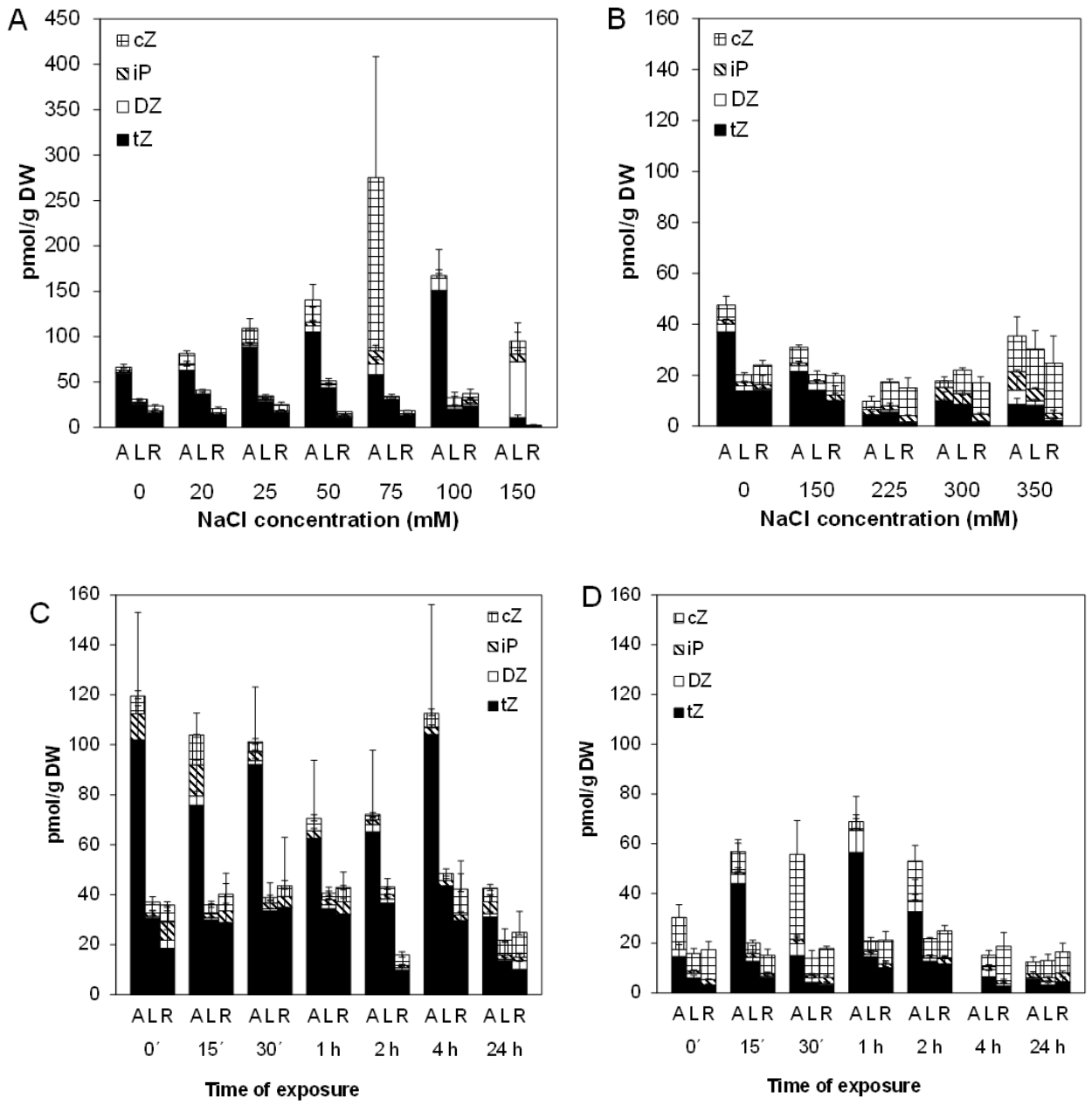


Fig. A.7. Levels of cytokinin (CK) ribosides in (A) *Arabidopsis* and (B) *Thellungiella* exposed to different salt concentrations for 1 week; and (C) *Arabidopsis* or (D) *Thellungiella* exposed to high NaCl concentration (150 or 350 mM, respectively) for short time period (from 15 min to 24 h). tZR: *trans*-zeatin riboside; DZR: dihydrozeatin riboside; iPR: isopentenyladenosine; cZR: *cis*-zeatin riboside. A – apices; L – leaves; R – roots.

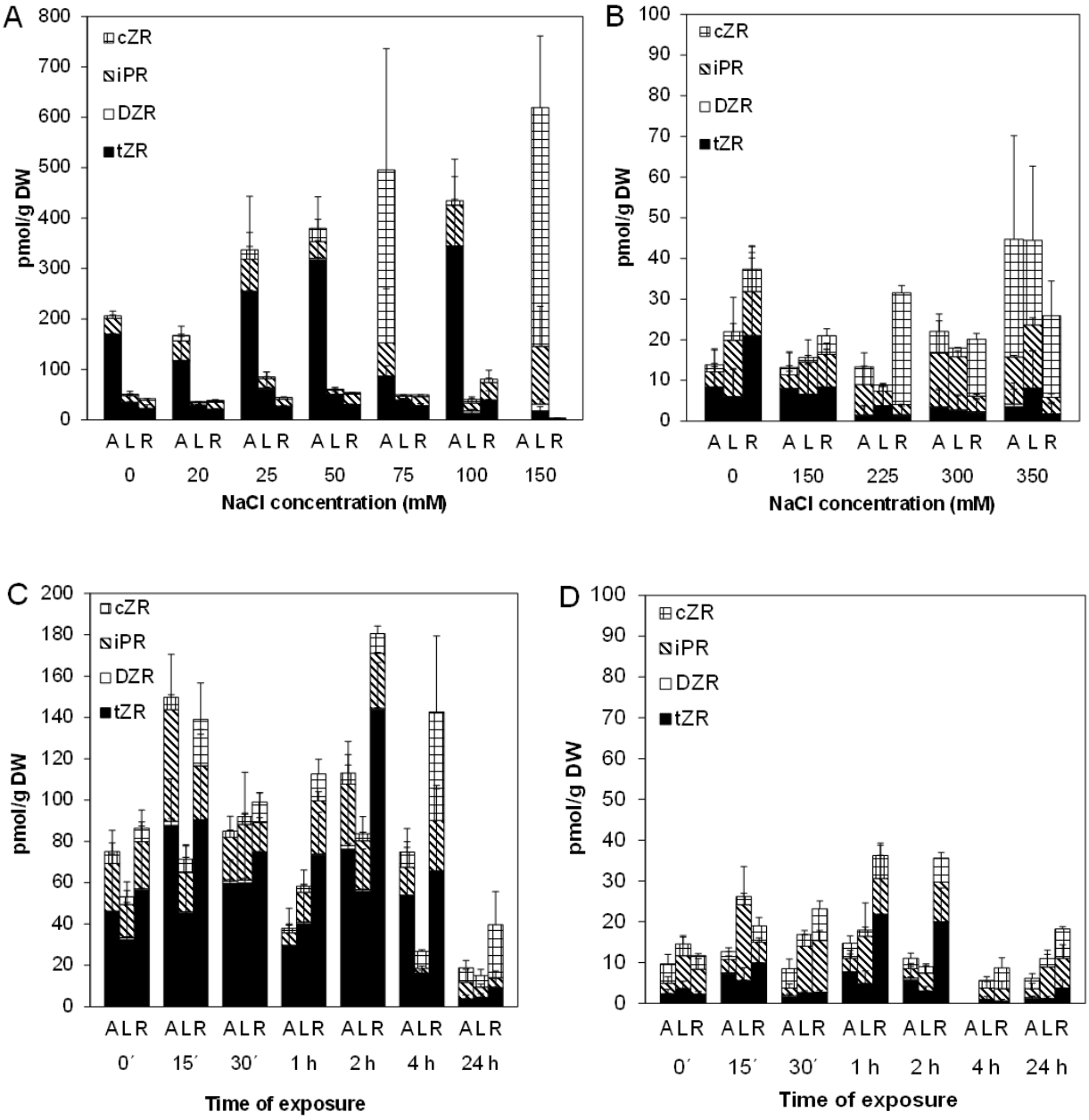


Fig. A.8. Levels of cytokinin (CK) precursors in (A) *Arabidopsis* and (B) *Thellungiella* exposed to different salt concentrations for 1 week; and (C) *Arabidopsis* or (D) *Thellungiella* exposed to high NaCl concentration (150 or 350 mM, respectively) for short time period (from 15 min to 24 h). tZRMP: *trans*-zeatin riboside monophosphate; DZRMP: dihydrozeatin riboside monophosphate; iPRMP: isopentenyladenosine monophosphate; cZRMP: *cis*-zeatin riboside monophosphate. A – apices; L – leaves; R – roots.

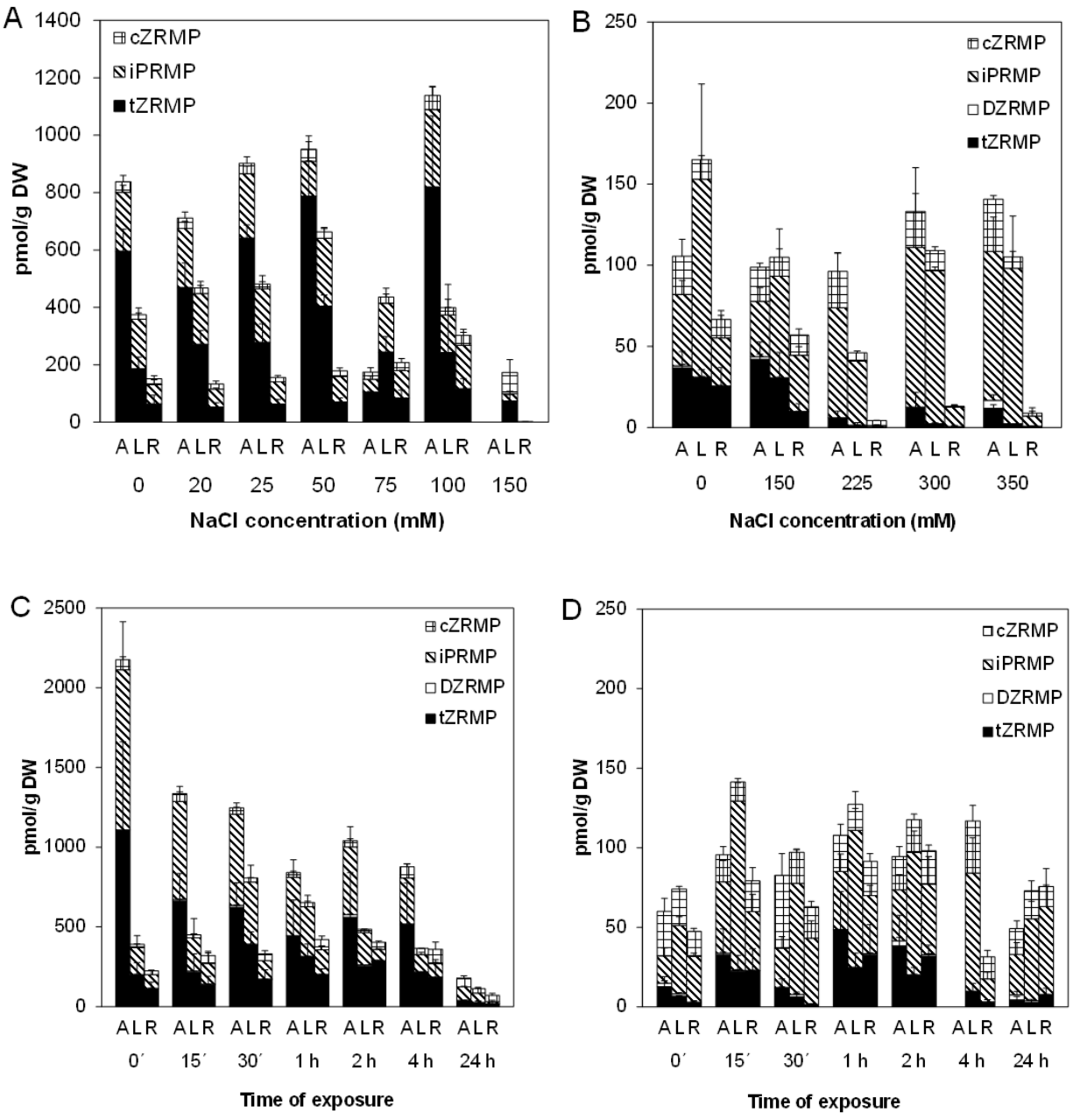


Fig. A.9. Levels of cytokinin (CK) O-glucosides in (A) *Arabidopsis* and (B) *Thellungiella* exposed to different salt concentrations for 1 week; and (C) *Arabidopsis* or (D) *Thellungiella* exposed to high NaCl concentration (150 or 350 mM, respectively) for short time period (from 15 min to 24 h). tZOG: *trans*-zeatin-O-glucoside; tZROG: *trans*-zeatin riboside-O-glucoside; DZROG: dihydrozeatin riboside-O-glucoside; cZOG: *cis*-zeatin-O-glucoside; cZROG: *cis*-zeatin riboside-O-glucoside. A – apices; L – leaves; R – roots.

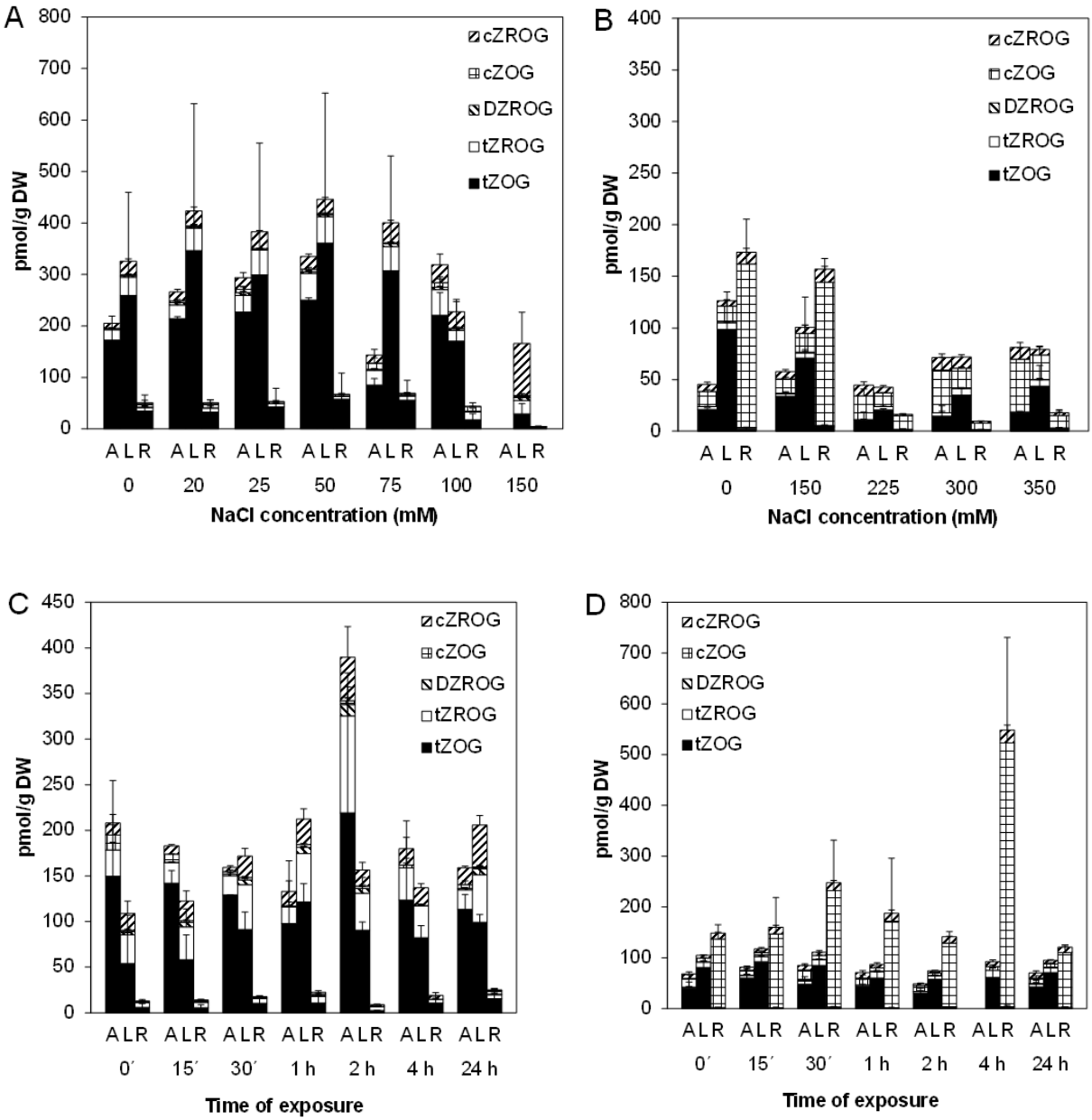


Fig. A.10. Levels of cytokinin (CK) N-glucosides in (A) *Arabidopsis* and (B) *Thellungiella* exposed to different salt concentrations for 1 week; and (C) *Arabidopsis* or (D) *Thellungiella* exposed to high NaCl concentration (150 or 350 mM, respectively) for short time period (from 15 min to 24 h). tZ7G: *trans*-zeatin-7-glucoside; tZ9G: *trans*-zeatin-9-glucoside; DZ9G: dihydrozeatin-9-glucoside; iP7G: isopentenyladenine-7-glucoside; iP9G: isopentenyladenine-9-glucoside; cZ7G: *cis*-zeatin-7-glucoside. A – apices; L – leaves; R – roots.

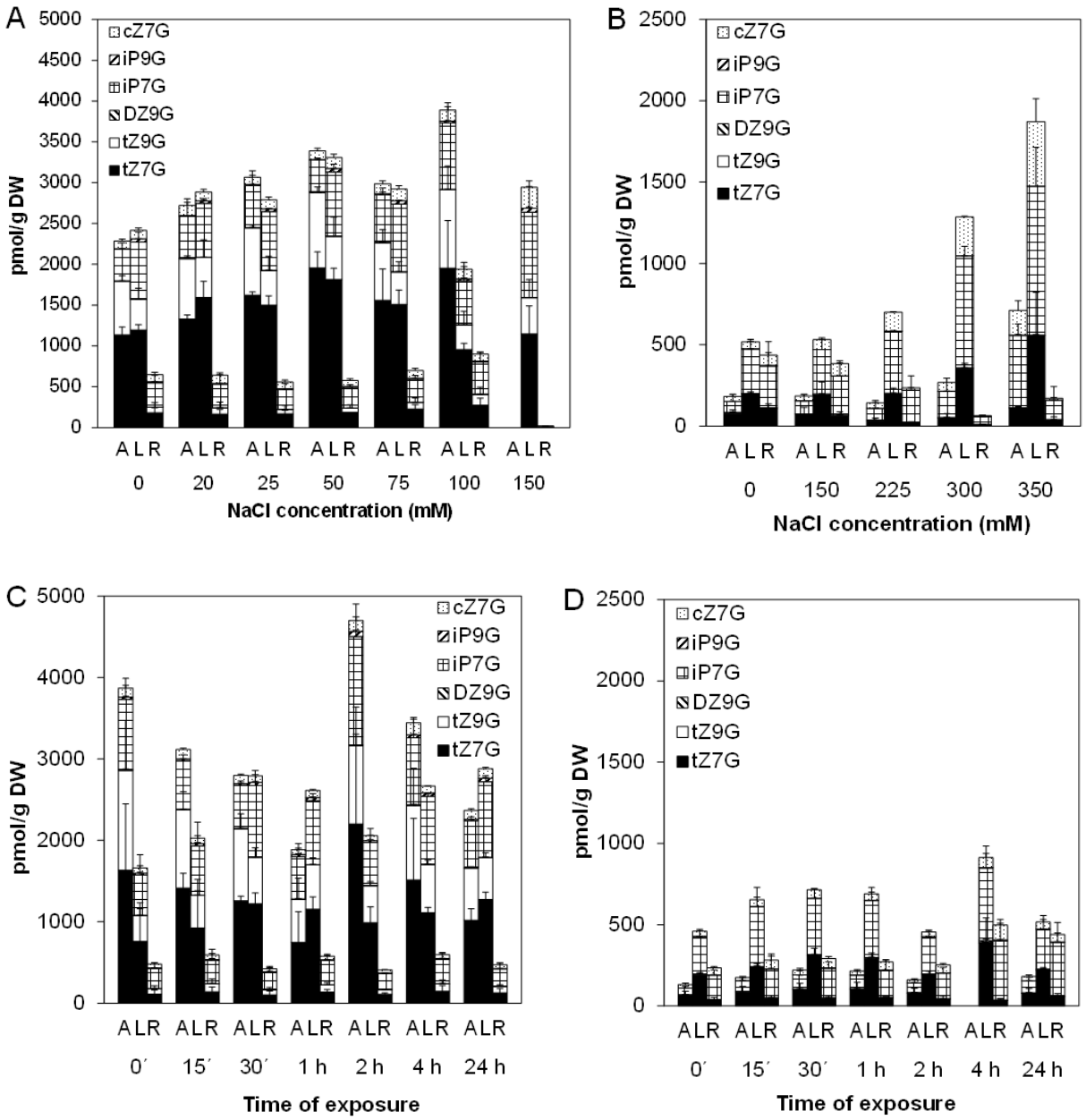
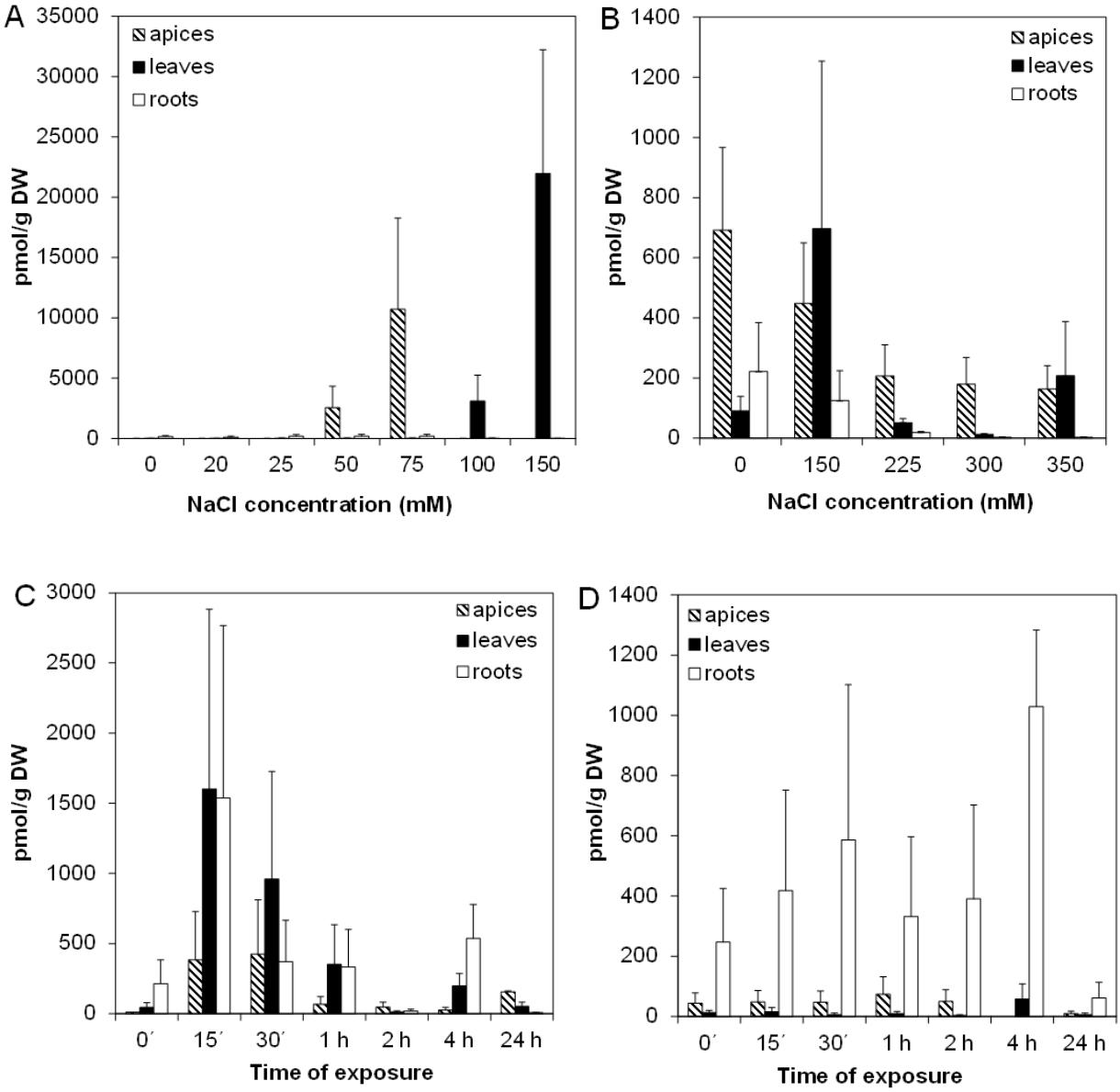


Fig. A.11. Levels of jasmonate-isoleucine in apices, leaves and roots of (A) *Arabidopsis* and (B) *Thellungiella* exposed to different salt concentrations for 1 week; and (C) *Arabidopsis* or (D) *Thellungiella* exposed to high NaCl concentration (150 or 350 mM, respectively) for short time period (from 15 min to 24 h).



2nd Article

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ORIGINAL RESEARCH
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Cytokinins: Their Impact on Molecular and Growth Responses to Drought Stress and Recovery in *Arabidopsis*

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Our phenotyping and hormonal study has characterized the role of cytokinins (CK) in the drought and recovery responses of *Arabidopsis thaliana*. CK down-regulation was achieved by overexpression of the gene for CK deactivating enzyme cytokinin oxidase/dehydrogenase (CKX): constitutive (35S:CKX) or at the stress onset using a dexamethasone-inducible *pOp/LhGR* promoter (DEX:CKX). The 35S:CKX plants exhibited slow ontogenesis and higher expression levels of stress-associated genes, e.g., *AtP5CS1,* already at well-watered conditions. CK down-regulation resulted during drought in higher stress tolerance (indicated by relatively low up-regulation of the expression of drought stress marker gene *AtRD29B*) accompanied with lower leaf water loss. Nevertheless, these plants exhibited slow and delayed recovery after re-watering. CK levels were increased at the stress onset by stimulation of the expression of CK biosynthetic gene *isopentenyl transferase* (*ipt*) (DEX:IPT) or by application of exogenous CK *meta*-topolin. After water withdrawal, long-term CK elevation resulted in higher water loss in comparison with CKX transformants as well as with plants overexpressing *ipt* driven by senescence-inducible *SAG12* promoter (SAG:IPT), which gradually enhanced CKs during the stress progression. In all cases, CK up-regulation resulted in fast and more vigorous recovery. All drought-stressed plants exhibited growth suppression associated with elevation of abscisic acid and decrease of auxins and active CKs (with the exception of SAG:IPT plants). Apart from the *ipt* overexpressers, also increase of jasmonic and salicylic acid was found.

Keywords: abscisic acid, auxin, cytokinin, cytokinin oxidase/dehydrogenase, drought stress, isopentenyl transferase, phytohormone

INTRODUCTION

Drought belongs to the most frequent abiotic stresses which worldwide reduce crop yields (Daryanto et al., 2016). This stress may occur in nearly all climatic regions. Plants had to evolve different mechanisms for sensing and responding to drought (Zwack and Rashotte, 2015). Their interactions with the environment as well as their growth and development are regulated by plant hormones (Ha et al., 2012).

Abscisic acid (ABA) is the most important hormone controlling plant water loss, and hence their water status and performance under water-limited conditions (de Ollas and Dodd, 2016). ABA induces closure of stomata, the crucial water loss regulation site, as well as stimulates substantial transcriptional changes, associated with growth suppression and activation of defense pathways. During water stress responses, ABA exhibits a complex cross-talk with other plant hormones. For example, jasmonic acid (JA) activates synergistically several branches of ABA signaling pathway (especially MYC/MYB and ANAC transcription factors; de Ollas and Dodd, 2016). Ethylene exhibits antagonism with ABA in regulation of shoot and root growth in drought (Sharp and LeNoble, 2002). Participation of salicylic acid (SA) in drought responses is indicated by elevation of this hormone after water withdrawal as well as by positive effects of exogenous SA application on plant tolerance (Miura and Tada, 2014). Brassinosteroids enhance tolerance to abiotic stresses, probably via the effect on the antioxidant system (Wani et al., 2016). Auxins can influence plant adaptation to adverse environmental conditions by control of plant growth (Rowe et al., 2016). Thus, a complex cross-talk among different phytohormones is underlying drought stress responses.

Cytokinins (CKs) have decisive impact on regulation of plant growth as well as on the stabilization of photosynthetic machinery during stress progression. Both exogenous application and modulation of CK levels were reported to have positive effect on drought tolerance (Rulcova and Pospisilova, 2001; Rivero et al., 2007). In case of exogenous CK application, it is advantageous to use aromatic CK(s), e.g., N6-benzyladenine or *meta*-topolin, as the isoprenoid ones undergo rapid degradation by CK deactivating enzymes – cytokinin oxidase/dehydrogenases (CKX). However, the widely used aromatic CK N6-benzyladenine is quickly N-glucosylated in plants and the accumulated N7- and N9-glucosides may inhibit growth (Werbrouck et al., 1996). In contrast, its hydroxylated derivative, *meta*-topolin, is deactivated by glucosylation at the side chain. The resulting O-glucoside serves as a CK storage form that can be gradually hydrolyzed, releasing the active compound (Werbrouck et al., 1996; Werbrouck, 2010) and prolonging considerably the CK effect. For these reasons, the aromatic CK *meta*-topolin was selected for our experiments.

Taking into account hormone functions in drought responses, attempts to increase stress tolerance by manipulating of plant hormone content/signaling have been intensively studied. Major attention has been paid to ABA. Both up-regulation of ABA biosynthesis (Estrada-Melo et al., 2015) and overexpression of ABA signaling component (Mao et al., 2010) proved to enhance drought tolerance. In the former case, both decreased stomata aperture and stress-induced transcriptome changes were reported, in the latter one only transcriptome changes were found. Stress tolerance was promoted also by modulations of the content or signaling of other hormones, e.g., through overexpression of the ethylene response factor *JERF1* (Zhang et al., 2010) or elevation of auxin levels (Shi et al., 2014).

Extensive attention has been paid to CKs as well. Surprisingly, both CK down- and up-regulation were reported to enhance drought tolerance (Rivero et al., 2007; Werner et al., 2010; Nishiyama et al., 2011). Down-regulation of CK content has been mostly achieved by overexpression of *cytokinin oxidase/dehydrogenase* (*CKX*;Werner et al., 2010). Constitutive *CKX* expression resulted in slow growth rate and elevated content of protective compounds, which contributed to strongly increased drought tolerance in *Arabidopsis* (Werner et al., 2001; Nishiyama et al., 2011), tobacco (Macková et al., 2013), and barley (Pospíšilová et al., 2016), manifested e.g., by higher drought survival rate.

On the other hand, increase of CK content may also help plants to tolerate drought, as demonstrated by several studies. CK elevation has been mostly achieved by overexpression of CK biosynthetic gene *isopentenyl transferase (IPT*), driven by senescence- (*SAG12*) or stress-inducible (*SARK*) promoters. Elevation of CKs in *SAG12:ipt* creeping bentgrass (Merewitz et al., 2012; Xu et al., 2016) highly increased tolerance to drought or heat, enhancing the activity of the antioxidant system. Drought tolerance was promoted by *SARK:ipt* construct in tobacco (Rivero et al., 2007, 2009, 2010), peanut (Qin et al., 2011), or cotton (Kuppu et al., 2013). CK elevation during stress progression diminished the negative stress effects on photosynthesis. In contrast, constitutive overexpression of *ipt* under *Pssu* promoter was associated with high drought sensitivity as well as disproportion of the shoot and root system (strong root suppression; Synková et al., 1999). The above mentioned findings demonstrate that the timing and extent of CK elevation exhibits a decisive impact on plant performance.

When evaluating the CK effect on drought tolerance, it should be kept in mind that transformants may have considerably changed phenotype, e.g., 35S:CKX overexpresser shows enhanced root system, dwarf shoots, changed leaf morphology and slow growth rate (Werner et al., 2010). Due to smaller leaf surface, they have lower transpiration rate, which together with lower stomata conductance results in maintenance of higher relative water content (Lubovská et al., 2014). So, the open questions remain: What are the specific functions of CKs

Abbreviations: 35S:CKX, *AtCKX1* gene under constitutive promoter *35S*; ABA, abscisic acid; ACC, aminocyclopropane carboxylic acid; CK, cytokinin; CKX, cytokinin oxidase/dehydrogenase; Col-0 CK, *Arabidopsis thaliana* plants sprayed with exogenous cytokinin; cZ, *cis*-zeatin; DAS, days after sowing; DEX:IPT, *ipt* gene under dexamethasone-inducible promoter; DEX:CKX, *HvCKX2* gene under dexamethasone-inducible promoter; DZ, dihydrozeatin; IAA, indole-3-acetic acid; iP, isopentenyladenine; iPR, isopentenyladenosine; IPT, isopentenyl transferase; JA, jasmonic acid; JA-Ile, jasmonate-isoleucine; NCED3, 9-cis-epoxycarotenoid dioxygenase 3; OxIAA 2-oxindole-3-acetic acid; P5CS1, δ-1-pyrroline-5-carboxylate synthase 1; RD29B, responsive to desiccation 29B; SA, salicylic acid; SAG, senescence-associated gene; SAG:IPT, *ipt* gene under senescence-inducible promoter *SAG12*; SARK, senescence-associated receptor-like kinase; tZ, *trans*-zeatin; tZR, *trans*-zeatin riboside; WT, wild-type.

in drought stress responses and what may be an indirect effect mediated by changed morphology?

The aim of this study has been to elucidate specific role(s) of CKs during drought stress and subsequent recovery. The impact of CK down-regulation was compared in constitutive *CKX* transformant (35S:CKX) and dexamethasone-inducible one (DEX:CKX), in order to distinguish the consequence of changed morphology from the effect of CK suppression. In parallel, CK up-regulation driven by different promoters was used to characterize the effect of timing of CK increase on the stress response – CK elevation at the stress onset (DEX:IPT or application of exogenous CK *meta*-topolin) in comparison with the stress-induced one (SAG:IPT). Complex phenotyping mapping supported by phytohormonal and transcriptomic analyses allowed characterization of the behavior of individual variants in detail.

MATERIALS AND METHODS

Experimental Setup and Stress Conditions

Transformant lines used in this study originated from *Arabidopsis thaliana* ecotype Columbia (Col-0): *AtCKX1* overexpressing line under *35S* promoter (35S:CKX; Werner et al., 2001); dexamethasone-inducible lines *CaMV35S*>GR>*HvCKX2* expressing *CKX2* from *Hordeum vulgare* (DEX:CKX; Černý et al., 2013), and *CaMV35S*>GR>*ipt* (pOp^{BK}-*ipt*; DEX:IPT; Craft et al., 2005); as well as the senescence inducible *ipt* transformant overexpressing *ipt* from *Agrobacterium tumefaciens* under control of *SAG12* promoter (SAG:IPT; Reusche et al., 2013). Col-0 plants were used as a control (wild-type; WT).

The genotypes were evaluated in pot experiments in the PhyTec Experimental Greenhouse at the Institute of Bio- and Geosciences, Plant Sciences (IBG-2), Forschungszentrum Jülich GmbH, Germany (50°54′36″N, 6°24′49″E). Seeds were sown on a mixture of clay and high moor peat (Pikiererde type CL P, Einheitserdewerk, Germany) with the PhenoSeeder robot system (Roussel et al., 2016). After 4 days of stratification at 4°C, plants were grown in a climate chamber at 8/16 h light/dark period, at 22/18°C, and 50% humidity. The plant germination was monitored daily by an automated germination detection system over a period of 5 days. Fourteen days after sowing (DAS), 640 *Arabidopsis* plants were transplanted into pots (7 × 7 × 7 cm) filled with a mixture (0.3/0.5/0.2) of peat, sand and pumice (SoMi 513 Dachstauden, Hawita, Germany) and watered to 60% soil water capacity (soil matric potential = 0 MPa). In the case of 35S:CKX seeds, sowing was done 14 days before the other variants (due to the delayed ontogenesis), in order to compare plants of similar rosette size. During the experiment, the plants at control conditions were watered every 2 days to a soil water capacity 30% (soil matric potential = −0.03 MPa), whereas the plants subjected to drought stress were watered for the last time 25th DAS in the morning and the water was withdrawn until 37th DAS. Soil water capacity reached 10% (soil matric potential = −1.5 MPa) in the case of WT at the time point of re-irrigation (38 DAS). The optimization of drought treatment was recently described by Barboza-Barquero et al. (2015). The photosynthetic active radiation was between 350 and 450 μmol m^{-2} s^{-1} at canopy level.

Two independent biological experiments were performed in subsequent years. In each experiment, 42 plants of each experimental variant were grown under drought stress conditions, and 56 at control conditions. The plants were randomly arranged in 16 trays (5 × 8 plants) with 7 trays at drought stress conditions and nine trays at control conditions.

Phenotypic measurements started 17 DAS (31 days in the case of 35S:CKX line) using the SCREEN Chamber system which consists of a climate chamber with an installed robot. The robot delivered the trays with plants to the GROWSCREEN FLUORO (Jansen et al., 2009) to scan leaf area by RBG measurement and chlorophyll fluorescence of dark adapted plants (F_v/F_m) at different time points during the experiment. The projected leaf area was used for calculation of the growth rate. Leaf area images were used for determination of number of leaves, compactness, stockiness, symmetry and diameter of rosettes. Measurements were performed every morning, before spraying and watering.

The induction of the *CKX* and *ipt* expression, respectively, in the dexamethasone-inducible lines was done by spraying with 20 μM dexamethasone (dissolved in DMSO to final concentration 0.1%, 0.01% Silwet). The spraying was carried out just before the drought stress initiation at 25 DAS, as well as on the 5th and 10th day of the stress progression. As an additional experimental variant, exogenous CK *meta*-topolin (10 μM dissolved in 0.1% DMSO with 0.01% Silwet) was sprayed on Col-0 plants at the same time as dexamethasone spraying. The other variants were sprayed with solution of 0.1% (v/v) DMSO with 0.01% Silwet. During each spraying treatment, approximately 3.2 ml was applied by sprayer on the surface of one pot and the respective plant (**Figure 1**).

Samples for hormone analyses, RT-qPCR, fresh and dry weight determination were taken in the morning just before the drought stress initiation (25 DAS), at the end of the drought stress period (38 DAS morning), and at the end of the recovery phase (44 DAS morning). In total 6 biological replicates were analyzed for each experimental variant. In the case of fresh and dry weight determination, each rosette was immediately weighed after cutting, dried in oven at 60°C for 24 h, and weighed again. Whole rosettes for hormone analyses and RT-qPCR were frozen in liquid nitrogen and stored at −80°C.

Plant Hormone Determination

Plant hormones were purified and analyzed according to Dobrev and Kaminek (2002) and Dobrev and Vankova (2012). Samples (ca 100 mg FW) were homogenized and extracted with methanol/water/formic acid (15/4/1, v/v/v). Internal standards (10 pmol per sample) were added: $^2H_6$-ABA, $^2H_3$-PA, $^2H_3$-DPA, $^2H_4$-7OH-ABA, $^2H_5$-ABA-GE, $^2H_5$-*trans*Z, $^2H_5$-*trans*ZR, $^2H_5$-*trans*Z7G, $^2H_5$-*trans*Z9G, $^2H_5$-*trans*ZOG, $^2H_5$-*trans*ZROG, $^2H_5$-*trans*ZRMP, $^2H_3$-DZ, $^2H_3$-DZR, $^2H_3$-DZ9G, $^2H_3$-DZRMP, $^2H_7$-DZOG, $^2H_6$-iP, $^2H_6$-iPR, $^2H_6$-iPRMP, $^2H_6$-iP7G, $^2H_6$-iP9G, $^{13}C_6$-IAA, $^2H_2$-OxIAA, $^2H_4$-SA, $^2H_5$-JA (Olchemim). Extracts were purified and separated on a reverse-phase cation exchange SPE column (Oasis-MCX, Waters). The first hormone fraction was eluted with methanol (contains ABA and other acidic

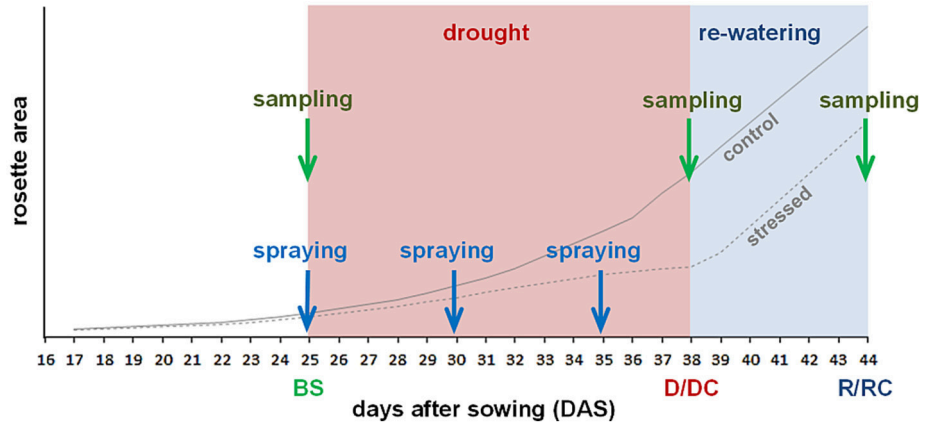

FIGURE 1 | Scheme of the experiment. Drought started 25 DAS; resumption of watering was in 38 DAS. Leaf treatment by spraying was done 25, 30 and 35 DAS. Samplings were made before stress (BS), after stress (D/DC) and 6 days after re-watering (R/RC). Lines illustrate growth curves of watered control plants and drought stressed plants.

hormones); the second fraction, eluted with 0.35 M NH_4OH in 70% methanol, contained CK metabolites. Both fractions were separated by HPLC (Ultimate 3000, Dionex; column Luna C18(2), 100 × 2 mm, 3 µm, Phenomenex); and the hormones were quantified using a hybrid triple quadrupole/linear ion trap mass spectrometer (3200 Q TRAP, Applied Biosystems) operated in selected reaction monitoring mode. The concentration of phytohormones was calculated relative to the corresponding internal standard or to the internal standard with a similar chemical structure.

RT-qPCR

Total RNA was extracted with the RNeasy Plant Kit (Qiagen) from samples homogenized in liquid nitrogen with mortar and pestle (2 × 3 repetitions). RNA was treated with rDNase from NucleoSpin RNA Plant kit (Machery-Nagel). cDNA was synthesized using M-MLV Reverse Transcriptase (RNase H Minus, Point Mutant, Promega), oligo dT primers and the Protector RNase Inhibitor (Roche Applied Science). cDNA (20x diluted) was mixed with the LightCycler 480 DNA SYBR Green I Master (Roche Applied Science) and 500 nM of respective primers to a final volume 10 µl. The RT-qPCR was performed with the Light Cycler 480 (Roche Applied Science). qPCR program was set on initial denaturation (5 min, 95°C), followed by 45 cycles of primer denaturation (10 s, 95°C), annealing (10 s, 60°C) and elongation (10 s, 72°C). Relative content of RNA was calculated according to Hellemans et al. (2007). *AtUBQ10* was used as the reference gene.

Primers were designed according to sequences retrieved from TAIR database (Lamesch et al., 2012) using Primer3Plus program (Untergasser et al., 2007). The quality of primers was verified by AlleleID (PREMIER Biosoft; Apte and Singh, 2007) and the probability of folding secondary structures was predicted in mfold (Zuker et al., 1999). Primer sequences are shown in Table S1.

Statistical Analyses and Calculations

Data exceeding interval of ± three standard deviations (SD) from the mean were excluded as outliers. The relative growth rate was calculated as $[\ln(x_2)-\ln(x_1)]/(t_2-t_1)$, where x_1 and x_2 are projected leaf areas measured in t_1 or t_2 time points with the statistical software R (R Development Core Team, 2011). Data from hormonal analysis, RT-qPCR and growth rate determination were tested by two-sample *t*-test in the program PAST 3.01 (Hammer et al., 2001). The principal component analysis (PCA) was performed using OriginPro 2014 (http://www.originlab.com/).

RESULTS

Wild-Type (WT)

The drought and recovery response of the wild-type Col-0 (WT) was characterized in detail using high throughput phenotyping system. Drought stress resulted in the decrease of leaf water content (**Figure 2**). Stress reaction was associated with growth suppression (Figure S1, **Table 1**, and Table S2). The first noticeable reduction of growth was detected after 4 days of drought stress (29 DAS), then the growth rate gradually decreased. The growth suppression was accompanied by the decrease of active CKs (by 39% after 13-day drought; **Figure 3**), predominantly of *trans*-zeatin (tZ). Drought caused also substantial decrease of CK ribosides (by ca 67%), CK precursors (CK phosphates; by ca 65%) and CK N-glucosides (by ca 40%; Table S4). The only active CK moderately enhanced under stress conditions was *cis*-zeatin (cZ; **Figure 3**), accompanied by an increase of its N- and O- glucosides. In accordance with the stress-induced decrease of active CK levels, transcription of the most abundant CK catabolic enzyme *AtCKX1* was up-regulated by drought (**Table 2**).

Growth suppression was also associated with down-regulation of auxin indole-3-acetic acid (IAA; **Figure 4**) and simultaneous elevation of IAA catabolites 2-oxindole-3-acetic acid (OxIAA;

Table S5) and its glucosyl ester (not shown), which due to biological variation did not reach statistical significance ($p < 0.05$). Similarly to IAA, the levels of another, weaker auxin phenylacetic acid also decreased (to minor extent) following the water withdrawal (Table S5). Stress response involved strong up-regulation of ABA (**Figure 5**), as well as of ABA catabolites: phaseic acid, dihydrophaseic acid, and 9′-hydroxy-ABA (Table S5). ABA levels correlated well with

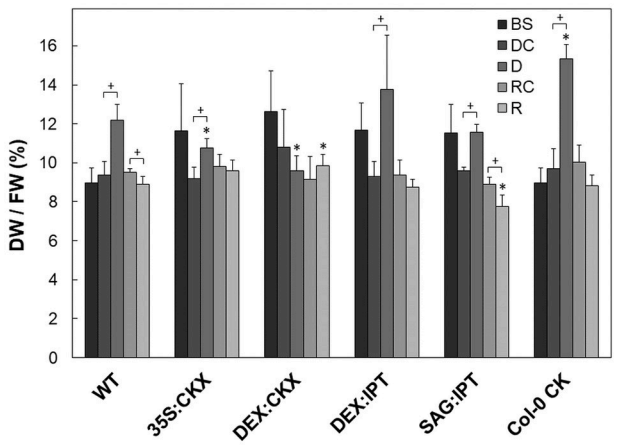

FIGURE 2 | Leaf water content expressed as leaf dry weight/fresh weight ratio. The experimental variants include *Arabidopsis thaliana* wild-type (WT), 35S:CKX, DEX:CKX, DEX:IPT, SAG:IPT plants as well as Col-0 after *meta*-topolin treatment (Col-0 CK). Before stress application–BS (25 DAS), well-watered plants at the end of drought period–DC (38 DAS), drought stressed plants–D (38 DAS), well-watered plants at the end of recovery period–RC (44 DAS), re-watered plants–R (44 DAS). Statistically significant differences ($p < 0.05$, two-sample *t*-test) between the corresponding variants of WT and transformants are marked (*), and between D/DC or R/RC variants within each genotype (+).

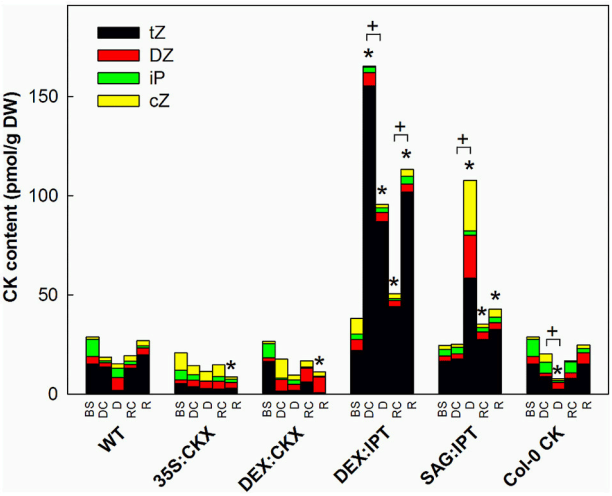

FIGURE 3 | The content of active cytokinins (CKs). Specification of genotypes and treatments is as described in **Figure 2**. tZ - *trans*-zeatin, DZ–dihydrozeatin, iP–isopentenyladenine, cZ–*cis*-zeatin. Data represent means ($n = 6$) in pmol per g DW. Statistically significant differences ($p < 0.05$, two-sample *t*-test) in total active CK content between the corresponding variants of WT and transformants are marked (*), and between D/DC or R/RC variants within each genotype (+).

TABLE 1 | The impact of drought stress on growth rate of *Arabidopsis thaliana* wild-type (WT), 35S:CKX, DEX:CKX, DEX:IPT, SAG:IPT plants and Col-0 (WT) after *meta*-topolin treatment (Col-0 CK).

DAS	WT	35S:CKX	DEX:CKX	DEX:IPT	SAG:IPT	Col-0 CK	
22	99.7	98.0	101.1	100.4	100.7	96.5	
23	96.5	94.8	98.0	105.7	96.7	98.0	
24	94.0	97.6	93.2	100.0	101.7	91.6	
25	106.1	96.0	103.9	92.4	99.8	95.2	
28	97.6	102.3	93.0	90.2	88.5	89.9	Drought
29	82.2	72.6	89.8	65.5	80.6	55.3	
30	71.8	50.7	79.1	64.1	87.2	68.1	
31	102.3	66.3	100.3	83.4	88.5	69.8	
32	62.3	50.9	68.7	40.5	59.6	52.2	
35	56.8	17.1	57.2	32.7	51.3	49.3	
36	27.2	8.6	68.5	14.5	23.7	21.4	
37	19.2	10.9	23.1	9.7	13.5	4.6	
38	14.7	1.1	13.3	13.0	8.5	15.0	
39	115.1	9.1	57.9	116.7	86.0	147.0	Re-watering
42	192.3	141.7	127.9	175.2	190.2	155.1	
44	177.9	138.9	123.9	215.2	164.6	203.9	

Ratio of mean growth rate of stressed plants to mean growth rate of the corresponding non-stressed plants within each time point was expressed in %. Growth rate of each plant was calculated from rosette area in mm^2 determined by RGB measurement ($n = 15–40$). Individual growth rates ± SD are shown in Table S2.

TABLE 2 | The impact of drought stress on transcription of selected genes (determined by RT-qPCR) in *Arabidopsis thaliana* wild-type (WT), 35S:CKX, DEX:CKX, DEX:IPT, SAG:IPT plants and Col-0 (WT) after *meta*-topolin treatment (Col-0 CK).

		HvCKX2	***IPT Agro***	***CKX1***	***IPT3***	***RD29B***	***P5CS1***	***NCED3***
WT	BS			1	1	1	1	1
	DC			0.125 ± 0.074	0.129 ± 0.013	1.363 ± 0.430	0.130 ± 0.072	0.728 ± 0.081
	D			**0.916 ± 0.617**	0.111 ± 0.058	**8.390 ± 4.535**	**1.052 ± 0.171**	**5.690 ± 2.337**
	RC			0.637 ± 0.274	0.139 ± 0.054	5.029 ± 0.632	0.003 ± 0.001	7.378 ± 0.911
	R			0.647 ± 0.327	0.305 ± 0.117	**0.757 ± 0.128**	**0.357 ± 0.128**	**4.155 ± 1.182**
35S:CKX	BS			260.999 ± 95.301*	1.478 ± 0.034*	3.471 ± 0.414*	1.380 ± 0.240*	1.503 ± 0.246*
	DC			NA	NA	NA	NA	NA
	D			168.162 ± 65.985*	0.842 ± 0.097*	1.225 ± 0.159*	0.506 ± 0.107*	3.936 ± 0.739
	RC			121.291 ± 42.753*	0.524 ± 9.7E-05*	7.517 ± 1.879	1.001 ± 0.163*	3.253 ± 0.281*
	R			**0.334 ± 0.182**	**0.370 ± 6.8E-05**	ND	**0.499 ± 0.097**	3.372 ± 0.318
DEX:CKX	BS			1.273 ± 0.449	0.932 ± 1.7E-04	0.843 ± 0.211	0.611 ± 0.099*	1.576 ± 0.136*
	DC	1		0.192 ± 0.083	0.470 ± 0.122*	0.443 ± 0.057*	0.453 ± 0.082*	1.697 ± 0.664*
	D	1.124 ± 0.280		0.413 ± 0.164	0.529 ± 0.081*	**1.808 ± 0.309***	0.425 ± 0.070*	**5.668 ± 0.787***
	RC	0.303 ± 0.073		0.063 ± 0.022*	0.068 ± 1.3E-05*	0.047 ± 0.012*	0.126 ± 0.020*	0.463 ± 0.040*
	R	**0.039 ± 0.018**		ND	ND	ND	0.095 ± 0.125	ND
DEX:IPT	BS			1.410 ± 0.497	0.746 ± 1.4E-04*	1.309 ± 0.327	0.911 ± 0.148	1.433 ± 0.124*
	DC		1	0.494 ± 0.193	0.243 ± 0.044*	0.768 ± 0.337	0.358 ± 0.062*	4.535 ± 0.449*
	D		**2.573 ± 1.057**	1.175 ± 0.486	**0.076 ± 0.005**	**59.850 ± 6.585***	**1.279 ± 0.219**	**7.027 ± 1.629**
	RC		0.599 ± 0.231	0.385 ± 0.192*	0.367 ± 0.117*	0.093 ± 0.033*	0.276 ± 0.051*	0.559 ± 0.048*
	R		**2.135 ± 0.757**	0.662 ± 0.310	**0.075 ± 0.009***	0.086 ± 0.009*	**0.003 ± 7.3E-04***	**2.804 ± 0.483**
SAG:IPT	BS		ND	0.504 ± 0.177*	0.756 ± 1.4E-04*	0.316 ± 0.079*	0.790 ± 0.129*	1.427 ± 0.123*
	DC		0.206 ± 0.073	0.753 ± 0.323*	0.310 ± 0.065*	1.148 ± 0.737	0.416 ± 0.097*	4.721 ± 1.895*
	D		0.283 ± 0.101	1.223 ± 0.493	0.167 ± 0.063	**5.583 ± 0.621**	0.679 ± 0.147*	3.676 ± 0.579
	RC		0.072 ± 0.026	0.788 ± 0.278	0.671 ± 1.2E-04*	0.364 ± 0.091*	0.373 ± 0.061*	1.902 ± 0.164*
	R		**0.607 ± 0.215**	1.520 ± 0.536*	**0.708 ± 1.3E-04***	0.566 ± 0.142	**0.657 ± 0.107***	**2.980 ± 0.257**
Col-0 CK	BS			1	1	1	1	1
	DC			0.712 ± 0.315*	0.141 ± 0.024	4.532 ± 0.539*	0.424 ± 0.079*	3.701 ± 0.497*
	D			0.458 ± 0.161	**0.048 ± 9.0E-06***	**3.671 ± 0.089***	0.477 ± 0.078*	**1.097 ± 0.272***
	RC			0.732 ± 0.258	0.436 ± 8.1E-05*	0.253 ± 0.063*	0.326 ± 0.053*	0.871 ± 0.075*
	R			1.203 ± 0.424	**0.755 ± 1.4E-04***	**0.730 ± 0.183**	**0.468 ± 0.076**	**1.327 ± 0.114***

HvCKX2, cytokinin oxidase/dehydrogenase 2 from Hordeum vulgare; IPT Agro, isopentenyl transferase from Agrobacterium; CKX1, cytokinin oxidase/dehydrogenase 1; IPT3, isopentenyl transferase 3; RD29B, responsive to desiccation 29B; P5CS1, δ-1-pyrroline-5-carboxylate synthase 1; NCED3, 9-cis-epoxycarotenoid dioxygenase 3. The expression was determined before stress application–BS (25 DAS); in well-watered plants at the end of drought period–DC (38 DAS); in drought stressed plants–D (38 DAS); in well-watered plants at the end of recovery period–RC (44 DAS); and in re-watered plants–R (44 DAS). Transcription values represent means ± SD (n = 6) normalized to WT before stress (25 DAS). Statistically significant (p < 0.05, two-sample t-test) differences between the corresponding variants of WT and transformants are marked (*), and between D/DC or R/RC variants within each genotype (bold). NA, not analyzed; ND, not detected.

up-regulated transcription of the gene for the rate-limiting biosynthetic enzyme *9-cis-epoxycarotenoid dioxygenase NCED3* (**Table 2**). Drought resulted in mild elevation of JA as well as of its active conjugate jasmonate-isoleucine (JA-Ile; **Figure 6**). Significant increase was observed in the case of salicylic acid (SA; **Figure 7**), while no significant change was observed in the level of the ethylene precursor aminocyclopropane carboxylic acid (ACC; **Figure 8**). Expression of two selected drought stress marker genes, *responsive to desiccation 29B* (*AtRD29B*) and *δ-1-pyrroline-5-carboxylate* synthase (*P5CS1*) were strongly up-regulated in drought (**Table 2**).

Re-watering was accompanied by vigorous growth, at a rate exceeding the growth rate of the corresponding control plants (reaching about 180% of control; **Table 1**). Growth rate acceleration was accompanied by high levels of tZ and *trans*-zeatin riboside (tZR), as well as of IAA, generally high above those in the corresponding controls (**Figures 3**, **4**). Increase of tZ-type CKs corresponded with up-regulation of *AtIPT3* due to re-watering (**Table 2**). ABA content was substantially diminished (**Figure 5**). However, the levels of the other analyzed hormones JA, JA-Ile, SA and ACC returned to control values (**Figures 6**–**8**).

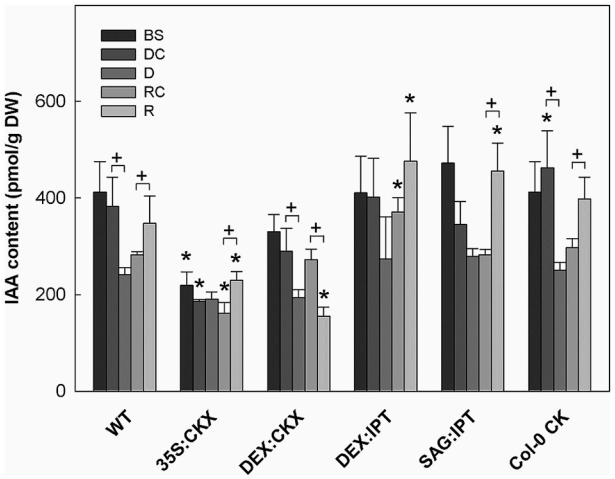

FIGURE 4 | The content of auxin indole-3-acetic acid (IAA). Specification of genotypes and treatments is as described in **Figure 2**. Data represent means ±SD ($n = 6$) in pmol per g DW. Statistically significant differences ($p < 0.05$, two-sample *t*-test) in IAA content between the corresponding variants of WT and transformants are marked (*), and between D/DC or R/RC variants within each genotype (+).

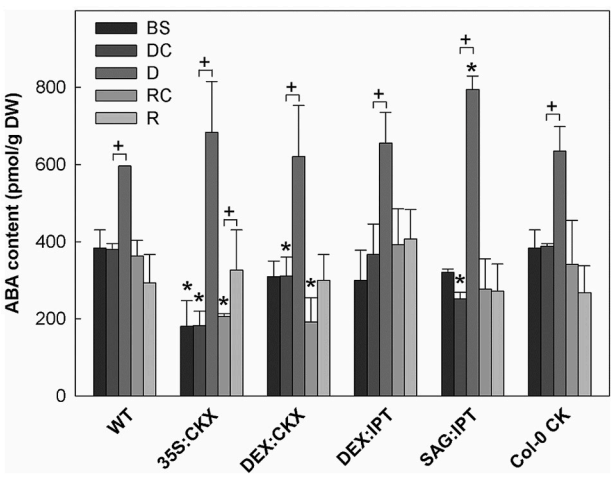

FIGURE 5 | The content of abscisic acid (ABA). Specification of genotypes and treatments is as described in **Figure 2**. Data represent means ±SD ($n = 6$) in pmol per g DW. Statistically significant differences ($p < 0.05$, two-sample *t*-test) in ABA content between the corresponding variants of WT and transformants are marked (*), and between D/DC or R/RC variants within each genotype (+).

Constitutive CK Down-Regulation (35S:CKX)

At well-watered conditions, *AtCKX1* overexpression under *35S* promoter led to ca 150-fold increase of its transcript level in comparison with WT (**Table 2**). This resulted in substantially lowered levels of active CKs, especially of tZ and isopentenyladenine (iP; **Figure 3**), as well as of their transport forms – tZR and isopentenyladenosine (iPR; Table S4).

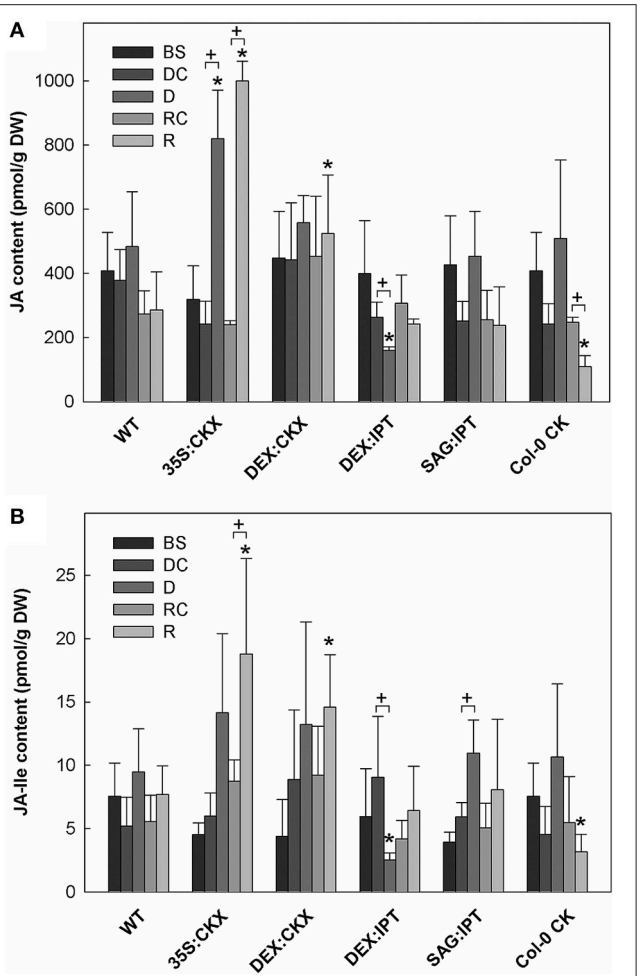

FIGURE 6 | The content of **(A)** jasmonic acid (JA); and **(B)** jasmonate-isoleucine (JA-Ile). Specification of genotypes and treatments is as described in **Figure 2**. Data represent means ±SD ($n = 6$) in pmol per g DW. Statistically significant differences ($p < 0.05$, two-sample *t*-test) in JA and JA-Ile content between the corresponding variants of WT and transformants are marked (*), and between D/DC or R/RC variants within each genotype (+).

Surprisingly, the content of cZ was higher than in WT. The enhanced *AtCKX1* expression resulted in strong down-regulation of the levels of all CK precursors (CK phosphates; to 14 % of WT) as well as of CK deactivation products — CK N- and O-glucosides (to ca 6 and 31% of WT, respectively; Table S4). Total CK levels reached only 20% of WT. Expression of *AtIPT3* was the highest from the followed genotypes (**Table 2**). Down-regulation of CKs was also associated with diminished content of other hormones — IAA (to ca 53% of WT), ABA (to ca 47%), to minor extent also of JA and JA-Ile (to ca 78%; **Figures 4**–**6**). Strong down-regulation of active CKs had severe impact on plant morphology – decrease of the growth rate (to ca 50% of WT; Tables S2, S3), smaller size of the rosettes, significantly higher compactness (wider blades and shorter petioles) and increase of leaf thickness. The basal expression of stress-associated genes, *AtRD29B* and *P5CS1*, was up-regulated in comparison with WT (**Table 2**).

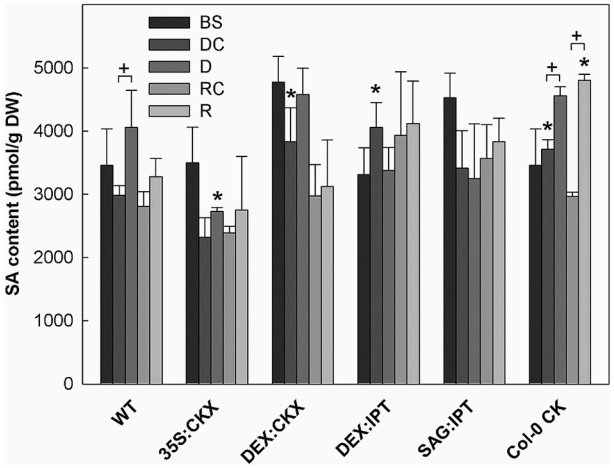

FIGURE 7 | The content of salicylic acid (SA). Specification of genotypes and treatments is as described in **Figure 2**. Data represent means ±SD ($n = 6$) in pmol per g DW. Statistically significant differences ($p < 0.05$, two-sample *t*-test) in SA content between the corresponding variants of WT and transformants are marked (*), and between D/DC or R/RC variants within each genotype (+).

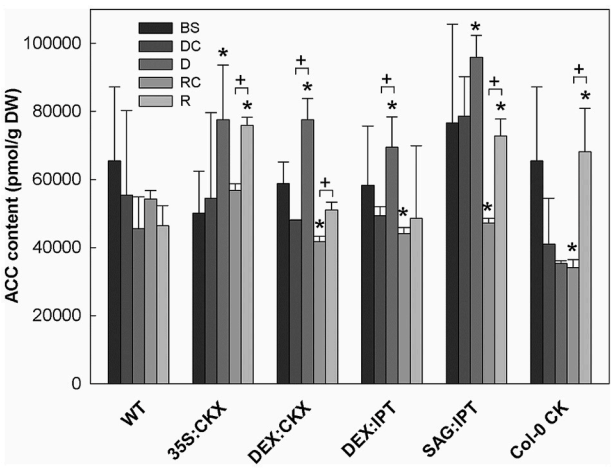

FIGURE 8 | The content of the ethylene precursor aminocyclopropane carboxylic acid (ACC). Specification of genotypes and treatments is as described in **Figure 2**. Data represent means ±SD ($n = 6$) in pmol per g DW. Statistically significant differences ($p < 0.05$, two-sample *t*-test) in ACC content between the corresponding variants of WT and transformants are marked (*), and between D/DC or R/RC variants within each genotype (+).

Drought response of 35S:CKX plants involved slight further decrease of the growth rate (**Table 1**), low transcription of dehydration-responsive gene *AtRD29B* and proline-biosynthetic gene *AtP5CS1* (**Table 2**) as well as relatively lower water loss (by 20% in comparison with watered control at the end of drought, *vs.* 23% in the case of WT; **Figure 2**). Stress had relatively minor effect on rosette area at the early phase of the drought response and growth rate was maintained (**Table 1**, Table S2). Fifth day after stress initiation, the growth rate of 35S:CKX plants started decreasing faster compared with the other genotypes. Higher drought stress tolerance was associated with enhanced quantum efficiency of photosystem II (F_v/F_m) in comparison with stressed WT (Figure S2). Drought had relatively low impact on active CKs (decrease by ca 20% of well-watered 35S:CKX plants; **Figure 3**), as well as CK ribosides (by ca 30%; Table S4). Stress-induced down-regulation of CK precursors was only negligible. Transcription of *AtIPT3* was relatively high in drought (**Table 2**). No significant change was observed in IAA content in comparison with well-watered conditions, which was still lower than in the other drought stressed variants (**Figure 4**). ABA was induced by drought to the level comparable to those reached in the other genotypes, in spite of considerably lower value under control conditions (**Figure 5**). Drought resulted in substantial elevation of JA and to minor extent also of its active conjugate JA-Ile (**Figure 6**). Only slight elevation was found in the case of SA (**Figure 7**). Elevation of ACC was the most profound from all genotypes (**Figure 8**).

Growth re-initiation during recovery was in 35S:CKX plants much slower and smaller in comparison with the other genotypes (substantial delay by 2 days; **Table 1**, Table S2). After 7 days, the growth rate reached about 37% of recovered WT (Table S3B). After re-watering, the levels of active CKs were even lower than in drought due to the strong decrease of cZ (**Figure 3**). Only CK precursors (CK phosphates) increased after re-watering by about ca 90% in comparison with drought-stressed plants (Table S4). The content of IAA was elevated moderately above the level in the corresponding control (**Figure 4**). ABA, ACC and especially JA and JA-Ile content stayed elevated well above the control level during the whole followed period (**Figures 5**, **6**, **8**).

CK Down-Regulation Induced at the Stress Onset (DEX:CKX)

Morphology of DEX:CKX plants was indistinguishable from WT before the stimulation of *HvCKX2* expression at the stress onset. Up-regulation of *HvCKX2* expression by dexamethasone in both well-watered and drought-stressed DEX:CKX plants led to substantial decrease of the growth rate (in the case of watered plants to ca 80% of watered WT; in the case of stressed plants to ca 72% of stressed WT; Table S3). Growth suppression at the end of stress was 87% in comparison with the activated control plants (**Table 1**). Up-regulation of *HvCKX2* expression was associated with strong reduction of tZ (in well-watered conditions and in drought to 9 and 15% of non-induced plants before stress, respectively; **Figure 3**), tZR (to ca 51 and 39%, respectively) and of all CK phosphates (to ca 17 and 13%, respectively; Table S4). Also CK N- and O-glucosides were substantially decreased (Table S4). Stimulated *HvCKX2* expression resulted in strong suppression of endogenous *AtCKX1* transcription (which was further promoted by drought), as well as in relatively high transcription of *AtIPT3*, not affected by stress (**Table 2**). CK suppression by *HvCKX2* overexpression was accompanied by mild decrease of IAA, which was substantially promoted by drought (**Figure 4**). The impact of drought on elevation of ABA or ACC content was high (**Figures 5**, **8**). Mild positive trend was found in the regulation of SA and JA/JA-Ile levels, not reaching

statistical significance (**Figures 6**, **7**). Nevertheless, transcription of the stress marker genes *AtRD29B* and *AtP5CS1* was low, similar to 35S:CKX transformant (**Table 2**). The water loss was the lowest in comparison with the other tested genotypes (by only ca 15% in comparison with well-watered control; **Figure 2**).

Re-watering resulted in only slight up-regulation of active CKs in comparison with the corresponding drought-stressed plants (by ca 17% at the end of recovery period; **Figure 3**), while CK ribosides were elevated much more (by ca 129%; Table S4). The growth reactivation was thus much slower and smaller than in WT. The growth rate reached just about 123% of the corresponding control at the end of recovery period (**Table 1**). No up-regulation of IAA in comparison with stress conditions was observed (**Figure 4**). ABA, JA-Ile and ACC content remained slightly higher than in the corresponding control (**Figures 5**, **6B**, **8**). JA, similarly to SA, was close to the control level (**Figures 6A**, **7**).

CK Up-Regulation Induced at the Stress Onset (DEX:IPT)

After induction with dexamethasone, the DEX:IPT transformants expressed CK biosynthetic gene *isopentenyl transferase* (*ipt)* from *Agrobacterium tumefaciens*. Transcription of endogenous *AtIPT3* was down-regulated (**Table 2**). The activation of *ipt* gene led in control plants to huge increase of tZ (ca 10-times compared to WT; **Figure 3**), tZR (ca 13-times) and tZR phosphate (ca 28-times), as well as of dihydrozeatin (DZ) riboside phosphate (ca 50-times), CK N- (ca 7-times) and O-glucosides (ca 14-times; Table S4). Stimulation of *ipt* under well-watered conditions resulted in the mild increase of ABA (as well as of its metabolites); while the other stress hormones JA and ACC were slightly decreased (**Figures 5**, **6**, **8**, Table S5).

During the drought progression, growth rate of DEX:IPT plants gradually declined, similarly as in WT (Figure S1, **Table 1**, and Table S2). Leaf water loss at the end of drought was higher than in WT (loss by 30% in comparison with 23% in WT; **Figure 2**). *AtIPT3* transcription was strongly suppressed and *AtCKX1* transcription was enhanced (**Table 2**). Transcription of stress-marker genes was high. Drought caused decrease of active CKs by 52% in comparison with activated well-watered control (**Figure 3**). Nevertheless, tZ highly prevailed. The IAA content decreased (**Figure 4**). Up-regulation of IAA catabolites – OxIAA and its glucosyl ester was observed (Table S5). ABA content was strongly up-regulated by drought, as well as its metabolites (**Figure 5**, Table S5). At the end of drought, JA and JA-Ile were diminished, while ACC was moderately increased (**Figures 6**, **8**).

Recovery was associated with fast growth activation, one of the most pronounced among the tested genotypes (comparable with the effect of exogenous CK application; **Table 1**). At the end of 6-day recovery period, growth rate reached about 215% of the corresponding control. The active CK content was elevated by ca 19%, and IAA content even by ca 75% in comparison with drought conditions (**Figures 3**, **4**). ABA content decreased to the level of induced control, remaining higher than in WT (**Figure 5**). SA was slightly enhanced in comparison with WT plants (**Figure 7**).

CK Up-Regulation Induced During Stress Progression (SAG:IPT)

Stimulation of *ipt* expression by *SAG12* promoter resulted in substantially different CK dynamics in comparison with DEX:IPT plants. In well-watered plants, no elevation of active CKs was observed 38 DAS (**Figure 3**). Only tZR and tZR phosphate were increased (by about 300 and 60%, respectively), which may indicate stimulation of *SAG12* promoter activity given by the initiation of the senescence program (Table S4). Significant increase of active CKs (by 46%) was observed at the end of experiment (44 DAS; **Figure 3**); *AtIPT3* transcription was down-regulated, while *AtCKX1* transcription increased with plant age (**Table 2**). Simultaneously, moderate decrease of ABA, JA, and SA was found in comparison with 25th DAS (**Figures 5**–**7**).

During drought progression, initial drop of growth rate occurred earlier than in DEX:IPT plants; however, in later stages (after 4 days, 29 DAS) relatively higher growth rate was maintained (Figure S1, **Table 1**, and Table S2). Decrease of water content was the same as in WT (**Figure 2**). The transcription of stress marker genes was low (**Table 2**). Under drought stress, SAG:IPT plants exhibited significantly higher F_v/F_m than WT (Figure S2). SAG:IPT was the only genotype, which responded to drought by the increase of active CKs (more than 4-times in comparison with well-watered control). Apart from tZ, also DZ and cZ were elevated (**Figure 3**). The *AtIPT3* transcription was diminished, while *CKX1* transcription was increased in comparison with the corresponding well-watered control (**Table 2**). Drought stress resulted in down-regulation of IAA, which, however, remained slightly higher than in the other stressed genotypes (**Figure 4**). Stress response was associated with strong ABA elevation, the highest among the tested genotypes (**Figure 5**). Simultaneously, increase of JA and especially of JA-Ile was observed (**Figure 6**). In the case of ACC mild elevation was also observed in stressed plants (**Figure 8**).

Recovery was associated with increased growth rate (by 165% of the corresponding well-watered control; recovered SAG:IPT grew by 7% faster than recovered WT; Figure S1, **Table 1**, and Table S3B). Simultaneously, active CK levels still remained higher than in WT (ca 159% of WT; **Figure 3**). Up-regulation of active CKs was observed at the end of experiment also in well-watered plants (to ca 182% of WT), probably due to undergoing senescence. The transcription of *AtIPT3* increased to the level of young plants (25 DAS; **Table 2**). Recovery was associated with IAA elevation, high above the level of the corresponding control (**Figure 4**). The contents of stress hormones ABA and JA were low (**Figures 5**, **6**).

CK Up-Regulation Achieved by Application of Exogenous CK (Col-0 CK)

The exogenous application of aromatic CK *meta*-topolin had minor negative effect on endogenous active CKs (with exception of cZ), which coincided with substantial suppression of *AtIPT3* transcription (**Figure 3**, **Table 2**). Under control conditions, IAA synthesis was stimulated, while level of JA, and to minor extent also of JA-Ile, was decreased after abrupt CK up-regulation (**Figures 4**, **6**).

Drought imposed gradual decrease of the growth rate, similar to WT (Figure S1, **Table 1**, and Table S2). Application of CK resulted in higher water loss than in WT (37 vs. 23% at the end of drought; **Figure 2**). Drought response of CK treated plants was associated with the decrease of endogenous active CK levels (by 40% in comparison with the well-watered control; **Figure 3**), CK ribosides (by 32%) and CK phosphates (by 50%; Table S4). The most profound effect was observed in the case of tZ-type and iP-type CKs. Transcription of *AtIPT3* was strongly suppressed (**Table 2**). Drought stress caused substantial down-regulation of IAA, associated with mild up-regulation of OxIAA (**Figure 4**, Table S5) and more profound increase of its glucosyl ester (results not shown). ABA was strongly up-regulated by drought, together with its catabolites (mainly phaseic acid and 9′-hydroxy-ABA; **Figure 5**, Table S5). The other stress hormones, SA, JA and JA-Ile were only slightly elevated (**Figures 6**, **7**), while ACC decreased (**Figure 8**).

After re-watering, the growth rate of CK treated plants increased fastest of all tested variants (**Table 1**). At the end of recovery period, the growth rate was by 21% higher than that of WT, reaching about 204% of the corresponding treated control (**Table 1**, Table S3B). The level of active CKs increased substantially (by ca 20% in comparison with the control and ca 4-times in comparison with the corresponding drought stressed plants). CK phosphates increased more than 4-times in comparison with drought stressed-plants, which correlated well with highly increased *AtIPT3* transcription (**Figure 3**, **Table 2**, and Table S4). Recovery was associated with IAA elevation, well above the level in the corresponding control (**Figure 4**). Recovery led to reduction of ABA, JA, and JA-Ile contents (**Figures 5**, **6**).

The Impact Modulated Cytokinin Content on the Other Hormones

Modulation of the content of one hormone usually results in changes of the other hormone levels, in order to keep the desirable hormone ratio. Close relationship exists between CKs and auxins. Both CKs and auxins are indispensable for cell cycle progression and thus for cell division (Laureys et al., 1998). They are necessary for coordination of the growth of the above- and under-ground parts of the plant. Their ratio controls morphology of growing tissues (Miller et al., 1955). Under control conditions, CK suppression by *CKX* overexpression was accompanied by decrease of IAA (**Figure 4**). This feature was evident especially in case of long-term CK down-regulation in 35S:CKX transformant. Accordingly, the application of exogenous CK promoted IAA formation.

Another hormone, which exhibits an intensive cross-talk with CKs, is ABA. CK/ABA ratio affects stomata aperture (e.g., Skalák et al., 2016), which is crucial for regulation of the water loss, but also for the supply of carbon dioxide for photosynthesis. In well-watered conditions, the lowest ABA content was determined in 35S:CKX plants (decrease of ABA by ca 53% in comparison with WT correlated well with decrease of active CKs by ca 50%; **Figure 5**).

Multivariate Analyses Highlight Similarities in Observed Patterns

We performed a set of multivariate analyses to identify similarities in observed patterns. The hierarchical clustering and PCA analyses of leaf area dynamics showed a clear separation of stressed plants from their respective controls with the exception being 35S and DEX-inducible *CKX* overexpresser lines, which exhibited relatively minor stress impact. Analyses also showed that the total leaf areas of DEX:IPT and Col-0 CK plants had the best pattern match and their close similarity is reflected also in the drought:control ratios of leaf area and growth rates (data not shown). The most informative proved to be the PCA of growth rates (**Figure 9**) that separates drought stress along the PC2 and individual genotypes/treatments along the PC1.

PCA analysis of active hormones (namely active CKs, IAA, ABA, JA, SA, and ACC; **Figure 10**) revealed distinct response of drought-stressed SAG:IPT plants, which enhanced CK levels during stress progression (PC1). The clustering of stressed DEX:IPT plants with well-watered plants of all genotypes showed the effect of enhanced CK levels on delay in activation of stress defense, which may diminish drought impact (at least in the stress strength used in our experiment). Distribution along PC1 illustrates a link between the stress hormones and low active CKs - cZ and dihydrozeatin. Separation along PC3 demonstrated the importance of the stress hormones, namely of ABA, JA, ACC, and SA in drought response. Clustering of 35S:CKX plants in drought stress and after rewatering reflected maintenance of enhanced defense mechanisms in this genotype also after the stress release (at least for some period).

PCA analysis of all determined hormone-related metabolites (**Figure 11**) showed clear separation of tested genotypes

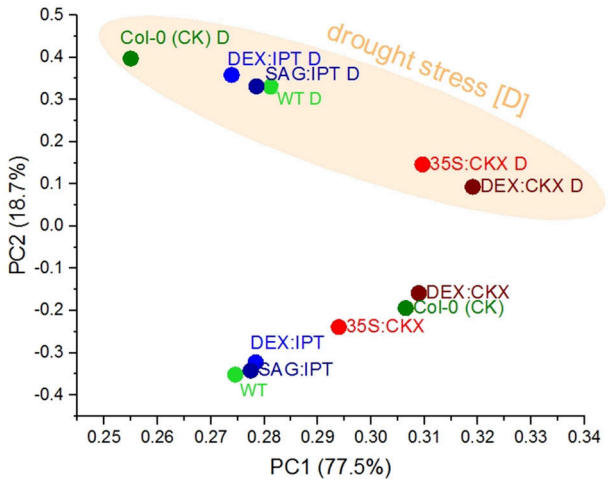

FIGURE 9 | Effects of drought and cytokinin pool size on growth rates of *Arabidopsis* plants. The growth rates of individual *Arabidopsis* lines recorded during the drought stress and recovery period and of their respective controls were analyzed by principal component analysis (PCA). The PCA clearly separates the drought stressed plants from the control groups along the PC2, and PC1 illustrates a similarity in the growth rate dynamics between lines with the constitutive and inducible CKX expression.

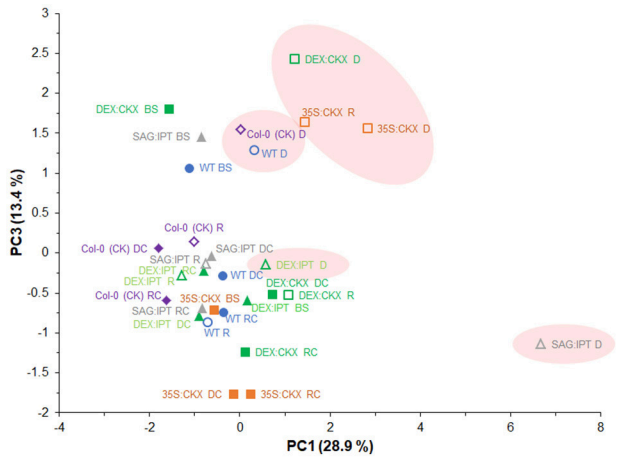

FIGURE 10 | Effects of drought and modulation of cytokinin metabolism on the levels of active hormones (active CKs, IAA, ABA, JA, SA and ACC) in *Arabidopsis* plants. The hormone levels in individual variants determined before stress (BS), during the drought stress (D) and recovery period (R) and of their respective controls (DC, RC) were analyzed by principal component analysis (PCA). PC1 illustrates a link among the stress hormones ABA, JA, ACC and cZ as well as dihydrozeatin. Separation along PC3 clusters stressed and non-stressed plants according to the content of stress hormones ABA, JA, SA and ACC, showing distinct behavior of SAG:IPT and DEX:IPT.

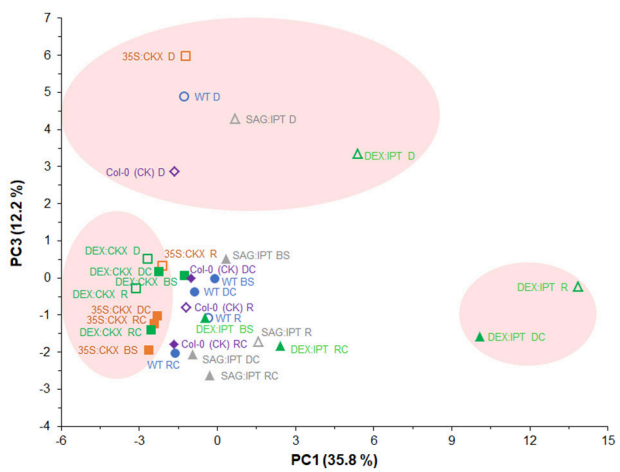

FIGURE 11 | Effects of drought and modulation of cytokinin metabolism on the content of all determined hormone-related metabolites in *Arabidopsis* plants. Metabolite profiling recorded before stress (BS), during the drought stress (D) and recovery period (R) and of their respective controls (DC, RC) were analyzed by principal component analysis (PCA). PC1 clustered variants according to CKs content. The PCA confirmed the importance of ABA (and its metabolites) and of JA/JA-Ile in drought response of all experimental variants (PC3). Clustering of DEX:IPT plants after re-watering (R) and of drought-stressed control (DC) along the PC1 indicates positive effect of CKs on the recovery.

according to CK content along the PC1. Separation along PC3 confirmed the importance of ABA (and its metabolites) and of JA/JA-Ile in drought response of all experimental variants. Clustering of DEX:CKX plants close to its well-watered control with strongly stimulated CK degradation indicates that down-regulation of active CKs is an inherent stress response. Clustering of DEX:IPT plants after re-watering (R) and of drought-stressed control (DC) indicates positive effect of CKs in recovery.

DISCUSSION

Tendency of Transgenic Plants to Re-establish Hormonal Homeostasis

Regulation of Endogenous Cytokinin Levels

Modification of phytohormone levels in plants is usually achieved by overexpression of the genes for biosynthetic or degradation enzymes (Gan and Amasino, 1995; Werner et al., 2001). However, the final effect on hormonal pool depends not only on the activity of the introduced genes but also on the plant response to the disturbed hormonal homeostasis (**Figures 10**, **11**).

In activated DEX:CKX plants, the overexpression of CK catalytic enzyme *HvCKX2* led to suppression of endogenous *AtCKX1* expression and up-regulation of expression of CK biosynthetic gene *AtIPT3* (**Table 2**). Thus, expression of CK biosynthetic gene(s) was promoted by intensive CK degradation, in order to diminish CK imbalance. Low levels of CK precursors seemed to indicate high CK turn-over due to the fast degradation rather than down-regulated CK biosynthesis (Table S4). Simultaneously, the other deactivation pathways (CK N- and O-glucosylation) were down-regulated, which might suggest tendency to maintain CK homeostasis as much as possible (Table S4).

Accordingly, overexpression of *ipt* in DEX:IPT or SAG:IPT genotypes led to the inhibition of endogenous *AtIPT3* transcription and in the case of SAG:IPT also to stimulation of expression of endogenous *AtCKX1* (**Table 2**). In both genotypes O-glucosylation pathway was promoted (Table S4). Similar effects as *ipt* expression were imposed by application of exogenous CK *meta*-topolin. The modulation of CK metabolism had significant effect on the resulting levels of active CKs in all genotypes.

Apart from the introduced gene, important role is played by the promoter (**Figures 9**–**11**). Constitutive *AtCKX1* expression under 35S promoter strongly decreased the growth rate of the transformed plants (Figure S1, **Table 1**), having also significant impact on plant morphology (enhanced root growth, thicker and darker leaves; Werner et al., 2001). Use of dexamethasone-inducible promoter allowed distinguishing of the impact of slow growth from the morphological effects. The *ipt* induction by dexamethasone at the drought stress onset (supported by two stimulations during the stress progression) and stress-induced *ipt* expression under *SAG12* promoter enabled to compare the impact of the timing of CK elevation. Our data indicated better performance of the transformant with the construct containing senescence-inducible promoter *SAG12* than the dexamethasone-inducible one.

Plant Responses to Drought

In all tested genotypes, drought response was associated with suppression of growth, accompanied with down-regulation of active CK levels (especially of tZ), even in transformants with

strongly stimulated *ipt* gene expression (**Figures 3**, **9–11**, Figure S1, **Table 1**, and Table S2). This is in accordance with other reports on different species, e.g., tomato (Kudoyarova et al., 2007). The only exception in CK regulation was SAG:IPT genotype, due to the gradual activation of *SAG12* promoter by drought-strengthened senescence (Vanková et al., 2012).

During drought progression, all genotypes gradually diminished their growth rate (Figure S1, **Table 1**). Metabolism of 35S:CKX plants was adjusted to slow growth even under control conditions, which was accompanied by enhanced basal level of the stress associated transcripts (e.g., *AtP5CS1*; **Table 2**). These results evidence high "preparedness" of 35S:CKX transformants for the unfavorable conditions (**Figure 9**). 35S:CKX plants were not affected by mild stress; however, they responded very effectively to severe drought (fast and strong decrease of the growth rate – ca 32% of stressed WT, as well as strong increase of ABA levels; **Figure 5**, Figure S1, Table S2). Dexamethasone-induced stimulation of *HvCKX2* substantially down-regulated the growth rate, both under control and stress conditions (**Figure 9**). After the initial drop caused by *CKX* activation, the growth rate inhibition followed the same trend as in WT (at the level of ca 75% of stressed WT; Table S2). Sudden decrease of active CKs at the stress onset might contribute to stomata closure, as this variant preserved the water content best from the studied genotypes.

Stimulation of *ipt* with dexamethasone slightly promoted plant growth under control conditions (Figure S1, Table S2). However, in drought, the growth rate was suppressed even faster than in WT (**Table 1**). Mild increase of ABA was found in DEX:IPT plants, in comparison with WT (by ca 10%; **Figure 5**), probably in order to balance, at least partially, the elevated CK content. Nevertheless, CK/ABA ratio was still enhanced (ca 0.15 vs. ca 0.03), which might be the reason of higher water loss in this genotype in comparison with WT and especially with CKX overexpressers. Similar negative effect on water relations was observed also after application of exogenous CK *meta*-topolin.

SAG:IPT plants were the only genotype, which exhibited elevation of the active CK content during drought progression (in comparison with non-stressed control more than 4-times; **Figure 3**). This increase was given by promotion of *SAG12* promoter activity by drought, which was shown in detail in tobacco (Vanková et al., 2012). Stimulation of *ipt* expression led to elevation of active CKs as well as of iPR phosphate and cZ riboside O-glucoside together with down-regulation of *AtIPT3* and up-regulation of *AtCKX1* transcription (**Figure 3**, **Table 2**, and Table S4). The CK dynamics of SAG:IPT differed from the dynamics of DEX:IPT. No sudden CK increase took place in SAG:IPT plants, which might be, together with the most profound ABA elevation (**Figure 5**), the reason of the lower water loss. In the case of SAG:IPT, the up-regulation of CKs occurred also in well-watered plants at the end of the experiment (**Figure 3**), probably due to the promoter activation by the onset of natural senescence.

In spite of the fact that JA is the key hormone in the defense to necrotroph or herbivore attack, the increasing evidence has shown that it plays an important role also in drought response (Djilianov et al., 2013), which was confirmed by our PCAs (**Figures 10**, **11**). Increase of JA and/or JA-Ile (to higher or lower extent) was observed in all genotypes with exception of DEX:IPT plants, which might reflect antagonistic relationship between JA and CKs in regulation of senescence program, chlorophyll content and cell division (e.g., Liu et al., 2016; **Figure 6**).

Plant Response During the Recovery Phase (After Re-watering)

Recovery was associated with vigorous growth, accompanied by high elevation of active CKs (**Figure 3**). Within 1 day after re-watering, growth rates of all variants accelerated and exceeded those of corresponding well-watered plants, with the exception of both CKX overexpressers (Figure S1, **Table 1**). These genotypes with down-regulated CK levels (predominantly 35S:CKX) maintained elevated ABA levels and exhibited high levels of JA as well as of JA-Ile (higher than controls; **Figures 5**, **6**). PCA analyses (**Figures 10**, **11**) indicated that CKX plants (especially 35S:CKX) remained (at least for some time) prepared for another stress period.

In contrast, the fastest recovery and the highest growth rate reached in the end of recovery period were found in the variant sprayed with *meta*-topolin and in DEX:IPT plants (**Figure 9**, Figure S1, and **Table 1**). These lines with up-regulated CKs (especially DEX:IPT) enhanced predominantly tZ-type CKs, including tZ–the most physiologically active CK in the stimulation of cell division (**Figure 3**, Table S4). Similar response was exhibited also by WT. The plants treated with exogenous CK maintained significantly higher SA levels than the corresponding controls (**Figure 7**). Together with DEX:IPT, they strongly down-regulated JA content during recovery (**Figure 6**).

Both *ipt* transformants maintained high expression level of the introduced gene also during the recovery period, which was associated with very high content of CK phosphates (mainly tZR phosphate) as well as of CK transport form tZR (**Tables 2**, S4). Simultaneously, CK deactivation pathways were active, which indicates tight regulation of CK homeostasis.

CONCLUSIONS

The possibility to enhance plant drought tolerance by modulation of CK levels depends on the stress duration, severity (soil water potential) and speed of dehydration. The actual conditions substantially affect performance of the transformants.

Various determined parameters, e.g., shoot area expansion rate, indicated that the most drought tolerant genotype was 35S:CKX line. In this case, both low CK levels and permanent adaptation to slow growth (plant morphology, up-regulated basal transcription of stress-related genes, e.g., *P5CS1*) contributed to the stress tolerance. Constant up-regulation of defense (before and after stress period) indicated better "preparedness" of this CKX genotype to stress. Dwarf shoot phenotype, however, is not in favor of potential application in agriculture.

Dexamethasone-induced down-regulation of active CKs resulted in the decrease of the growth rate. Transient character

of *CKX* overexpression was associated with earlier switch-off of defense mechanisms after stress release, in comparison with 35S:CKX plants. However, sudden CK decrease after *HvCKX2* stimulation had a significant positive effect on plant water relations, probably due to the promotion of stomata closure.

Up-regulation of CK levels at the stress onset (repeated twice during the stress progression) by induction of *ipt* expression or by application of stable aromatic CK *meta*-topolin, had highly significant positive effect on plant recovery after rehydration, diminishing the stress effects. However, CK elevation was associated with higher water loss, which might be disadvantageous in case of severe long-term stress.

The up-regulation of CKs driven by senescence inducible promoter (SAG:IPT plants) led to slower recovery in comparison with DEX:IPT plants (comparable with WT). However, the water loss was substantially diminished. SAG:IPT transformant has been shown to be the most advantageous at least in conditions of relatively short-term drought stress.

Our results illustrate different plant strategies to cope with drought and demonstrate that CKs play an important role in both stimulatory and "quiescent" plant approach.

AUTHOR CONTRIBUTIONS

RV, FF, NK, US, and SP contributed conception and design of this study; RV, RP, US, and FF arranged for financial support; BB, MČ, LS, and JH provided Arabidopsis seeds and information about plant physiology; SP, NK, FF, and JH performed the experiment; VK, PD, AG, SP, and NK analyzed samples; RV, SP, FF, MČ, and TV evaluated results; RV, SP, and FF wrote the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00655/full#supplementary-material

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Cytokinins: their impact on molecular and growth responses to drought stress and recovery in *Arabidopsis*

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Table S1. The list of primers used in RT-qPCR.

Gene	**Forward**	**Reverse**
AtUBQ10 (At4g05320)	gaagttcaatgtttcgtttcatgt	ggattatacaaggccccaaaa
HvCKX2 (AF490591)	aacgctcactgaagggaa	attctcggggcagcaag
ipt from *Agrobacterium*	atcctccctcaagaataagc	ctgaaaggaacgacgc
AtCKX1 (At2g41510)	caccttggcaattctacat	tgtccttgaagcgagtga
AtIPT3 (At3g63110)	ttttcaggaacgagcagt	ttggaccttcgctttgta
AtRD29B (At5g52300)	caggatcaacggtgatgaca	tgcgtctccttcactccac
AtP5CS1 (At2g39800)	aacgccagcacaagattc	cctctcattatccatctcgttgt
AtNCED3 (At3g14440)	gacgagaatctcaagagtg	ccgagcatgtttctgttgac

Table S2. Daily growth rates of all experimental variants. Growth rate of each plant was calculated from rosette area in mm^2 determined by RGB measurement. Mean growth rate ± SD was calculated from growth rates of each variant in corresponding time (n = 15-40). Measurement was performed before stress (22-25 DAS), during drought (28-38 DAS) and during recovery (39-44 DAS). Statistically significant differences ($p < 0.01$, two-sample t-test) between the corresponding variants (drought or control) of WT and transformants within each time-point are marked by *; and between stressed and control variants within each genotype in each time-point are marked in bold.

DAS	WT		35S:CKX		DEX:CKX	
	control	stressed	control	stressed	control	stressed
22	0.248 ± 0.017	0.247 ± 0.019	0.124 ± 0.023*	0.122 ± 0.016*	0.245 ± 0.022	0.247 ± 0.018
23	0.245 ± 0.047	0.236 ± 0.028	0.141 ± 0.026*	0.133 ± 0.033*	0.243 ± 0.038	0.238 ± 0.035
24	0.227 ± 0.029	0.213 ± 0.027	0.132 ± 0.025*	0.128 ± 0.033*	0.228 ± 0.026	0.213 ± 0.035
25	0.212 ± 0.032	0.225 ± 0.025	0.125 ± 0.026*	0.120 ± 0.033*	0.225 ± 0.032	0.234 ± 0.029
28	0.183 ± 0.043	0.178 ± 0.030	0.089 ± 0.023*	0.091 ± 0.031*	0.139 ± 0.023*	0.130 ± 0.021*
29	0.193 ± 0.036	**0.159 ± 0.036**	0.089 ± 0.022*	**0.064 ± 0.028***	0.137 ± 0.030*	0.123 ± 0.031*
30	0.182 ± 0.028	**0.130 ± 0.032**	0.091 ± 0.027*	**0.046 ± 0.016***	0.152 ± 0.024*	**0.120 ± 0.022**
31	0.145 ± 0.034	0.148 ± 0.038	0.075 ± 0.025*	**0.050 ± 0.024***	0.105 ± 0.029*	0.105 ± 0.025*
32	0.179 ± 0.036	**0.111 ± 0.028**	0.067 ± 0.024*	**0.034 ± 0.019***	0.118 ± 0.027*	**0.081 ± 0.021***
35	0.152 ± 0.035	**0.086 ± 0.032**	0.070 ± 0.023*	**0.012 ± 0.016***	0.109 ± 0.018*	**0.063 ± 0.023***
36	0.130 ± 0.030	**0.035 ± 0.026**	0.038 ± 0.022*	**0.003 ± 0.017***	0.067 ± 0.024*	**0.046 ± 0.022**
37	0.194 ± 0.032	**0.037 ± 0.026**	0.085 ± 0.016*	**0.009 ± 0.019***	0.114 ± 0.025*	**0.026 ± 0.026**
38	0.138 ± 0.031	**0.020 ± 0.029**	0.082 ± 0.016*	**0.001 ± 0.015***	0.120 ± 0.024*	**0.016 ± 0.026**
39	0.124 ± 0.024	0.143 ± 0.033	0.059 ± 0.012*	**0.005 ± 0.032***	0.091 ± 0.023*	**0.053 ± 0.027***
42	0.118 ± 0.034	**0.227 ± 0.024**	0.059 ± 0.017*	**0.083 ± 0.018***	0.097 ± 0.018	**0.125 ± 0.024***
44	0.083 ± 0.013	**0.148 ± 0.022**	0.036 ± 0.019*	0.049 ± 0.016*	0.069 ± 0.027	0.085 ± 0.024*

DAS	DEX:IPT		SAG:IPT		Col-0 CK	
	control	stressed	control	stressed	control	stressed
22	0.241 ± 0.022	0.242 ± 0.021	0.246 ± 0.025	0.248 ± 0.013	0.248 ± 0.019	0.239 ± 0.021
23	0.236 ± 0.035	0.249 ± 0.037	0.255 ± 0.057	0.247 ± 0.023	0.241 ± 0.037	0.236 ± 0.034
24	0.218 ± 0.028	0.218 ± 0.027	0.220 ± 0.025	0.224 ± 0.026	0.224 ± 0.024	**0.205 ± 0.027**
25	0.234 ± 0.038	0.216 ± 0.032	0.234 ± 0.037	0.234 ± 0.029	0.227 ± 0.029	0.216 ± 0.029
28	0.186 ± 0.035	0.168 ± 0.029	0.196 ± 0.032	**0.173 ± 0.019**	0.164 ± 0.034	0.147 ± 0.042*
29	0.220 ± 0.039*	**0.144 ± 0.031**	0.205 ± 0.030	**0.166 ± 0.019**	0.208 ± 0.044	**0.115 ± 0.063***
30	0.200 ± 0.029	**0.128 ± 0.026**	0.189 ± 0.030	**0.165 ± 0.026***	0.166 ± 0.034	**0.113 ± 0.042**
31	0.155 ± 0.029	**0.130 ± 0.044**	0.158 ± 0.028	0.140 ± 0.035	0.173 ± 0.034*	**0.121 ± 0.048**
32	0.175 ± 0.037	**0.071 ± 0.021***	0.186 ± 0.030	**0.111 ± 0.028**	0.141 ± 0.032*	**0.074 ± 0.034***
35	0.137 ± 0.029	**0.045 ± 0.020***	0.150 ± 0.034	**0.077 ± 0.030**	0.129 ± 0.028*	**0.064 ± 0.028***
36	0.139 ± 0.028	**0.020 ± 0.027**	0.140 ± 0.027	**0.033 ± 0.027**	0.116 ± 0.031	**0.025 ± 0.028**
37	0.177 ± 0.034	**0.017 ± 0.027***	0.205 ± 0.040	**0.028 ± 0.027**	0.153 ± 0.043*	**0.007 ± 0.041***
38	0.134 ± 0.038	**0.017 ± 0.027**	0.150 ± 0.039	**0.013 ± 0.036**	0.124 ± 0.034	**0.019 ± 0.031**
39	0.130 ± 0.039	0.152 ± 0.066	0.136 ± 0.031	0.117 ± 0.041	0.126 ± 0.038	**0.185 ± 0.044***
42	0.119 ± 0.035	**0.209 ± 0.037**	0.133 ± 0.028	**0.252 ± 0.033**	0.129 ± 0.041	**0.200 ± 0.048**
44	0.080 ± 0.025	**0.173 ± 0.036**	0.096 ± 0.026	**0.158 ± 0.021**	0.088 ± 0.030	**0.179 ± 0.043**

Table S3. A) Ratio of mean growth rate of non-stressed transformants and mean growth rate of non-stressed WT (expressed in %). B) Ratio of mean growth rate of stressed genotype and mean growth rate of stressed WT (in %). The individual mean growth rates are shown in Table S2.

A)

DAS	35S:CKX	DEX:CKX	DEX:IPT	SAG:IPT	Col-0 CK	
22	50.1	98.8	97.5	99.5	100.0	
23	57.5	99.1	96.4	104.4	98.5	
24	58.0	100.7	96.1	97.2	99.0	
25	58.9	106.0	110.1	110.3	106.9	
28	48.9	76.3	101.7	107.1	89.6	drought
29	46.0	70.9	113.8	106.4	107.8	
30	50.0	83.7	109.9	104.0	91.5	
31	51.6	72.1	107.0	108.8	119.1	
32	37.3	65.8	98.3	104.3	79.2	
35	46.0	72.1	90.5	98.7	85.2	
36	29.3	51.7	106.7	107.2	89.5	
37	43.8	58.7	91.5	105.5	79.1	
38	59.3	86.7	96.8	108.5	90.0	
39	47.3	73.3	105.2	109.6	101.5	re-watering
42	49.8	82.5	101.0	112.2	109.2	
44	42.8	82.7	96.9	115.6	105.7	

B)

DAS	35S:CKX	DEX:CKX	DEX:IPT	SAG:IPT	Col-0 CK	
22	49.3	100.2	98.2	100.4	96.7	
23	56.4	100.7	105.6	104.6	100.0	
24	60.3	99.9	102.3	105.2	96.5	
25	53.3	103.9	96.0	103.7	96.0	
28	51.3	72.7	94.1	97.2	82.6	drought
29	40.6	77.4	90.6	104.4	72.4	
30	35.3	92.2	98.1	126.4	86.8	
31	33.5	70.8	87.3	94.2	81.3	
32	30.5	72.6	63.8	99.8	66.4	
35	13.8	72.6	52.1	89.1	73.8	
36	9.3	72.4	56.9	93.5	70.5	
37	24.7	70.4	45.9	73.8	18.8	
38	4.3	78.3	85.6	62.4	91.5	
39	3.7	36.9	106.7	81.9	129.6	re-watering
42	36.7	54.9	92.0	111.0	88.0	
44	33.4	57.6	117.2	107.0	121.2	

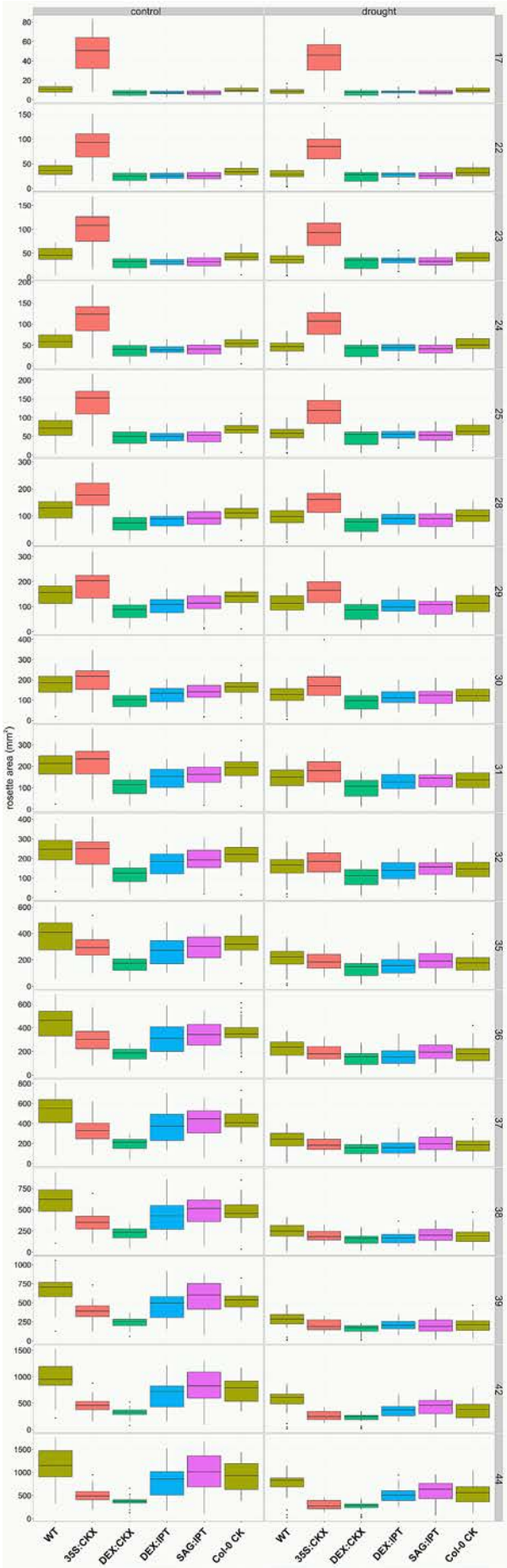
Table S4. The impact of drought stress and recovery on cytokinin (CK) metabolites in different genotypes. CK Ribosides: *trans*-zeatin riboside, dihydrozeatin riboside, isopentenyladenosine, *cis*-zeatin riboside. CK Phosphates: *trans*-zeatin riboside phosphate, dihydrozeatin riboside phosphate, isopentenyladenosine phosphate, *cis*-zeatin riboside phosphate. CK N-glucosides: *trans*-zeatin-7-glucoside, *trans*-zeatin-9-glucoside, dihydrozeatin-7-glucoside, dihydrozeatin-9-glucoside, isopentenyladenine-7-glucoside, isopentenyladenine-9-glucoside, *cis*-zeatin-9-glucoside. CK O-glucosides: *trans*-zeatin-O-glucoside, *trans*-zeatin riboside-O-glucoside, dihydrozeatin riboside-O-glucoside, *cis*-zeatin riboside-O-glucoside. Values give mean concentrations of phytohormones in pmol/g DW ± SD (n = 6). Statistically significant ($p < 0.05$, two-sample t-test) differences between the corresponding variants of WT and transformant are marked by *, and between D/DC or R/RC variants within each genotype in bold.

		CK Ribosides	CK Phosphates	CK N-glucosides	CK O-glucosides
WT	**before stress**	274.8 ± 18.5	25.2 ± 7.3	1810.3 ± 411.5	122.0 ± 49.9
WT	**drought control**	241.3 ± 39.5	19.8 ± 2.8	1342.1 ± 281.6	161.7 ± 65.9
WT	**drought**	**90.9 ± 11.7**	**7.4 ± 2.2**	1087.8 ± 156.3	156.5 ± 32.1
WT	**recovery control**	201.2 ± 50.3	20.0 ± 6.0	644.7 ± 193.4	89.9 ± 36.0
WT	**recovery**	296.1 ± 59.5	**36.0 ± 7.2**	**1400.9 ± 131.7**	165.1 ± 71.5
35S:CKX	**before stress**	38.2 ± 12.9*	12.7 ± 1.4*	102.3 ± 25.1*	38.2 ± 15.37*
35S:CKX	**drought control**	37.7 ± 20.7*	10.3 ± 3.1*	116.8 ± 60.2*	77.4 ± 42.9
35S:CKX	**drought**	41.0 ± 9.5*	9.0 ± 3.6	140.5 ± 28.3*	**204.8 ± 36.7**
35S:CKX	**recovery control**	66.5 ± 12.8*	10.7 ± 1.5*	131.1 ± 16.9*	100.2 ± 17.5
35S:CKX	**recovery**	71.9 ± 12.8*	**17.1 ± 4.0***	125.2 ± 12.5*	84.0 ± 24.9
DEX:CKX	**before stress**	56.1 ± 14.0*	14.6 ± 4.4	372.9 ± 111.9*	194.9 ± 78.0
DEX:CKX	**drought control**	10.3 ± 2.6*	3.0 ± 0.9*	360.5 ± 17.3*	134.0 ± 44.5
DEX:CKX	**drought**	7.4 ± 1.8*	1.8 ± 0.6*	**239.9 ± 65.7***	**86.4 ± 3.0***
DEX:CKX	**recovery control**	79.2 ± 2.9*	16.1 ± 1.6	362.5 ± 11.1*	96.0 ± 5.3
DEX:CKX	**recovery**	**31.1 ± 1.4***	**10.5 ± 1.4***	**205.5 ± 15.9***	**49.3 ± 4.0***
DEX:IPT	**before stress**	251.3 ± 10.7	18.4 ± 5.5	1425.6 ± 307.3	125.5 ± 56.2
DEX:IPT	**drought control**	3356.8 ± 839.2*	191.2 ± 57.4*	10155.3 ± 3046.6*	1866.0 ± 746.4*
DEX:IPT	**drought**	**1523.6 ± 380.9***	84.9 ± 25.5*	7319.6 ± 2195.9*	1568.3 ± 627.3*
DEX:IPT	**recovery control**	856.9 ± 214.2*	91.2 ± 27.4*	4167.4 ± 1250.2*	740.8 ± 296.3*
DEX:IPT	**recovery**	**2786.2 ± 696.6***	300.0 ± 90.0*	17075.0 ± 5122.5*	3198.9 ± 1279.6*
SAG:IPT	**before stress**	295.9 ± 74.0	6.4 ± 1.9*	2237.8 ± 671.4	111.3 ± 44.5
SAG:IPT	**drought control**	361.9 ± 90.5	29.0 ± 1.3*	1374.1 ± 412.2	197.5 ± 38.9
SAG:IPT	**drought**	345.8 ± 23.1*	**89.4 ± 26.8***	1506.6 ± 124.5*	**623.7 ± 53.2***
SAG:IPT	**recovery control**	641.7 ± 18.7*	79.7 ± 3.9*	1250.3 ± 69.2*	321.2 ± 9.8*
SAG:IPT	**recovery**	**1006.6 ± 21.1***	**175.3 ± 9.8***	**1984.2 ± 595.3**	**497.2 ± 37.3***
WT	**before stress**	274.8 ± 18.5	25.2 ± 7.3	1810.3 ± 411.5	122.0 ± 49.9
Col-0 CK	**drought control**	50.4 ± 2.6*	6.3 ± 0.2*	310.4 ± 16.5*	143.6 ± 29.1
Col-0 CK	**drought**	**34.4 ± 1.8***	**3.2 ± 0.3***	**404.1 ± 52.6***	110.1 ± 19.7
Col-0 CK	**recovery control**	175.9 ± 17.3	29.2 ± 5.5	397.6 ± 39.5*	79.8 ± 5.8
Col-0 CK	**recovery**	194.3 ± 8.1*	**14.8 ± 1.1***	**519.4 ± 10.1***	**133.9 ± 19.2**

Table S5. Auxin and abscisic acid (ABA) metabolites. OxIAA – 2-oxindole-3-acetic acid; PAA – phenylacetic acid; DPA – dihydrophaseic acid; PA – phaseic acid; 9OH-ABA – 9´-hydroxy-ABA. Values give mean concentrations of phytohormones in pmol/g DW ± SD (n = 6). Before stress application – BS (25 DAS); well-watered plants at the end of drought period – DC (38 DAS); drought stressed plants – D (38 DAS); well-watered plants at the end of recovery period – RC (44 DAS); re-watered plants – R (44 DAS). Values give mean concentrations of phytohormones in pmol/g DW ± SD (n = 6). Statistically significant ($p < 0.05$, two-sample t-test) differences between the corresponding variants of WT and transformant are marked by *, and between D/DC or R/RC variants within each genotype in bold.

		OxIAA	**PAA**	**DPA**	**PA**	**9OH-ABA**
WT	**BS**	3719.6 ± 1859.8	4578.4 ± 667.4	1478.5 ± 198.6	261.9 ± 65.5	192.3 ± 57.7
WT	**DC**	4370.1 ± 2501.1	4236.1 ± 804.0	1109.5 ± 100.5	355.6 ± 131.6	159.3 ± 47.8
WT	**D**	5790.7 ± 2895.3	3572.1 ± 1071.9	**1573.2 ± 216.7**	**817.6 ± 37.9**	**293.8 ± 74.2**
WT	**RC**	4257.8 ± 2128.9	1648.4 ± 576.9	709.0 ± 148.9	347.2 ± 72.9	113.7 ± 60.5
WT	**R**	8555.7 ± 4277.8	2191.5 ± 724.5	**1834.2 ± 904.0**	378.0 ± 85.0	106.6 ± 21.8
35S:CKX	**BS**	5101.1 ± 2550.6	5130.8 ± 382.7	661.4 ± 138.9*	76.0 ± 18.4*	32.7 ± 9.8*
35S:CKX	**DC**	11408.6 ± 5704.3	4412.1 ± 783.3	812.9 ± 359.3	169.7 ± 35.4*	46.3 ± 5.1*
35S:CKX	**D**	18532.0 ± 9266.0*	3989.5 ± 816.7	**2100.3 ± 879.8**	**1187.8 ± 249.4***	**287.7 ± 119.1**
35S:CKX	**RC**	13642.6 ± 6821.3*	2026.9 ± 709.4	595.9 ± 125.1	200.6 ± 96.9	34.8 ± 10.4*
35S:CKX	**R**	19837.7 ± 9918.9	4197.1 ± 1958.4	1026.2 ± 334.5	146.4 ± 32.2*	53.7 ± 16.7*
DEX:CKX	**BS**	1108.3 ± 554.2*	4333.9 ± 1201.9	1461.4 ± 306.9	114.5 ± 24.1*	128.3 ± 38.5
DEX:CKX	**DC**	4081.8 ± 902.5	8346.8 ± 2921.4*	1399.1 ± 103.9*	201.1 ± 19.2	102.7 ± 19.9
DEX:CKX	**D**	2449.8 ± 1182.9	**3053.6 ± 960.1**	**971.3 ± 171.4***	306.4 ± 103.8*	164.1 ± 69.4
DEX:CKX	**RC**	2225.9 ± 1112.9	906.2 ± 267.9	422.2 ± 13.7*	145.7 ± 24.5*	46.5 ± 1.2*
DEX:CKX	**R**	1623.1 ± 619.6*	831.2 ± 202.0*	**670.7 ± 13.0***	**99.1 ± 3.0***	41.7 ± 1.3*
DEX:IPT	**BS**	3004.1 ± 1502.1	3993.1 ± 4897.6	1176.7 ± 261.2	111.2 ± 53.9*	92.1 ± 27.6*
DEX:IPT	**DC**	7115.5 ± 2878.3	3335.5 ± 973.9	1341.5 ± 272.3	345.8 ± 135.5	148.9 ± 44.7
DEX:IPT	**D**	10115.7 ± 3981.2	2569.7 ± 716.4	2398.5 ± 894.4	**1117.3 ± 110.2***	**338.2 ± 65.5**
DEX:IPT	**RC**	8822.4 ± 5586.4	2623.7 ± 918.3	916.4 ± 191.4	505.6 ± 148.5	151.0 ± 77.0
DEX:IPT	**R**	15318.0 ± 7166.6	3813.6 ± 1029.0	**1972.1 ± 296.9**	707.3 ± 276.9	223.2 ± 102.6
SAG:IPT	**BS**	2131.8 ± 1065.9	5060.4 ± 771.1	1885.3 ± 395.9	237.7 ± 49.9	141.8 ± 42.5
SAG:IPT	**DC**	1468.8 ± 453.3	4952.3 ± 1733.3	630.9 ± 42.7*	187.3 ± 24.2*	102.1 ± 27.0
SAG:IPT	**D**	**4268.2 ± 257.9**	**2089.2 ± 326.8***	**1440.2 ± 141.9**	**955.9 ± 168.0**	**312.5 ± 36.7**
SAG:IPT	**RC**	1568.7 ± 784.4	690.6 ± 20.3*	871.6 ± 89.6	363.9 ± 23.9	144.9 ± 43.5
SAG:IPT	**R**	**2925.6 ± 527.7***	**2749.8 ± 170.4**	**1604.4 ± 246.1**	375.5 ± 78.9	146.6 ± 12.3*
WT	**BS**	3719.6 ± 1859.8	4578.4 ± 667.4	1478.5 ± 198.6	261.9 ± 65.5	192.3 ± 57.7
Col-0 CK	**DC**	2326.4 ± 771.9	1626.8 ± 569.4*	1466.6 ± 64.3*	425.6 ± 17.2	194.0 ± 26.4
Col-0 CK	**D**	2850.0 ± 358.7	2021.4 ± 651.9	1459.7 ± 49.5	**934.3 ± 64.3***	**344.4 ± 48.7**
Col-0 CK	**RC**	1587.7 ± 320.6*	443.7 ± 73.2*	946.2 ± 160.4	469.7 ± 47.6*	168.1 ± 4.5
Col-0 CK	**R**	**4599.7 ± 811.8**	**2039.1 ± 142.1**	**1513.9 ± 94.3**	431.9 ± 25.8	156.5 ± 10.3*

Figure S1. Rosette area of WT, 35S:CKX, DEX:CKX, DEX:IPT, SAG:IPT plants, and Col-0 after *meta*-topolin treatment (Col-0 CK) in mm^2. Drought period lasted from 25 to 38 DAS; recovery period lasted from 38 to 44 DAS. Whiskers represent ± 1.5 x interquartile range (IQR).


control
drought
rosette area (mm²)
17
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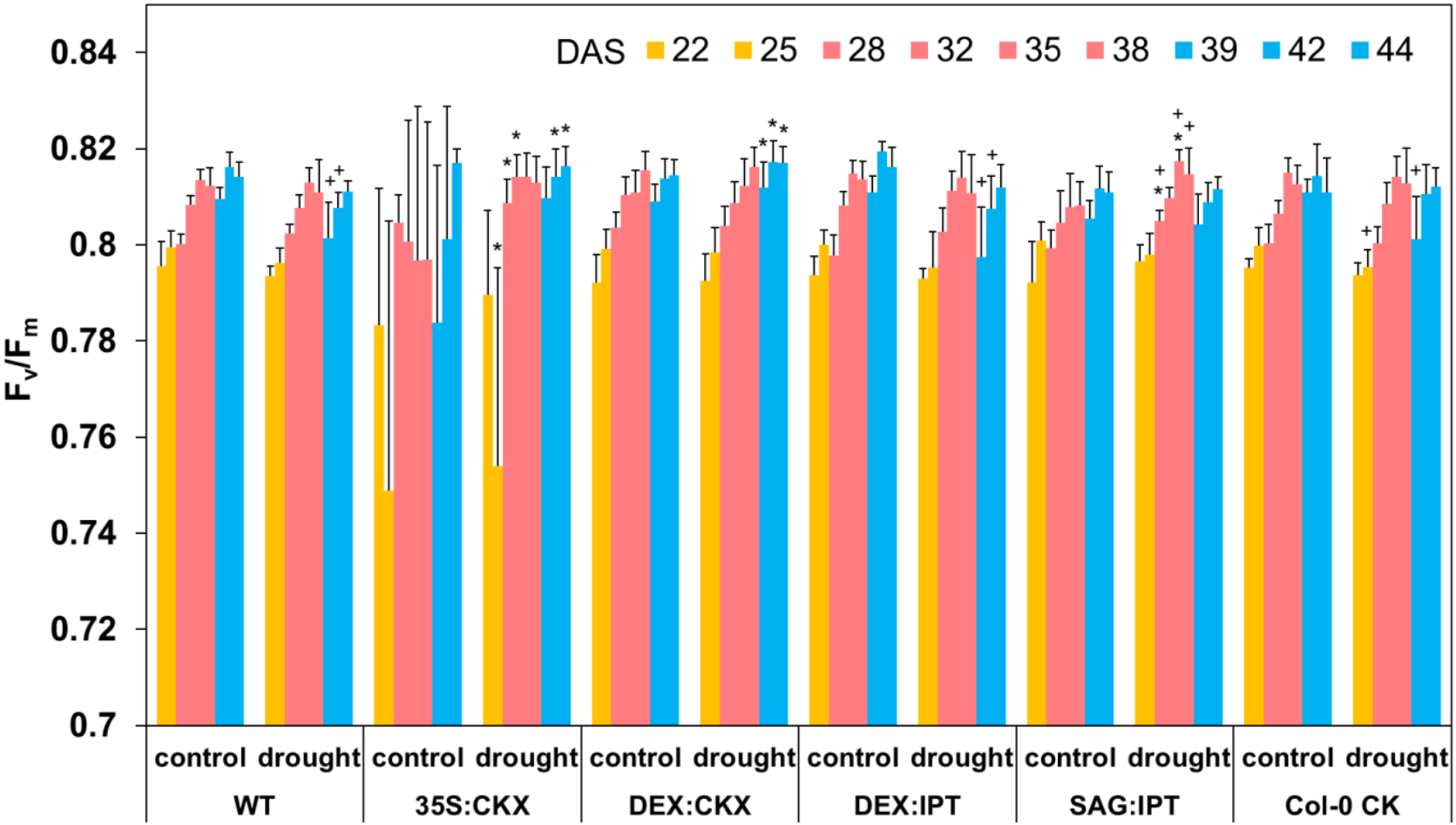
42
44
WT
35S:CKX
DEX:CKX
DEX:IPT
SAG:IPT
Col-0 CK

Figure S2: Chlorophyll fluorescence parameter Fv/Fm changes in time. Before stress – 22 and 25 DAS; Drought – 28, 32, 35, 38 DAS; Recovery – 39, 42, 44 DAS. Data represent means ± SD (n = 6-13). Statistically significant differences ($p < 0.01$, two-sample t-test) between WT and a transformant of according variant (drought or control) in each time-point are marked by *; and between D/DC or R/RC variants within each genotype in each time-point are marked by +.



3rd Article

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ZnO nanoparticle effects on hormonal pools in *Arabidopsis thaliana*

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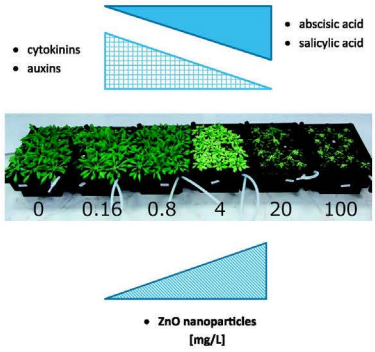
HIGHLIGHTS

- Growth suppression of Arabidopsis plants correlates with ZnO nanoparticle content.
- Growth inhibition is associated with decrease of cytokinins and auxins in apices.
- Nanoparticle stress effects are indicated by abscisic and salicylic acids elevation.

GRAPHICAL ABSTRACT



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ABSTRACT

At present, nanoparticles have been more and more used in a wide range of areas. However, very little is known about the mechanisms of their impact on plants, as both positive and negative effects have been reported. As plant interactions with the environment are mediated by plant hormones, complex phytohormone analysis has been performed in order to characterize the effect of ZnO nanoparticles (mean size 30 nm, concentration range 0.16–100 mg L^{-1}) on *Arabidopsis thaliana* plants. Taking into account that plant hormones exhibit high tissue-specificity as well as an intensive cross-talk in the regulation of growth and development as well as defense, plant responses were followed by determination of the content of five main phytohormones (cytokinins, auxins, abscisic acid, salicylic acid and jasmonic acid) in apices, leaves and roots. Increasing nanoparticle concentration was associated with gradually suppressed biosynthesis of the growth promoting hormones cytokinins and auxins in shoot apical meristems (apices). In contrast, *cis*-zeatin, a cytokinin associated with stress responses, was elevated by 280% and 590% upon exposure to nanoparticle concentrations 20 and 100 mg L^{-1}, respectively, in roots. Higher ZnO nanoparticle doses resulted in up-regulation of the stress hormone abscisic acid, mainly in apices and leaves. In case of salicylic acid, stimulation was found in leaves and roots. The other stress hormone jasmonic acid (as well as its active metabolite jasmonate isoleucine) was suppressed at the presence of nanoparticles.
The earliest response to nanoparticles, associated with down-regulation of growth as well as of cytokinins and auxins, was observed in apices. At higher dose, up-regulation of abscisic acid, was detected. This increase, together with elevation of the other stress hormone - salicylic acid, indicates that plants sense nanoparticles as severe stress. Gradual accumulation of *cis*-zeatin in roots may contribute to relatively higher stress resistance of this tissue.

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0048-9697/

1. Introduction

The rapid development of nanotechnology has resulted in production and utilization of a number of nanoparticles of varying composition, size and shape characteristics (Nair and Chung, 2014a). The nanoparticles have been applied in multiple industrial, agricultural as well as medicinal areas. Their wide-spread use is inevitably associated with their release into the environment, creating a potential risk, as the knowledge of their interactions with biological systems is mostly lacking. Plants, as the main source of food and feed, represent an important component of the food chain. Thus, an understanding of the fate of nanoparticles in plants and of their impact on plant growth, metabolism and yield is highly desirable.

Both positive and negative effects of nanoparticles on plants have been reported (for review, see Arruda et al., 2015). Seed germination and root growth were found promoted, e.g., in case of zinc oxide nanoparticles (Prasad et al., 2012; Adhikari et al., 2016), silver nanoparticles (Syu et al., 2014) or carbon nanotubes (Martinez-Ballesta et al., 2016). Toxic effects were reported as well, e.g., in case of zinc oxide nanoparticles (Zhang et al., 2015a, 2015b). Thus, not only the particle composition, but many other factors like their concentration, size, shape, charge, exposure time, plant species and age, as well as type of the substrate should be taken into consideration.

Generally, nanoparticle application leads to an increase of the level of reactive oxygen species (e.g., H_2O_2) with subsequent activation of the antioxidant system (Zhao et al., 2012). The oxidative stress may be accompanied with DNA damage, as indicated by transient stimulation of the expression of DNA mismatch repair genes (Nair and Chung, 2014b). Transcriptomic studies revealed upon nanoparticle application also up-regulation of the expression of genes related to abiotic and biotic stress responses (Thiruvengadam et al., 2015; Landa et al., 2015). Nanoparticle exposure may result in the change of transcription of genes involved in biosynthesis or signal transduction of plant hormones, e.g., of auxin repressor or auxin response genes, abscisic acid (ABA) biosynthetic genes or ethylene signaling components (Kaveh et al., 2013; Syu et al., 2014).

Zinc oxide nanoparticles (ZnO NP) are produced in high amounts. At present their production reaches approximately 550 tons per year (Piccinno et al., 2012). Due to a wide application of zinc oxide, e.g., in cosmetics (UV-protection in sunscreens), in paints or as anticorrosive, antibacterial and antifungal agents, further increase of ZnO NP use can be anticipated. Therefore we focused on evaluation of the impact of ZnO NP on *Arabidopsis thaliana* plants. As plant growth and development as well as plant interactions with the environment are regulated by plant hormones, we followed the response of Arabidopsis to ZnO NP at the level of cytokinins (CKs), auxin, ABA, salicylic acid (SA) and jasmonic acid (JA). The changes in hormone pools were correlated with the physiological performance of the plants.

2. Materials and methods

2.1. Plant material and growth conditions

Arabidopsis thaliana Columbia 0 plants were cultivated in hydroponic conditions in cultivation containers (Araponics SA, Belgium) using 25% Hoagland solution as a cultivation medium. The pH of the medium was adjusted to 6.2–6.3 prior to autoclaving. Plants were cultivated at 21 °C with 8/16 h light/dark cycle, at a light intensity of 130 μmol m^{-2} s^{-1} in a growth cabinet (MLR-350, Sanyo Electric Co., Japan) with aeration of cultivation media every 3 h for 15 min.

2.2. Treatment of plants with ZnO NPs

Four-week old plants were exposed to ZnO NPs (average particle size 30 nm, specific surface area 70 m^2 g^{-1}, purity 99.9%; MKnano, Canada). The nanoparticles were added to the cultivation media to reach final concentrations of 0.16, 0.8, 4, 20, and 100 mg L^{-1}. After two-week exposure to ZnO NPs, the weight of rosettes and length of roots were determined. Samples of apices, leaves and roots were quickly cut, weighed, flash frozen in liquid nitrogen and stored at −80 °C until further analysis.

2.3. Hormone analysis

Samples were purified and analyzed as described earlier (Dobrev and Kaminek, 2002; Dobrev and Vankova, 2012). Samples (ca 20 mg fresh weight) were homogenized with a ball mill and extracted with cold (−20 °C) methanol/water/formic acid (15/4/1 v/v/v). The following stable isotope-labeled internal standards (10 pmol/sample) were added: $^{13}C_6$-IAA (indole-3-acetic acid, Cambridge Isotope Laboratories); $^2H_4$-SA (Sigma-Aldrich); $^2H_3$-PA (phaseic acid), $^2H_3$-DPA (dihydrophaseic acid, NRC-PBI), $^2H_6$-ABA, $^2H_5$-JA, $^2H_5$-transZ (*trans*-zeatin), $^2H_5$-transZR (*trans*-zeatin riboside), $^2H_5$-transZ7G (*trans*-zeatin N7-glucoside), $^2H_5$-transZ9G (*trans*-zeatin N9-glucoside), $^2H_5$-transZOG (*trans*-zeatin O-glucoside), $^2H_5$-transZROG (*trans*-zeatin riboside O-glucoside), $^2H_5$-transZRMP (*trans*-zeatin riboside phosphate), $^2H_3$-DHZ (dihydrozeatin), $^2H_3$-DHZR (dihydrozeatin riboside), $^2H_3$-DHZ9G (dihydrozeatin N9-glucoside), $^2H_6$-iP (isopentenyladenine), $^2H_6$-iPR (isopentenyladenosine), $^2H_6$-iP7G (isopentenyladenine N7-glucoside), $^2H_6$- iP9G (isopentenyladenine N9-glucoside), $^2H_6$-iPRMP (isopentenyladenosine phosphate) (Olchemim). Extract was applied to a mixed mode reverse phase–cation exchange SPE column (Oasis-MCX, Waters). Two hormone fractions were sequentially eluted: (1) fraction A, eluted with methanol (auxins, ABA, SA, JA); and (2) fraction B, eluted with 0.35 M NH_4OH in 60% methanol (CKs). Fractions were evaporated to dryness, dissolved in 10% methanol and analyzed using high-performance liquid chromatograph (HPLC) (Ultimate 3000, Dionex) coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer (3200 Q TRAP, Applied Biosystems), column Luna C18(2) (100 × 2 mm, 3 μm, Phenomenex), flow rate 0.25 mL min^{-1}. Quantification of hormones was done by isotope dilution with multilevel calibration curves ($r^2 > 0.99$) (Ljung et al., 2005). Data processing was carried out with the Analyst 1.5 software (Applied Biosystems).

2.4. Statistical analysis

Data on the hormone values (n = 6; two independent biological experiments, in each three biological replicates) were analyzed using a multi-factorial ANOVA. The significance of the differences among the measured hormone values was assessed using a *t*-test.

3. Results

3.1. Effect of ZnO NPs on growth of Arabidopsis plants

Exposure to different concentrations of ZnO NPs in the range 0.16–100 mg L^{-1} resulted in substantial phenotypic changes in Arabidopsis plants (Fig. 1). The lowest ZnO NP concentration of 0.16 mg L^{-1} had an only negligible influence on shoot growth in comparison to control plants. Application of 0.8 mg L^{-1} had already a negative effect, which, however, did not reach statistical significance (Table 1). Substantial negative impact was observed at 4 mg L^{-1} ZnO NP, very strong growth inhibition being found in case of 20 and 100 mg L^{-1}. The two highest ZnO NP concentrations resulted in substantial elevation of anthocyanin content in leaves

Fig. 1. *Arabidopsis thaliana* plants exposed for two-weeks to ZnO nanoparticles in concentration range 0–100 mg L^{-1}.

Table 1
The effect of two-week exposure to ZnO nanoparticles (in concentration range 0–100 mg L^{-1}) on the biomass of *Arabidopsis thaliana* rosettes and the root length. Statistical differences from the control values ($p \leq 0.01$) are indicated by *.

ZnO NP concentration (mg L^{-1})	Rosette fresh mass (g)	Root length (cm)
0	1.01 ± 0.29	20.98 ± 1.29
0.16	0.98 ± 0.25	20.05 ± 4.84
0.8	0.82 ± 0.20	18.63 ± 2.62
4	0.46 ± 0.13*	12.66 ± 2.12*
20	0.19 ± 0.04*	12.97 ± 1.26*
100	0.13 ± 0.04*	10.35 ± 1.78*

(Fig. 1). Dependence of the root length on ZnO NP concentration followed a similar profile as the mean rosette biomass (Table 1). The nanoparticle effect, however, was not so strong (maximal inhibition by 50%, in comparison with a ca 87% inhibition in the case of shoot biomass).

3.2. Cytokinins

Plant cell division and growth are regulated predominantly by plant hormones CKs and auxins. The active CKs include *trans*-zeatin, isopentenyladenine, dihydrozeatin and *cis*-zeatin. As *cis*-zeatin, a CK with relatively low physiological activity (ca 20–50 times lower in comparison to the most active *trans*-zeatin), is often involved in stress responses, its content was not summarized with the other active CKs. The increasing content of ZnO NPs in media had the most significant effect on active CK levels in apices (Fig. 2). Severe growth inhibition at 20 and 100 mg L^{-1} was associated with strong suppression of active CK content in this meristematic tissue. The effect of ZnO NPs on active CKs in leaves was relatively moderate (with exception of the highest NP concentration). Surprisingly, active CK content in roots was increasing at higher ZnO NP levels, mainly due to accumulation of *cis*-zeatin.

CK phosphates represent direct precursors of active CKs. Their levels in apices gradually decreased with increasing stress strength (Fig. 3). In leaves, CK phosphate homeostasis was more-or-less maintained. In roots, elevation of CK biosynthesis was observed during mild and moderate stress (0.8 and 4 mg L^{-1}, respectively). Strong stress was accompanied with CK phosphate suppression.

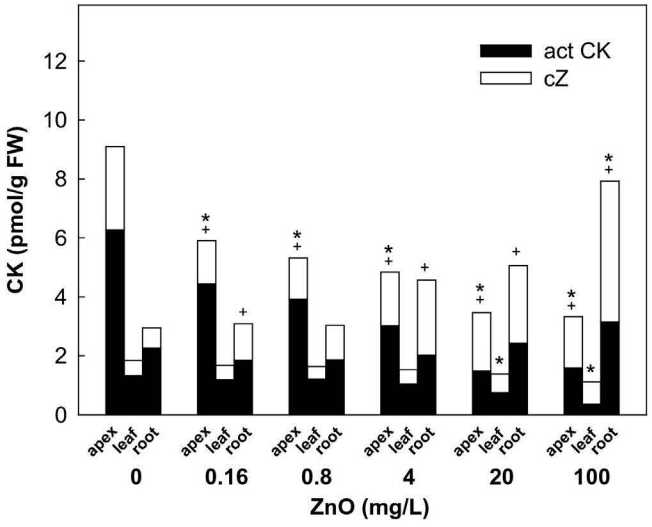

Fig. 2. The content of active cytokinins (*trans*-zeatin, isopentenyladenine, dihydrozeatin) in *A. thaliana* apices, leaves and roots after two-week exposure to ZnO nanoparticles (dashed filling). Stress-related active cytokinin *cis*-zeatin is shown separately (no pattern). Asterisks indicate significant differences in active cytokinins and crosses significant differences in *cis*-zeatin values among control and ZnO NP treated plants ($p \leq 0.01$) according to ANOVA.

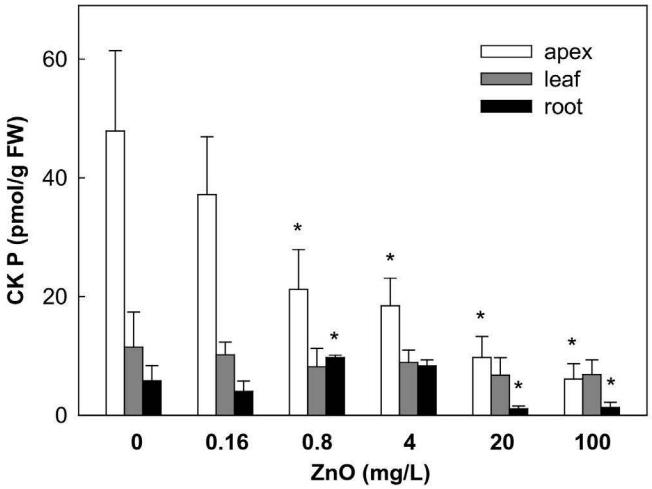

Fig. 3. The content of cytokinin phosphates (phosphates of *trans*-zeatin, isopentenyladenine, dihydrozeatin and *cis*-zeatin) in *A. thaliana* apices, leaves and roots after two-week exposure to ZnO nanoparticles. Bars indicate SD. Asterisks indicate significant differences among control and ZnO NP treated plants ($p \leq 0.01$) according to ANOVA.

The levels of active CKs may be diminished by their glucosylation, either at the purine ring (N-glucosylation) or at the side chain (O-glucosylation). CK N-glucosylation was high in apices at control conditions (Table 2). Both in apices and in leaves, CK N-glucosylation was diminished at mild/moderate stress (4 mg L^{-1}) and at very strong stress (100 mg L^{-1}). In roots, transient up-regulation was observed at 4 and 20 mg L^{-1}. O-glucosides exhibited a similar profile as N-glucosides (Table 2), showing a transient maximum in leaves at 0.8 mg L^{-1} and in roots at 20 mg L^{-1} ZnO NP. Taking into account the high elevation of *cis*-zeatin during stress progression, its glucosides are shown separately (Table 2). High levels of *cis*-zeatin(riboside) glucosides in roots correlated very well with elevation of the corresponding base. CK ribosides (the transport forms) significantly decreased upon ZnO NP treatment (Table 2).

Table 2
The content of cytokinin N-glucosides (N-glc: sum of N7- and N9-glucosides of *trans*-zeatin, isopentenyladenine and dihydrozeatin), cytokinin O-glucosides (O-glc: *trans*-zeatin O-glucoside and *trans*-zeatin riboside O-glucoside), *cis*-zeatin glucosides (cZ-glc: *cis*-zeatin(riboside) O-glucoside and 7N- and 9N-glucoside) and cytokinin ribosides (CK-R: ribosides of *trans*-zeatin, isopentenyladenine, dihydrozeatin and *cis*-zeatin) in *A. thaliana* apices, leaves and roots after two-week exposure to ZnO nanoparticles. Mean values (pmol per g fresh weight) with standard deviation are presented. Statistical differences from the control values ($p \leq 0.01$) are indicated by *.

ZnO NP concentration (mg L^{-1})		N-glc (pmol/g FW)	O-glc	cZ-glc	CK-R
0	Apex	277.82 ± 95.36	19.49 ± 4.14	18.33 ± 7.77	5.02 ± 1.54
0	Leaf	183.47 ± 17.20	6.52 ± 1.56	11.84 ± 0.82	4.43 ± 2.31
0	Root	59.97 ± 18.14	1.73 ± 0.86	6.39 ± 1.61	2.20 ± 0.54
0.16	Apex	104.58 ± 43.00*	6.65 ± 2.24*	7.44 ± 2.51	1.86 ± 1.07*
0.16	Leaf	135.05 ± 16.61	3.88 ± 0.65	12.96 ± 2.2	1.65 ± 0.33
0.16	Root	47.53 ± 4.33	0.88 ± 0.33	7.37 ± 1.10	1.26 ± 0.34
0.8	Apex	83.11 ± 21.50*	6.94 ± 1.76*	9.87 ± 4.26	1.75 ± 1.33*
0.8	Leaf	161.70 ± 30.17	8.18 ± 3.57	10.4 ± 3.29	1.72 ± 0.95
0.8	Root	48.02 ± 15.08	0.93 ± 0.12	5.60 ± 1.65	2.15 ± 0.44
4	Apex	93.20 ± 27.89*	7.07 ± 3.08*	11.91 ± 4.11	1.88 ± 0.69*
4	Leaf	71.34 ± 12.87*	3.39 ± 1.25	11.68 ± 2.05	1.64 ± 1.03
4	Root	106.47 ± 18.56*	3.39 ± 1.61	24.07 ± 1.64*	2.50 ± 0.82
20	Apex	162.43 ± 35.72	4.75 ± 2.18*	20.04 ± 5.99	1.43 ± 1.11*
20	Leaf	134.86 ± 27.05	2.71 ± 0.88*	20.21 ± 2.38*	1.59 ± 1.09
20	Root	88.76 ± 13.94	8.32 ± 3.02*	39.99 ± 10.29*	2.66 ± 0.87
100	Apex	66.90 ± 22.06*	0.94 ± 0.25*	16.59 ± 1.33	0.26 ± 0.18*
100	Leaf	41.65 ± 13.16*	4.92 ± 2.01	17.93 ± 2.76*	2.57 ± 1.34
100	Root	59.68 ± 6.58	0.92 ± 0.21	40.35 ± 14.27*	1.70 ± 1.01

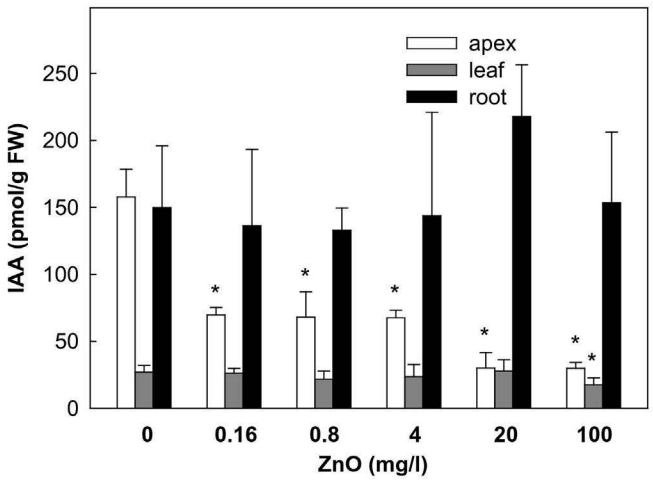

Fig. 4. The content of auxin – indole-3-acetic acid (IAA) in *A. thaliana* apices, leaves and roots after two-week exposure to ZnO nanoparticles. Bars indicate SD. Asterisks indicate significant differences among control and ZnO NP treated plants ($p \leq 0.01$) according to ANOVA.

3.3. Indole-3-acetic acid

The presence of ZnO NPs in the medium had a very negative effect on the level of the active auxin, indole-3-acetic acid (IAA) in apices (Fig. 4). Very strong inhibition was found especially at 20 and 100 mg L^{-1}. The auxin content in leaves was maintained during increasing stress strength, with exception of the highest ZnO NP concentration, where a significant down-regulation was observed. IAA levels were relatively high in roots, showing mild transient elevation at 20 mg L^{-1} ZnO NPs.

The levels of IAA conjugates with amino acids (IAA-aspartate and IAA-glutamate) were relatively low (Table 3). The IAA-aspartate exhibited a maximum in leaves at 4 mg L^{-1} ZnO NP and in roots at 20 mg L^{-1}, while IAA-glutamate had the highest concentrations in roots at the range 4–100 mg L^{-1} (Table 3). In addition, the concentration of IAA glucosyl-ester was very low, especially under control conditions. It was enhanced in roots at 4 and 20 mg L^{-1} and in apices at 0.8–100 mg L^{-1}. The oxo-IAA and especially its glucosyl-ester were the most abundant IAA catabolites. Oxo-IAA reached the highest value in apices at 20 mg L^{-1}, its glucosyl-ester at 100 mg L^{-1}. In leaves, oxo-IAA maximum was found already at 4 mg L^{-1}, while glucosyl-ester accumulation was observed at 20–100 mg L^{-1}. In roots, the highest concentration of oxo-IAA glucosyl-ester was observed at 0.16 mg L^{-1} (Table 3).

The IAA precursor indole-3-acetonitrile (IAN) was much more abundant than indole-pyruvic acid (IPyA). Their content was decreased at the presence of low NP levels. Transient elevation (in all tissues) was found at 4 mg L^{-1} ZnO NPs. Nevertheless, the values in apices did not reach the control levels, in leaves and roots moderate increase above control levels was found. Enhanced levels in leaves were maintained also at 20 mg L^{-1} ZnO NPs.

The concentration of phenylacetic acid (PAA) was lower in apices exposed to ZnO NPs. A transient maximum was detected at 4 mg L^{-1} (still substantially lower in comparison to control levels). A moderate increase in the level of PAA was observed in leaves in the presence of 4–100 mg L^{-1} ZnO NPs, whereas in roots, concentrations were highest at 4–20 mg L^{-1}.

3.4. Abscisic acid

The stress hormone ABA did not exhibit profound changes in Arabidopsis apices up to 4 mg L^{-1} ZnO NPs (Fig. 5). ABA levels, however, highly increased in apices as well as in leaves at strong stress (at 20 and 100 mg L^{-1}). In roots, transient elevation of ABA was observed at moderate stress (4 mg L^{-1}). This effect pertained also at 20 mg L^{-1}. At very severe stress (100 mg L^{-1}), ABA levels in roots were diminished.

The most abundant ABA metabolite under control conditions was dihydrophaseic acid (DPA). The DPA profile in apices more-or-less followed the changes in ABA content (Table 4) with the exception of 100 mg L^{-1} ZnO NPs, where the high increase of ABA levels in apices seems to be accompanied by a strongly diminished formation of DPA. In leaves and roots DPA generally followed the ABA dynamics, however, the maximum was reached at 20 mg L^{-1}, i.e. with stronger stress strength. The other highly abundant ABA metabolite under control conditions was phaseic acid (PA). In apices, it followed the same trend as DPA. In leaves, the high increase at 100 mg L^{-1} ZnO NP indicates that PA is associated with precise regulation of ABA levels under strong stress conditions in leaves. In roots, the highest concentration of PA coincided with that of ABA. The level of neophaseic acid was generally low in all tissues, with the exception of roots at 20 mg L^{-1} ZnO NP. The content of 9-hydroxy-abscisic acid (9OH-ABA) was also relatively low. It reached higher levels under strong stress (20–100 mg L^{-1}) in apices and leaves, in roots already at 4 mg L^{-1}. The ABA glucosyl-ester (ABA-GE) was very low at control conditions, but strongly accumulated in

Table 3
The content of auxin metabolites and precursors (OxIAA – oxo-indole-3-acetic acid, IAA-Asp – IAA-aspartate, IAA-GLU – IAA-glutamate, OxIAA-GE – OxIAA glucosyl-ester, IAA-GE – IAA glucosyl-ester, PAA – phenylacetic acid, IPyA – indole-pyruvic acid, IAN – indole-3-acetonitrile) in *A. thaliana* apices, leaves and roots after two-week exposure to ZnO nanoparticles. Mean values (pmol per g fresh weight) with standard deviation are presented. Statistical differences from the control values ($p \leq 0.01$) are indicated by *.

ZnO NP concentration (mg L^{-1})		IAA-Asp (pmol/g FW)	IAA-GLU	OxIAA	OxIAA-GE	IAA-GE	PAA	IPyA	IAN
0	Apex	8.04 ± 2.28	6.69 ± 2.16	247.42 ± 47.94	495.71 ± 114.67	0.50 ± 0.28	490.98 ± 11.64	44.37 ± 13.84	760.00 ± 287.72
0	Leaf	1.61 ± 0.14	0.33 ± 0.33	27.44 ± 15.87	177.53 ± 0.36	3.38 ± 2.37	92.85 ± 18.28	3.84 ± 0.11	131.09 ± 3.89
0	Root	10.56 ± 3.96	0.32 ± 0.45	213.52 ± 65.72	61.42 ± 32.56	0.79 ± 0.56	80.98 ± 40.07	17.46 ± 8.41	368.22 ± 140.08
0.16	Apex	4.08 ± 2.24	3.63 ± 1.80	8.72 ± 3.25*	323.35 ± 10.36	1.21 ± 0.66	172.60 ± 22.47*	22.04 ± 10.60	469.40 ± 34.22
0.16	Leaf	2.31 ± 0.70	1.02 ± 0.42	27.66 ± 7.96	212.04 ± 103.87	2.62 ± 0.32	97.09 ± 35.14	4.16 ± 0.91	92.02 ± 44.86
0.16	Root	11.52 ± 5.82	1.51 ± 0.07*	152.19 ± 58.52	137.01 ± 10.78*	1.27 ± 0.90	60.33 ± 5.35	16.37 ± 10.46	265.32 ± 116.76
0.8	Apex	3.68 ± 2.48	3.59 ± 0.23	7.13 ± 3.86*	351.43 ± 16.28	7.94 ± 2.18*	151.98 ± 20.04*	18.21 ± 4.20*	519.40 ± 120.04
0.8	Leaf	1.22 ± 0.64	1.36 ± 1.08	11.86 ± 2.06	152.60 ± 26.79	3.60 ± 2.51	88.68 ± 13.49	4.11 ± 3.94	55.66 ± 24.00*
0.8	Root	8.60 ± 3.47	3.41 ± 2.61*	112.3 ± 6.51*	82.67 ± 20.71	7.82 ± 3.32*	82.97 ± 4.94	18.27 ± 8.12	377.33 ± 68.44
4	Apex	6.15 ± 4.41	3.14 ± 1.17	67.95 ± 19.26*	378.05 ± 5.39	9.84 ± 0.45*	161.08 ± 22.67*	44.28 ± 7.73	555.22 ± 58.38
4	Leaf	2.37 ± 1.48	0.92 ± 0.81	124.65 ± 35.6*	125.57 ± 30.82	1.81 ± 1.67	163.49 ± 33.56*	8.84 ± 5.35	183.16 ± 115.2
4	Root	9.07 ± 0.78	11.07 ± 1.11*	140.59 ± 49.08	115.45 ± 48.43	21.87 ± 7.73*	283.18 ± 34.47*	39.41 ± 8.83*	241.40 ± 86.30
20	Apex	2.55 ± 0.39*	3.74 ± 0.55	113.36 ± 44.54*	449.58 ± 114.22	14.38 ± 9.87*	177.00 ± 17.23*	20.13 ± 4.42*	598.40 ± 193.15
20	Leaf	1.73 ± 1.61	1.35 ± 0.83*	103.27 ± 5.11*	492.70 ± 22.05*	2.50 ± 1.30	133.76 ± 75.09	1.97 ± 0.77*	591.38 ± 40.71*
20	Root	30.73 ± 0.58*	22.70 ± 4.21*	154.25 ± 7.14	33.02 ± 20.49	16.35 ± 8.40*	251.33 ± 30.57*	7.94 ± 1.30*	94.23 ± 77.88*
100	Apex	6.41 ± 2.50	8.41 ± 3.12	81.36 ± 36.05*	644.50 ± 225.04	12.22 ± 9.79*	187.44 ± 83.14*	25.86 ± 4.22*	967.98 ± 234.38
100	Leaf	0.71 ± 0.17*	2.13 ± 0.47*	44.38 ± 7.30	392.03 ± 15.76*	2.64 ± 0.75	144.74 ± 30.64	0.36 ± 0.08*	670.02 ± 251.65*
100	Root	12.4 ± 0.08	8.65 ± 0.23*	77.65 ± 3.65*	13.51 ± 7.88*	10.55 ± 0.38*	112.29 ± 15.32	7.63 ± 1.42*	22.81 ± 15.83*

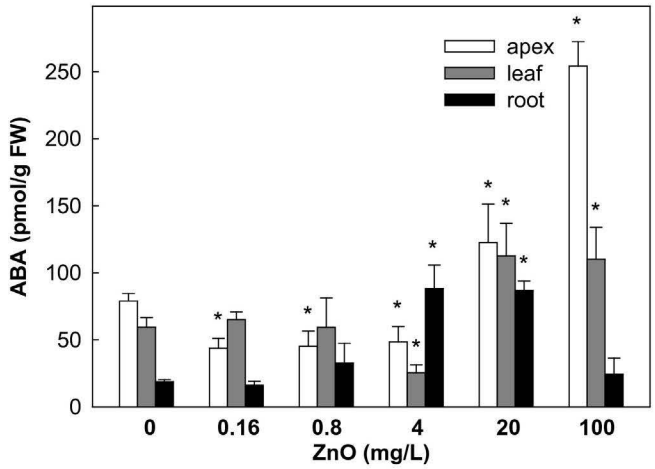

Fig. 5. The content of abscisic acid (ABA) in *A. thaliana* apices, leaves and roots after two-week exposure to ZnO nanoparticles. Bars indicate SD. Asterisks indicate significant differences among control and ZnO NP treated plants ($p \leq 0.01$) according to ANOVA.

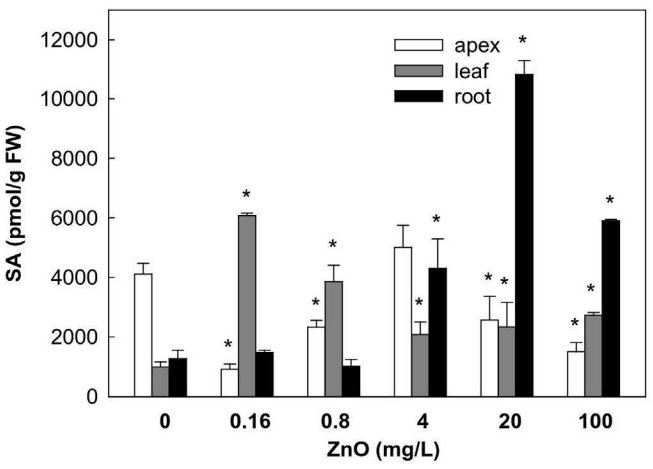

Fig. 6. The content of salicylic acid (SA) in *A. thaliana* apices, leaves and roots after two-week exposure to ZnO nanoparticles. Bars indicate SD. Asterisks indicate significant differences among control and ZnO NP treated plants ($p \leq 0.01$) according to ANOVA.

stress conditions, in apices and leaves at 20 and 100 mg L^{-1}, in roots its concentration increased already at 4 mg L^{-1} ZnO NPs.

3.5. Salicylic acid

The salicylic acid (SA) level was diminished in apices at low ZnO NP concentration (0.16 mg L^{-1}). It reached a transient maximum in apices at 4 mg L^{-1} ZnO NP (Fig. 6). The leaves responded earlier, transient maximum in SA concentration was observed already at low ZnO NP content. In roots, the SA profile was delayed, and substantial elevation was found at 4 mg L^{-1}, while the highest content was detected at 20 mg L^{-1}.

3.6. Jasmonate and its isoleucine conjugate

Jasmonic acid (JA) gradually decreased in apices at increasing ZnO NP concentration (Fig. 7). The minimum was reached at 4 mg L^{-1}. Jasmonate isoleucine (JA-Ile) generally followed in apices the same trend as JA (Fig. 8). In leaves, transient up-regulation of both JA and JA-Ile was found at low ZnO NP concentration (0.16 mg L^{-1}). In roots, mild down-regulation was observed at 0.16–4 mg L^{-1}, at higher ZnO NP concentrations strong suppression was found.

4. Discussion

The inverse relationship between biomass growth and concentration of ZnO NPs in media (Table 1) clearly indicates that nanoparticles function as stressors in Arabidopsis plants. It is an open question whether toxicity of the ZnO NPs can be attributed to released metal ions or to the small size of the nanoparticles (both options are supported by a number of reports). Transport of ions across the plasma membrane may be diminished due to the "ionic trap", i.e., a low membrane permeability for charged molecules, which may not apply to nanoparticles. The above-mentioned aspects, however, may also be interrelated. Due to their small size, nanoparticles can easily enter the plant body and the individual cells and then release ions (Xiao et al., 2015). Higher uptake of Zn from ZnO NPs than in case of Zn^{2+} treatment was reported in *Schoenoplectus tabernaemontani* (Zhang et al., 2015a) or in corn (Zhang et al., 2015b). Nanoparticles were reported to pass through the cell wall (Zhang et al., 2015a), through plasma membrane (Arruda et al., 2015) as well as to be transported via plasmodesmata (Kumari et al., 2011).

Table 4
The content of ABA metabolites (DPA – dihydrophaseic acid, PA – phaseic acid, ABA-GE – ABA glucosyl-ester, NeoPA – neophaseic acid, 9OH-ABA – hydroxy-ABA), benzoic acid (BzA) and JA precursor cisOPDA (*cis*-12-oxo-10,15-phytodienoic acid) in *A. thaliana* apices, leaves and roots after two-week exposure to ZnO nanoparticles. Mean values (pmol per g fresh weight) with standard deviation are presented. Statistical differences from the control values ($p \leq 0.01$) are indicated by *.

ZnO NP concentration (mg L^{-1})		DPA (pmol/g FW)	PA	ABA-GE	NeoPA	9OH-ABA	BzA	cisOPDA
0	Apex	136.18 ± 26.95	34.27 ± 6.95	15.78 ± 7.57	3.73 ± 0.44	13.70 ± 1.95	4198.77 ± 581.68	104.08 ± 36.06
0	Leaf	33.42 ± 11.02	34.21 ± 9.02	12.54 ± 4.80	1.15 ± 0.88	3.01 ± 0.96	805.49 ± 107.40	58.09 ± 22.12
0	Root	54.72 ± 0.38	1.55 ± 0.38	1.20 ± 0.90	1.27 ± 0.64	0.25 ± 0.03	1246.09 ± 540.25	153.65 ± 72.09
0.16	Apex	56.61 ± 16.66*	22.89 ± 6.66	1.15 ± 0.24*	2.03 ± 1.02	5.04 ± 2.97*	1461.09 ± 107.04*	117.23 ± 25.91
0.16	Leaf	25.38 ± 1.43	43.82 ± 10.43	15.38 ± 0.28	1.18 ± 0.73	4.72 ± 3.20	981.08 ± 189.01	37.89 ± 12.05
0.16	Root	51.52 ± 18.00	3.59 ± 2.00	1.31 ± 0.85	0.74 ± 0.63	0.26 ± 0.02	731.82 ± 208.93	78.76 ± 37.67
0.8	Apex	69.11 ± 23.12*	18.89 ± 8.12	1.26 ± 0.45*	1.90 ± 0.32*	4.84 ± 2.41*	1561.09 ± 270.12*	206.11 ± 86.05
0.8	Leaf	25.43 ± 12.48	38.99 ± 12.48	16.16 ± 4.24	2.38 ± 1.63	6.52 ± 1.18	861.58 ± 364.99	39.25 ± 22.84
0.8	Root	91.13 ± 43.33	4.56 ± 2.33	1.76 ± 0.49	1.57 ± 0.64	0.14 ± 0.10	1104.04 ± 214.07	101.37 ± 51.82
4	Apex	88.61 ± 29.00	25.20 ± 6.08	3.13 ± 0.93*	3.26 ± 0.31	6.75 ± 1.76*	1646.00 ± 104.46*	360.06 ± 18.83*
4	Leaf	30.55 ± 15.29	36.69 ± 12.29	11.90 ± 3.20	0.65 ± 0.70	7.06 ± 3.73	1274.90 ± 571.32	271.34 ± 109.41*
4	Root	73.40 ± 34.39	5.27 ± 3.39*	53.92 ± 9.42*	1.98 ± 0.53	7.57 ± 4.84*	2792.62 ± 270.89*	253.08 ± 99.80
20	Apex	146.39 ± 17.20	209.72 ± 17.20*	83.41 ± 4.71*	0.63 ± 0.26*	32.16 ± 12.42*	1618.01 ± 135.44*	302.48 ± 49.90*
20	Leaf	146.28 ± 51.62*	224.54 ± 51.62*	52.57 ± 20.85*	0.42 ± 0.36	29.15 ± 10.32*	1542.87 ± 966.02	256.91 ± 34.93*
20	Root	131.82 ± 7.15*	35.34 ± 7.15*	108.87 ± 21.81*	36.16 ± 2.15*	7.06 ± 2.13*	2608.69 ± 53.13*	59.75 ± 32.22*
100	Apex	71.99 ± 3.15*	136.14 ± 23.15*	74.44 ± 29.81*	10.14 ± 5.27*	29.27 ± 11.61*	2302.12 ± 753.64	170.37 ± 39.41*
100	Leaf	120.38 ± 23.04*	251.81 ± 13.04*	53.59 ± 24.32*	0.88 ± 0.67	30.81 ± 2.94*	1926.6 ± 504.85*	718.61 ± 120.83*
100	Root	31.62 ± 1.52*	9.33 ± 1.52*	22.11 ± 17.16	2.26 ± 0.59	5.26 ± 1.47*	816.27 ± 252.43	27.31 ± 10.41*

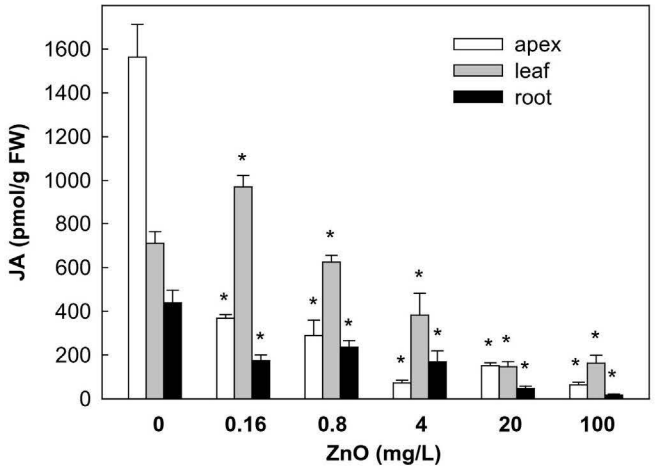

Fig. 7. The content of jasmonic acid (JA) in *A. thaliana* apices, leaves and roots after two-week exposure to ZnO nanoparticles. Asterisks indicate significant differences among control and ZnO NP treated plants according to ANOVA.

When the impact of the size of silver nanoparticles was compared, maximum up-take and cell damage was found in case of 25 nm particles (Mazumdar and Ahmed, 2011).

Metal containing NPs were found to cause systemic stress responses, including oxidative stress (as indicated by an elevation of ROS as well as of the activity of antioxidant enzymes) (Zhao et al., 2012), up-regulation of protective proteins (e.g., heat shock protein 70 - HSP70) or stimulation of the expression of genes involved in DNA repair and oxidative DNA damage bypass (Nair and Chung, 2014b). The other stress response is accumulation of anthocyanins (natural antioxidants), which we observed in leaves at high, growth inhibitory concentrations of ZnO NPs (Fig. 1). Our finding is in agreement with the impact of silver NPs on Arabidopsis (Nair and Chung, 2014c; Syu et al., 2014) or on *Brassica rapa* (Thiruvengadam et al., 2015).

The effect of nanoparticles strongly depends on their concentration. Thus, low NP concentrations have negligible or even positive effects on plant growth, e.g., 1000 ppm ZnO NPs in case of peanut (Prasad et al., 2012). This is probably caused by the fact that mild stresses may stimulate plant organisms and enhance their metabolic activity, so that the low stress-inducing compounds have anti-senescence effects (Nyitrai et al., 2009).

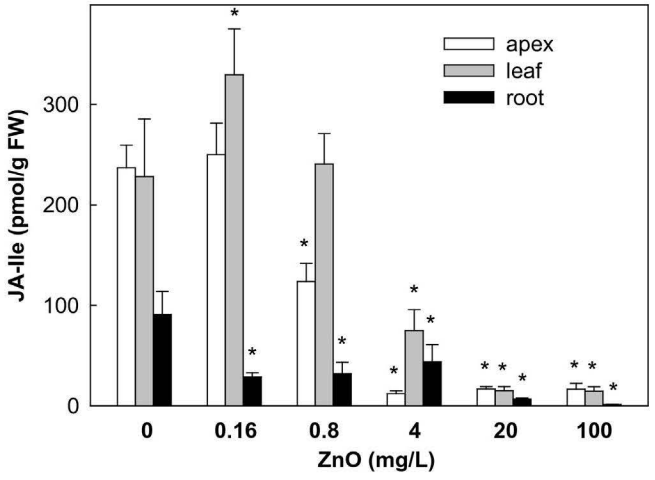

Fig. 8. The content of jasmonate isoleucine (JA-Ile) in *A. thaliana* apices, leaves and roots after two-week exposure to ZnO nanoparticles. Asterisks indicate significant differences among control and ZnO NP treated plants according to ANOVA.

As plant hormones play an important role in plant stress sensing, defense and adaptation, we aimed to correlate the physiological state of plants (biomass, root length) with the hormonal profiles. One of the most common stress responses is redirection of energy resources from the growth to plant defense. Fast growth suppression is usually associated with down-regulation of hormones stimulating cell division – CKs and auxins. CKs represent an important signal for cell cycle progression during both check points (G_1/S and G_2/M) (Laureys et al., 1998). When CK metabolites were followed, substantial differences were found between individual tissues. The most prominent changes were observed in case of apex, i.e. the shoot meristem. The levels of active CKs in apices (Fig. 2) correlated with the shoot growth rate. A similar profile was exhibited by their precursors – CK phosphates. CK deactivation by N- and O-glucosylation, which was high in apices at control conditions when intensive CK biosynthesis took place, was suppressed at lower ZnO NP concentrations, which may indicate the tendency to maintain CK homeostasis in plants, and minimize the stress impact. When the stress became severe, at 20 mg L^{-1}, CK N-glucosylation was increased, in order to contribute to fast down-regulation of active CKs and thus to the growth rate inhibition. At 100 mg L^{-1}, CK biosynthesis was so low, that there was no further need for deactivation of the excess of active CKs. The mature leaves more-or-less maintained active CK levels until severe stress (at 20 and 100 mg L^{-1}), when CKs dropped. Steady CK levels in leaves may be important for stabilization of the photosynthetic machinery. In roots, active CKs were not significantly affected by moderate stress, only the ratio of the stress-related CK *cis*-zeatin to the other active CKs increased at higher nanoparticle concentrations. The increase in the concentration of *cis*-zeatin was accompanied by an increase in the level of its glucosides. High levels of *cis*-zeatin metabolites may indicate enhanced degradation of specific tRNAs. In fact, *cis*-zeatin may be an integral part of the anticodon loop, which facilitates during translation codon-anticodon interaction and enhances the efficiency of specific tRNAs (Kaminek, 2015).

This paper is, up to our knowledge, the first report describing the effect of nanoparticles on different plant hormone groups simultaneously. The reports on the effect of nanoparticles on individual hormones have been rather rare, focused mainly on auxin. Thus, we can compare our data predominantly with the effects of metal ions. Cadmium stress was also found to decrease CK levels in leaves (only kinetin was followed) (Lomaglio et al., 2015). In contrast, $ZnSO_4$ was reported to increase the level of *trans*-zeatin riboside (and dihydrozeatin riboside, the only quantified CKs) in roots and leaves of Arabidopsis (Sofo et al., 2013). However, the method description does not allow to judge whether the authors distinguished in their analysis between the *trans*- and the *cis*-isomer, as both yield the same MS ions. Expression of CK related genes was suppressed by arsenic (Srivastava et al., 2009).

The other hormone indispensable for cell division is the auxin IAA. As in case of CKs, the most profound change upon ZnO NP application was suppression of auxin levels in apices. The decrease was observed already at the lowest ZnO NP concentration. Further substantial decrease occurred at severe stress (20 and 100 mg L^{-1}). These data indicate that apices are the tissue that is most sensitive to nanoparticle presence. As this meristematic tissue is essential for generative growth, and subsequently for the yield (in case of most crops), it is necessary to take this feature into account when evaluating the potential negative consequences of nanoparticle contamination in practice. In comparison with apices, the effect of ZnO NP on leaves was lower. In roots, mild elevation at 20 mg L^{-1} was observed. Similarly to CKs, the highest deactivation of IAA occurred at a ZnO NP concentration of 20 mg L^{-1}. Significant elevation of IAA catabolism was observed already at 4 mg L^{-1}.

A concentration dependent negative effect of $ZnSO_4$ on growth and auxin signal transduction of Arabidopsis was reported by Wang et al. (2015). In spite of the fact that auxin signaling was substantially suppressed in leaves and root tips, the effect of zinc on expression of auxin related genes was relatively small, only moderate up-regulation of transcription repressor *ARF2* was found in the roots. Similarly,

expression of the IAA repressor *IAA8* was found to be up-regulated after silver NP treatment (Syu et al., 2014). The expression of the auxin-response gene *ARGOS* was suppressed by silver NPs (Kaveh et al., 2013). Thus, it seems that nanoparticles suppress auxin signaling, in apices both at the levels of metabolites and signal transduction. The latter factor may play more important role in Arabidopsis leaves and roots.

Abscisic acid (ABA), the key hormone in abiotic stress defense, has been reported as an essential phytohormone involved in responses to heavy metal stresses (Shi et al., 2015). ABA seems to be involved in heavy metal stress sensing and response (Chaca et al., 2014). We found a high increase of ABA levels in apices and leaves at high ZnO NP levels (20 and 100 mg L^{-1}). In directly exposed roots, an elevation of ABA concentration was observed already at moderate stress. Our data on ABA content are in accordance with the report on the long-term effect of $ZnSO_4$ (Sofo et al., 2013), when an increase in the ABA level was detected both in roots and leaves. ABA level elevation also corresponds well with the enhanced expression of the gene coding the rate limiting biosynthetic enzyme *NCED3* upon Arabidopsis exposure to silver nanoparticles (Syu et al., 2014). Exogenous ABA was reported to decrease Zn levels in poplar by modulating the transcript levels of the key genes involved in Zn uptake and detoxification (Shi et al., 2015). Profound effect of ABA on the transcriptome can be deduced from the presence of ABA responsive elements (ABRE) in promoter regions of many metal stress induced genes (e.g., after exposure to gold nanoparticles, Shukla et al., 2014). Thus, it seems that plant hormonal responses to ZnO NPs overlap, at least partially, with the reactions to heavy metals.

Another stress hormone, known to be involved in the defense against heavy metal stresses, e.g. Pb, Cd or Cu – reviewed in Khan et al. (2015), is salicylic acid (SA). This hormone promotes antioxidant defense capacity by stimulation of the expression and activity of antioxidant enzymes (Kovacs et al., 2014) as well as expression of gluthahione synthetase and consequently the content of antioxidant glutathione (Guan et al., 2015). We found strong elevation of SA in the leaves already at low ZnO NP level (0.16 mg L^{-1}). In apices, the SA maximum was observed at 4 mg L^{-1}, while in roots, strong increase of SA was detected both at moderate and strong stress. SA decrease in apices and leaves at strong stress may be related to its antagonism with ABA. Exogenous SA was reported to prevent some nano-specific transcriptomic changes in Arabidopsis after TiO_2 and silver nanoparticle exposure (Garcia-Sanchez et al., 2015).

Further stress hormone, which was reported to play a role in heavy metal stress sensing and response, is JA (Chaca et al., 2014). Exogenous JA was found to increase the activity of antioxidant enzymes, namely ascorbate peroxidase in *Avicennia marina* and simultaneously to decrease the uptake of Cd (Yan et al., 2015a). Also exogenous methyl jasmonate enhanced the activity of catalase and reversed the negative effect of Cd on activity of superoxide dismutase (Yan et al., 2015b). When we followed the levels of JA and jasmonate-isoleucine, we found after 14-day gradual decrease of both compounds in dependence on the ZnO NP concentration. These data are in accordance with the results of Chen et al. (2014), who found decrease in endogenous JA in leaves of *Kandelia obovata* after 9-day Cd stress. The discrepancy between the effect of exogenous application and detected hormone levels may be explained by hormonal dynamics, it is possible that after 2-week ZnO NP exposure JA levels were already down-regulated. This assumption is supported by the fact that up-regulation of genes involved in jasmonate biosynthesis was detected after 3 h and 6 h exposure to arsenic (Srivastava et al., 2009).

5. Conclusions

The obtained results indicate that plants sense already low concentrations of ZnO NPs. At small doses, the presence of NPs seems to coincide with an elevation of CK biosynthesis, as may be deduced from the increase of CK precursors (CK phosphates). At moderate and high doses, ZnO NPs function as stressors. They stimulate plant defense, which is associated with a strong up-regulation of the levels of the stress hormones ABA and SA. The tissue most sensitive to NPs is the shoot apical meristem. Even moderate ZnO NP concentrations cause down-regulation of active CKs and auxin with subsequent growth inhibition. The inhibitory effect of NPs is shifted to higher concentrations in roots. This might reflect the tendency to maintain their growth in order to avoid contaminated substrate.

List of abbreviations

ABA	abscisic acid
CK	cytokinin
DPA	dihydrophaseic acid
DZ	dihydrozeatin
DZR	dihydrozeatin riboside
DZ9G	dihydrozeatin N9-glucoside
IAA	indole-3-acetic acid indole-3-acetonitrile
IPyA	indole-pyruvic acid
iP	isopentenyladenine
iPR	isopentenyladenosine
iPRMP	isopentenyladenosine phosphate
iP7G	isopentenyladenine N7-glucoside
iP9G	isopentenyladenine N9-glucoside
JA	jasmonic acid
JA-Ile	jasmonate isoleucine
NP	nanoparticle
PA	phaseic acid
PAA	phenylacetic acid
SA	salicylic acid
TransZ	*trans*-zeatin
TransZR	*trans*-zeatin riboside
TransZ7G	*trans*-zeatin N7-glucoside
TransZ9G	*trans*-zeatin N7-glucoside
TransZOG	*trans*-zeatin O-glucoside
TransZROG	*trans*-zeatin riboside O-glucoside
TransZRMP	*trans*-zeatin riboside phosphate

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.03.160.

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4th Article

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Dynamics of cold acclimation and complex phytohormone responses in *Triticum monococcum* lines G3116 and DV92 differing in vernalization and frost tolerance level

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ABSTRACT

Cold stress response was compared in the crowns, leaves, and roots of *Triticum monococcum* DV92 spring line and G3116 winter line. The cold exposure was associated with a rapid increase of water saturation deficit, which resulted in a strong up-regulation of abscisic acid. Simultaneously, other stress hormones: salicylic acid, aminocyclopropane carboxylic acid (precursor of ethylene), and jasmonic acid decreased. The stress application resulted in a decrease of hormones associated with stimulation of cell growth and division (gibberellins, cytokinins, and auxin). During the acclimation phase of the stress response, the plants increased their frost tolerance and started the accumulation of dehydrins. Active gibberellin, cytokinins, and auxin were elevated; more rapidly in the spring line. Abscisic acid decrease was accompanied by a gradual increase of the other stress hormones. Simultaneously, the up-regulation of phenolic acids was observed, including ferulic and sinapic acids, which may be involved in the stabilization of auxin levels as well as antioxidative functions. After 21 days, the spring line DV92 exhibited its maximum of active cytokinins, which indicates the onset of the early stage of reproductive development. The winter line fulfilled its vernalization requirement after 42 days, as indicated by a decrease of frost tolerance and dehydrin levels, accompanied by similar growth hormone changes as in DV92. The similarities and differences between einkorn and common wheat in a long-term cold response are discussed.

1. Introduction

Cold is an important abiotic stress factor, affecting agricultural productivity in temperate climates (Sakai and Larcher, 1985). Cereals from the tribe *Triticeae* grown in temperate climates have evolved two major mechanisms of cold response – cold acclimation and vernalization. Cold acclimation represents a complex dynamic process directed at enhancement of the plant tolerance to low temperatures (frost tolerance = FrT), while vernalization represents an adaptation of plant development in order to prevent the plant premature transition from a cold-tolerant vegetative phase to a cold-sensitive reproductive phase (e.g., Kosová et al., 2008).

Einkorn wheat (*Triticum monococcum*) represents an ideal model for genetic studies of frost tolerance due to its diploid genomic makeup (A^mA^m). In the *T. monococcum* genome, both the major vernalization and frost tolerance associated loci (*Vrn1/Fr1* locus, *Fr2* locus, and *Vrn2* locus) all lie on the long arm of chromosome 5A (5AL; Dubcovsky et al., 1998). The *T. monococcum* lines G3116 and DV92 have been used for genetic mapping studies, leading to the identification of the *Fr2* locus (Vágújfalvi et al., 2003), as well as the deciphering the structure of the *CBF* cluster at this locus (Knox et al., 2008). They exhibit significant differences in acquired frost tolerance, but do not differ in the major *Vrn1/Fr1* frost-tolerance locus (Vágújfalvi et al., 2003). However, they do differ at the *Vrn2* locus, as line G3116 possesses a dominant *Vrn-A^m2* allele and has a vernalization requirement, while line DV92 possesses a recessive *vrn-A^m2* allele and lacks vernalization (Dubcovsky et al., 1998; Vágújfalvi et al., 2003). Genetically, the G3116 line corresponds to a winter growth habit due to a

Abbreviations: ABA, abscisic acid; ACC, aminocyclopropane carboxylic acid; CK, cytokinin; FA, ferulic acid; FrT, frost tolerance; GA, gibberellin; IAA, indole-3-acetic acid; JA, jasmonic acid; JA-Ile, jasmonate isoleucine; LT50, lethal temperature for 50% of the sample; OP, osmotic potential; PA, phaseic acid; SA, salicylic acid; SiA, sinapic acid; WSD, water saturation deficit; WCS, wheat cold-specific (protein).

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combination of a recessive *vrn1* allele and a dominant *Vrn2* allele, while the DV92 line can be considered a spring line due to the absence of a vernalization requirement (presence of a recessive *vrn2* allele). Due to the combination of recessive *vrn1* and *vrn2* alleles, DV92 can also be described as a facultative growth habit (Von Zitzewitz et al., 2005).

The detailed study of the genetic basis of frost tolerance of the G3116 and DV92 lines has led to the identification of 11 *TmCBF* genes, mapped to the *Fr2* locus (Knox et al., 2008). Quantitative differences were observed in *T. monococcum TmCBF* genes between the cold-treated lines. The G3116 line exhibited higher levels of *TmCBF12*, *TmCBF15*, and *TmCBF16* transcripts than the DV92 line did. Moreover, allelic differences between the two lines have been characterized in the *TmCBF12* gene, mapped to the central part of the *Fr2* locus. These differences indicate that the *TmCBF12* allele, which is present in the DV92 line, cannot bind to the CRT/DRE elements in the promoter of the *COR* genes, due to a mutation resulting in the deletion of the AP2 DNA binding domain. The inability of DV92 *TmCBF12* to bind to CRT/DRE binding elements in the promoter of the *COR* genes has been confirmed by electrophoretic mobility shift assays (EMSA; Knox et al., 2008). The authors hypothesize that the differences in the structure and ability of *TmCBF12* to bind to the CRT/DRE promoter elements may underlie the differences in acquired FrT between the two lines. Thus, *T. monococcum* lines G3116 and DV92 represent a very interesting model for the study of cold acclimation as well as vernalization response during a long-term cold treatment.

The CBFs directly affect metabolism of plant hormones gibberellins by stimulation of the expression of their main deactivating enzyme (GA 2-oxidases) (Achard et al., 2008). Down-regulation of the level of active gibberellins is associated with accumulation of their repressors – DELLA proteins, which results in the suppression of growth and increase in cold tolerance (Achard et al., 2008). Decrease in the growth rate at the beginning of the cold stress response was found to be associated with the down-regulation of also other hormones indispensable for cell division and growth – cytokinins and auxins (Kosová et al., 2012). Plant hormones generally are involved in the regulation of plant interactions with the environment. Abscisic acid (ABA) is the key plant hormone involved in the response to abiotic stresses associated with dehydration, which include not only drought, but also salinity and cold stress (Gusta et al., 2005). ABA mediates both fast responses (stomata closure), which are necessary for the control of water balance, as well as longer-term changes in the expression of many stress-associated genes. The function of ABA in the stabilization of water status in the early stage of the cold stress response has been repeatedly studied (Galiba et al., 1993; Janowiak et al., 2002). In addition, the participation of other "stress hormones" such as ethylene, jasmonic acid (JA), and salicylic acid (SA) in the cold response has been recognized (Majlath et al., 2012, Kosová et al., 2012).

Long-term cold treatment has been described in common wheat (*T. aestivum*) under both natural as well as controlled conditions (Gusta and Fowler, 1977; Kosová and Prášil, 2011; Kosová et al., 2011, 2012). These studies have shown differences in the time course of the FrT as well as of hormone levels, and enabled distinguishing of the different stages of the dynamics of the cold response: the alarm reaction, cold acclimation, phase of resistance, and the loss of high FrT. Tracking changes in a number of characteristics and properties then enabled the possibility to study the mechanisms influencing and controlling the dynamics of the FrT as well as of hormones in a greater detail (Vítámvás et al., 2010; Kosová et al., 2013).

The intent of our study lies in the study of the impact of a long-term (0–42 days) cold treatment on *T. monococcum* lines DV92 and G3116 at the level of plant development (days to heading), water relationships (water saturation deficit, osmotic potential), acquired frost tolerance (lethal temperature of 50% of the sample), dehydrin relative accumulation, phytohormone and phenolic acid levels. Comparisons of the data acquired on *T. monococcum* with our previous results on cold acclimation in the common wheat spring cultivar Sandra and the winter cultivar Samanta (Kosová et al., 2012) have been done in order to pinpoint both similarities and differences in a long-term cold response between the hexaploid and diploid wheats.

2. Materials and methods

2.1. Plant materials and growth conditions

Seeds of einkorn wheat (*T. monococcum*), lines G3116 and DV92 were germinated on moist filter paper for 2 days at 22 °C in the dark. The germinated seeds were planted into pots filled with a mixture of field soil and sand (6:1), and grown under controlled conditions in a growth chamber (Tyler T-16/4, Budapest, Hungary) with a 12 h photoperiod, 350 μmol $m^{-2}s^{-1}$ irradiance, and regulated temperature. The temperature was set to 18–20 °C for the first three weeks. When the plants reached the three-leaf growth stage, the temperature was decreased to 4 °C. Leaf, crown, and root samples were taken in the middle of the light period at 0 (control, non-treated plants), 1, 3, 7, 21, 31, and 42 days of cold treatment for all analyses, except for the determination of days to heading. The leaf samples for hormone analyses consisted of a pool of middle parts of six youngest fully developed leaves. With the crowns, the non-green underground stem parts, containing shoot apical meristem, and the basal parts of the leaves from ca 10 to 15 plants were taken for the analyses; with the roots, the apical root parts (ca 10 cm) were taken from 6 to 10 plants.

2.2. Days to heading and shoot apex development

Days to heading, indicating the time of vernalization saturation, were determined for three plants of each line which were transferred from cold (4 °C) to control conditions (20 °C) at each subsequent week of the cold treatment (0, 7, 14, 21, 28, 35, and 42 days of cold) and let to grow until heading. The period of growth at 20 °C from the transfer from cold until heading was determined as the days to heading. The apical development was determined from the changes in the morphology of the shoot apex for three plants, sampled at the same time as the plants for heading day (for further details see Prášil et al., 2004).

2.3. Plant water relationships

Plant water status (tissue hydration) was determined as the water saturation deficit (WSD; %) and osmotic potential (OP) in the youngest fully developed leaf. WSD was determined on leaf segments 1 cm long, exposed to a water saturation treatment in a hydration chamber, according to Slavík (1963). Briefly, the WSD was calculated as the difference between the fresh weight of fully water-saturated leaf segments and the fresh weight of leaf segments sampled under the given experimental conditions, divided by the difference between the fresh weight of the fully water-saturated leaf segments and the dry weight of the same segments. Leaf OP was determined as the osmolality of thawed leaf segments, sampled in a syringe and stored at −25 °C using a VAPRO Dew Point Osmometer (WESCOR Inc., Logan, Utah, USA). OP values have been calculated from the osmolality values using the van't Hoff equation $\psi = -cRT$ (c is osmolality of the solution measured; R is an universal gas constant, 8.314 J mol^{-1} K^{-1}; T is a thermodynamic temperature in K).

2.4. Frost tolerance test

Leaf and crown segments (1 cm in length; 2 replicate samples for each freezing temperature; 10 leaf segments and 3 crown segments for every sample) were placed into glass test tubes, inoculated by a piece of ice, and exposed to a set of freezing temperatures ranging from −1.5 °C to −30 °C, all as described in Prášil and Zámečník (1998). The rate of cooling was 10 °C h^{-1}. The samples were subjected to the given freezing temperature for 30 min and were then transferred to a bath at 0 °C for 12 h. After thawing, 15 ml of deionized water were added to each sample, the samples were then shaken for 2 h, and the freezing damage of the samples were determined conductometrically, as described in Prášil and Zámečník (1998). Values for the lethal temperature for 50% of the samples, i.e., temperature causing 50% electrolyte leakage of the sample, were calculated according to Janáček and Prášil (1991).

2.5. Determination of WCS120 protein content

Leaf, crown, and root samples were frozen in liquid nitrogen and stored at −80 °C until protein extraction and boiling. The dehydrin extraction as well as the 1D SDS-PAGE and immunoblot analyses were performed according to Vítámvás et al. (2007). The relative accumulation of WCS120 protein was determined densitometrically using Quantity One software (Bio-Rad, v. 4.6.2., Munich, Germany).

2.6. Phytohormone analysis

Plant hormones were purified and analyzed according to Dobrev and Kaminek (2002), and Dobrev and Vanková (2012). Samples (ca 300 mg FW) were homogenized and extracted with methanol/water/formic acid (15/4/1, v/v/v). The following labelled internal standards (10 pmol per sample) were added: $^{13}C_6$-IAA (Cambridge Isotope Laboratories), $^{2}H_4$-SA (Sigma–Aldrich), $^{2}H_2$-GA_4, $^{2}H_2$-GA_{19}, $^{2}H_6$-ABA, $^{2}H_5$-*trans*Z, $^{2}H_5$-*trans*ZR, $^{2}H_5$-*trans*Z7G, $^{2}H_5$-*trans*Z9G, $^{2}H_5$-*trans*ZOG, $^{2}H_5$-*trans*ZROG, $^{2}H_5$-*trans*ZRMP, $^{2}H_3$-DHZ, $^{2}H_3$-DHZR, $^{2}H_3$-DHZ9G, $^{2}H_6$-iP, $^{2}H_6$-iPR, $^{2}H_6$-iP7G, $^{2}H_6$-iP9G, $^{2}H_6$-iPRMP (Olchemim). Extracts were purified using a SPE-C18 column (SepPak-C18, Waters), and separated on a reverse phase-cation exchange SPE column (Oasis-MCX, Waters). The first hormone fraction was eluted with methanol (contains ABA and other acidic hormones); the second fraction, eluted with 0.35 M NH_4OH in 70% methanol, contained CK metabolites. Both fractions were separated by HPLC (Ultimate 3000, Dionex); and the hormones were quantified using a hybrid triple quadrupole/linear ion trap mass spectrometer (3200 Q TRAP, Applied Biosystems) operated in selected reaction monitoring mode.

2.7. Phenolic acid analysis

Phenolic acid analysis included determination of salicylic, *p*-hydroxybenzoic, 2,3-dihydrobenzoic and 2,5-dihydrobenzoic, *p*-coumaric, protocatechuic, vanillic, caffeic, syringic, *p*-coumaric, sinapic, and ferulic acids according to Gruz et al. (2008). Samples (ca 500 mg FW) were homogenized and extracted with 80% methanol. The sample losses during purification were corrected using labelled internal standard [$^{2}H_4$]salicylic acid (i.e., [2-hydroxybenzoic-3,4,5,6-d4 acid], Olchemim]. After evaporation to the water phase, the samples were diluted with water, and the pH was adjusted to 2. The phenolic acids were repeatedly extracted into ether. After evaporation, the samples were diluted in 50% methanol, filtered, and analyzed by LC/MS (Ion Trap Mass Spectrometer Finnigan MAT LCQ-MSn) using a Synergi 4u Hydro-RP column in negative MS/MS mode.

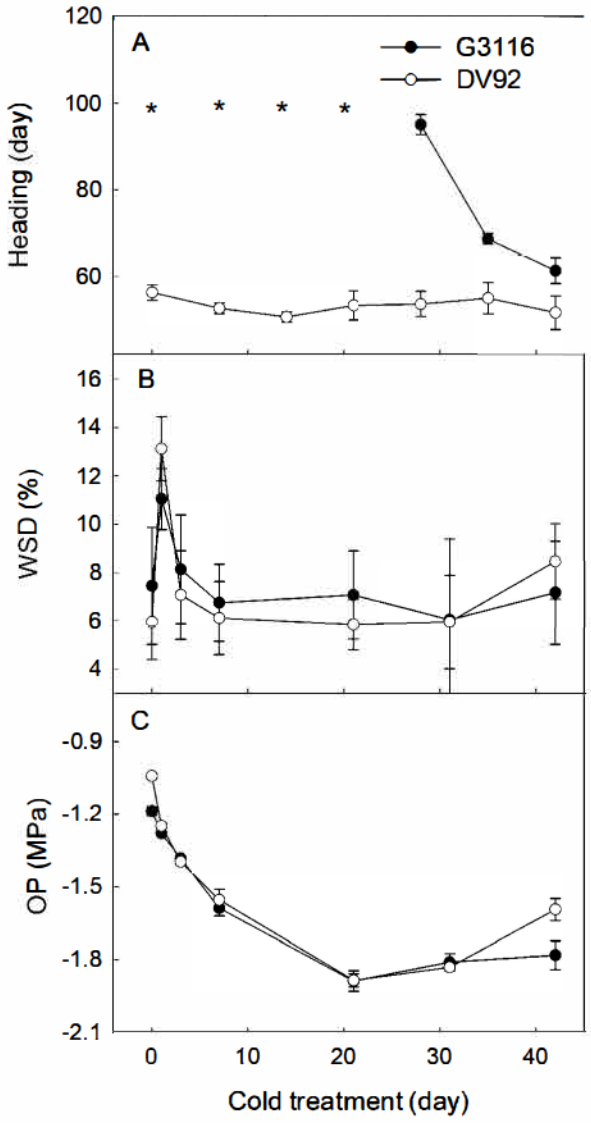

Fig. 1. Days to heading in plants (A), water saturation deficit (WSD) (B) and osmotic potential (OP) (C) in leaves of two einkorn wheat lines (G3116 and DV92) at 0, 7, 14, 21, 28, 35 and 42 (A); and 0, 1, 3, 7, 21, 31, and 42 (B and C) days of cold treatment (4 °C), respectively. Asterisks indicate that the plants had not reached heading by day 120.

2.8. Statistical analysis

Results were expressed as the means of three biological replicates, with at least two technical replicates, and compared using *ANOVA* analysis and Duncan's multiple range test at the 0.05 level (Statistica v. 10 software, StatSoft, Tulsa, OK, USA). Principal component analysis (PCA) was also carried out in Statistica software. Cluster analysis was carried out in PermutMatrix software, v. 1.9.3. (Caraux and Pinloche, 2005).

3. Results

3.1. Days to heading and shoot apex development

The cold treatment did not reveal any significant effect on days to heading in the DV92 line (Fig. 1A), confirming that the line did not have a vernalization requirement. In contrast, the G3116 line revealed a significant reduction in the days to heading parameter, along with the length of cold treatment; the number of days to heading was lowest in plants exposed to 42 days of cold. At this

time, the differences in heading time were insignificant between the two lines, indicating the vernalization saturation of the G3116 line (Suppl. Table 1A). Throughout the cold acclimation, the shoot apex of the G3116 line remained in a vegetative state (apex was short, of hemispherical shape, with leaf primordia at its base), while the beginnings of shoot apex elongation (when leaf primordia initiate elongation of the shoot apex) was observed in the DV92 line at 42 days of cold.

3.2. Plant water relationships

WSD exhibited a significant transient increase in both lines at the beginning of cold treatment (1 day of cold) (Fig. 1B; Suppl. Table 1B, C). A transient increase in WSD meant a transient leaf tissue dehydration, which indicated a shock caused by a sudden temperature decrease. However, the initial WSD level was very quickly restored in both lines. In contrast, leaf OP steadily decreased up to 21 days of cold, when an osmotic adjustment to the altered water relationship was reached. The G3116 winter line then maintained the low OP until the end of the experiment at 42 days of cold; while the spring line DV92 exhibited an increase in OP at 42 days of cold (Fig. 1C). There were no significant differences observed between the lines, either in the WSD or OP values, throughout the entire cold treatment, except for the OP values at 42 days of cold.

3.3. Frost tolerance tests

The lethal temperature for 50% of the samples (LT50 values) decreased during the first 21 days of cold treatment in the leaf and crown tissues in both lines, indicating the induction of FrT (Fig. 2A). During the next period, the FrT (LT50s) remained at the same level, and in the last (42nd) day of cold treatment the FrT slightly decreased (LT50 increased); primarily in the crown tissues of both lines. There were no significant differences in LT50 values in the leaf tissue between the two lines; however, in the crowns, the DV92 line showed significantly higher LT50s (lower FrT) than the G3116 line from 3 days of cold onwards.

3.4. Dehydrins

There were four distinct dehydrin bands detectable on 1-D immunoblots in both the G3116 and DV92 lines during the entire experiment (Figs. 3A and B). The bands corresponded to dehydrins from the WCS120 family in *T. aestivum*, according to their MW values (apparent molecular weight on 1-D SDS-PAGE), except for the absence of a homologue of the WCS66 protein; i.e., there were homologues of WCS200, WCS180, WCS120, and WCS40 detected in *T. monococcum* (hereafter referred to as WCS200, WCS180, WCS120, WSC40). Similar to *T. aestivum*, the homologue of WCS120 was the most abundant protein, followed by WCS40; while the homologues of WCS200 (and especially WCS180) were only present as very faint bands in the *T. monococcum* lines. The relative abundance of the homologue of *T. aestivum* WCS120 was used for quantitative analyses of dehydrin relative abundance during the cold treatment between the organs. The crowns accumulated larger amounts of dehydrins than the leaves, and in all samples WCS120 accumulation in the roots was below the detection limits (with the exception of the two last samplings in the G3116 line; see Fig. 3C; Suppl. Table 1B, C)).

The dynamics of quantitative changes in the relative abundance of WCS120 in both the leaf and crown tissues revealed an initial increase in WCS120 relative abundance in both lines, during the first 21 days of cold treatment (Fig. 2B). The lines became differentiated after 21 days of cold treatment, with the winter line G3116 revealing an increase in WCS120, while the DV92 line revealed a steady level from 21 days onwards. The WCS120 protein reached a

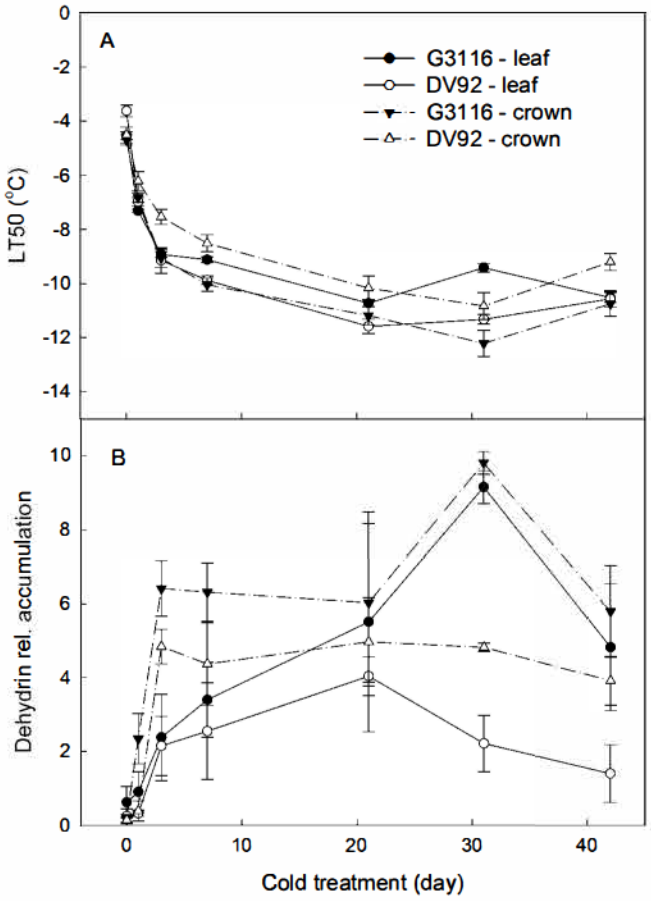

Fig. 2. Relative accumulation of WCS120 protein (A) and frost tolerance (expressed as LT50 values) (B) in leaves and crowns of two einkorn wheat lines (G3116 and DV92) at 0, 1, 3, 7, 21, 31, and 42 days of cold treatment (4 °C).

peak in the accumulation at 31 days of cold in the G3116 line; then in accordance with the fulfilment of the vernalization requirement its level declined. Both lines became significantly differentiated in WCS120 relative abundance only at 31 and 42 days of cold.

The level of WCS120 accumulation significantly correlated with the level of FrT (LT50 values), not only in all sample comparisons ($n = 28$; $r = -0.692$; $P < 0.01$), but also between leaf samples ($n = 14$; $r = -0.621$; $P < 0.05$), crown samples ($n = 14$; $r = -0.892$; $P < 0.01$), line G3116 samples ($n = 14$; $r = -0.796$; $P < 0.01$), and line DV92 samples ($n = 14$; $r = -0.612$; $P < 0.05$) (Suppl. Fig. 1).

3.5. Plant hormones

The dynamics of abscisic acid (ABA) levels (Fig. 4) during cold stress progression corresponded well to the profile of WSD. A sharp WSD peak was closely followed by a significant ABA increase in the leaves and crowns of both genotypes (after 1 day of cold stress). The ABA content returned to the basal level before day 3 of cold treatment in the crowns and leaves of winter genotype G3116; while in the spring genotype DV92, ABA remained elevated at this time-point. Under control conditions as well as during the alarm phase, ABA levels were higher in crowns than in leaves (with significant differences only in genotype G3116); the ABA basal level being higher in the crowns of G3116 compared with DV92 (Suppl. Table 1B, C). The ABA content was generally low in the roots, decreasing further in the later stages of the cold treatment. The levels of ABA catabolites (phaseic acid, dihydrophaseic acid, 9-hydroxy-ABA, and ABA glucosyl ester) are shown in Suppl. Fig. 2. The most abundant metabolite was dihydrophaseic acid. The

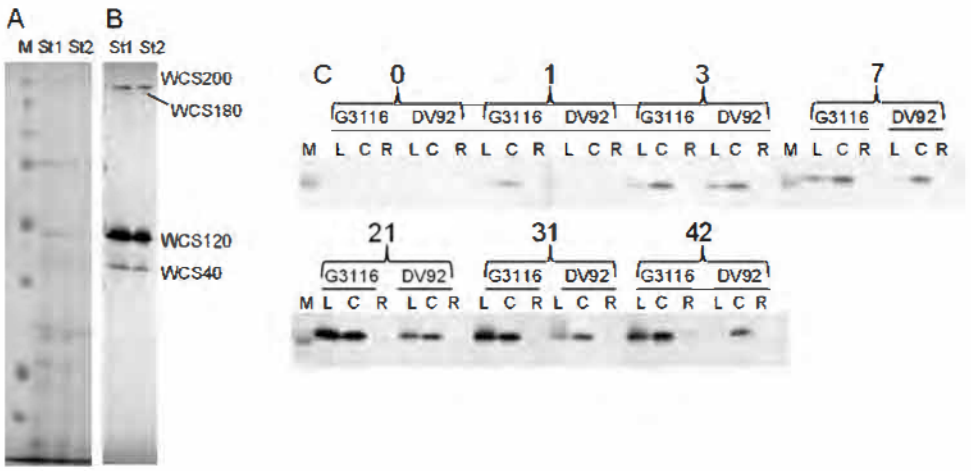

Fig. 3. The visualized proteins: (A) nitrocellulose membrane stained with Ponceau S; (B) protein hybridization with anti-dehydrin antibody; (C) the level of accumulation of WCS120 protein in leaves (L), crowns (C), and roots (R) of two einkorn wheat lines (G3116 and DV92) at 0, 1, 3, 7, 21, 31, and 42 days of cold treatment (4 °C). M – precision plus protein standards all blue (Bio-Rad). St1 – sample extracted from 31 days of cold-acclimated leaves of G3116 line loaded on each gel for standardization of data between each membrane; St2 – ½ of St1 loaded in the well.

highest ABA metabolite content was detected in the crowns, and the lowest in the roots. A prolonged cold period was associated with down-regulation of ABA catabolites, especially in the spring cultivar.

The cold stress was accompanied by the decrease of active gibberellin GA_4 levels; a rapid decrease was observed in the leaves, crowns and roots in both genotypes (Fig. 5). The crowns and roots of G3116 exhibited a slight delay, but the down-regulation was more profound. A deep GA_4 minimum was observed in DV92 leaves after 7 days of cold. Leaves of both genotypes showed maxima in the acclimation phase after 21 days of cold. The other active gibberellin, GA_1, was not detected in either genotype. The level of the GA_4 precursor, GA_{19}, was higher in the crowns than in leaves, with the roots exhibiting relatively low levels of this metabolite. In all organs of both genotypes a transient maximum was observed after 21 days of cold.

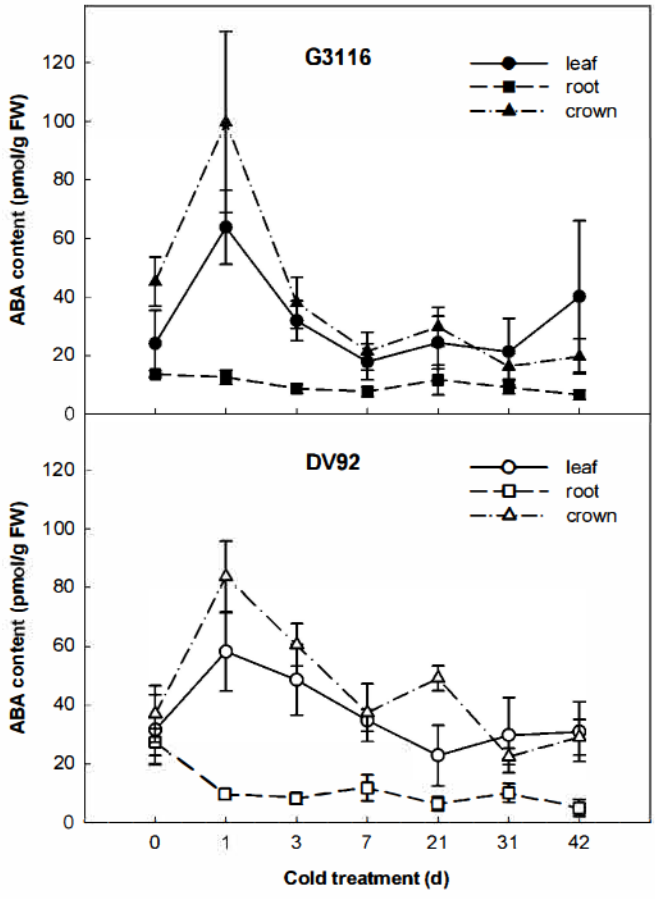

Fig. 4. Levels of abscisic acid (ABA) in leaves, roots and crowns of two einkorn wheat lines (G3116 and DV92) during cold treatment (at 4 °C).

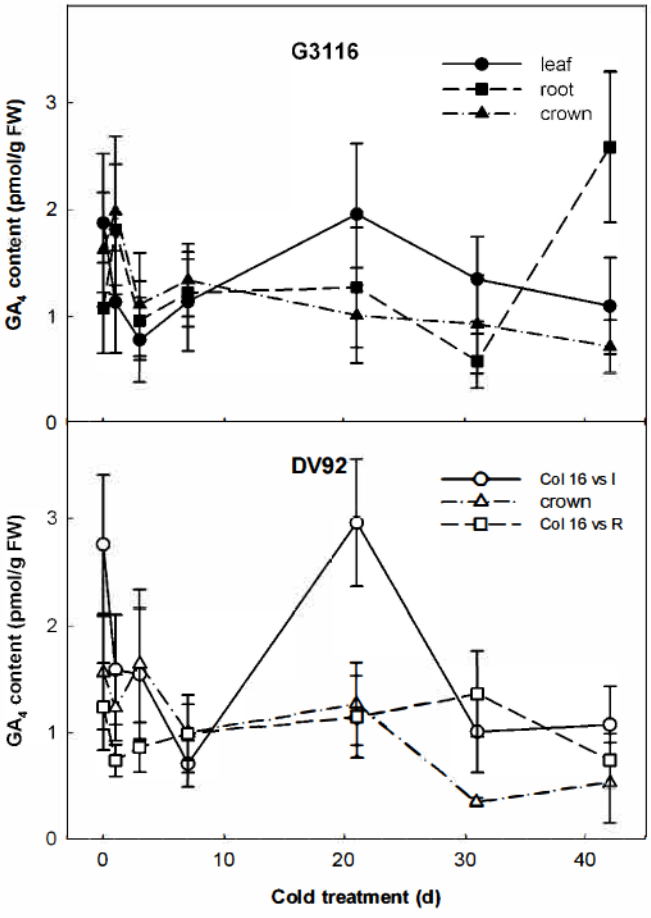

Fig. 5. Levels of active gibberellin GA_4 in leaves, roots and crowns of two einkorn wheat lines (G3116 and DV92) during cold treatment (at 4 °C).

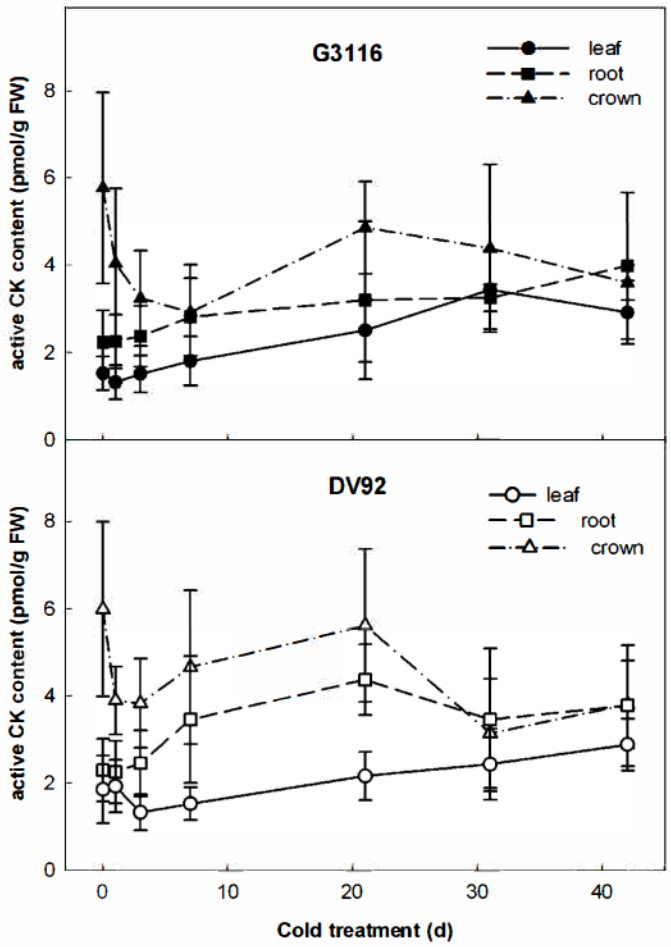

Fig. 6. Levels of active cytokinins (*trans*-zeatin, isopentenyladenine, *cis*-zeatin, dihydrozeatin and the corresponding ribosides) in leaves, roots and crowns of two einkorn wheat lines (G3116 and DV92) during cold treatment (at 4 °C).

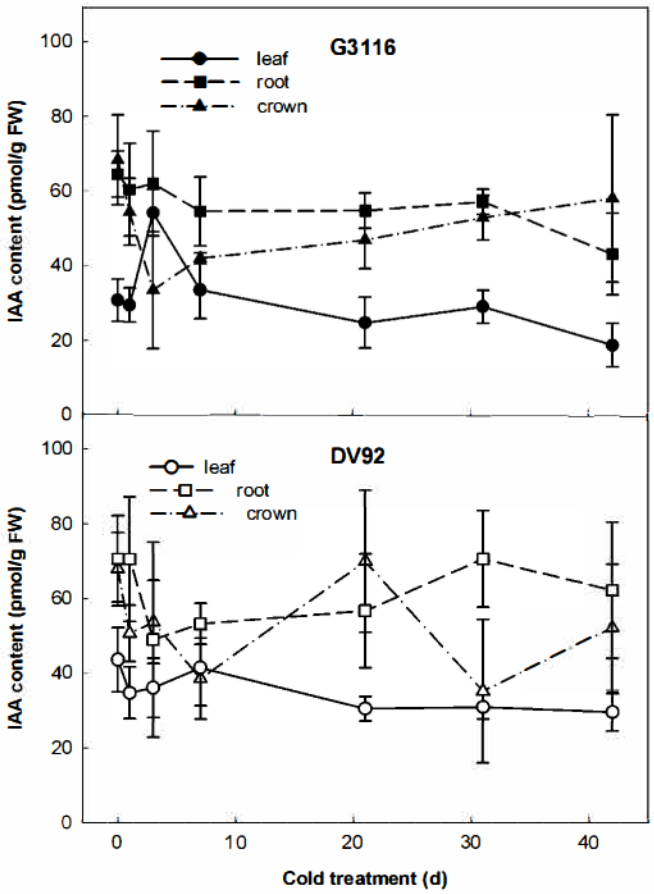

Fig. 7. Levels of auxin (indole-3-acetic acid, IAA) in leaves, roots and crowns of two einkorn wheat lines (G3116 and DV92) during cold treatment (at 4 °C).

The active CKs were more abundant in the crowns compared with the roots and leaves in both genotypes (Fig. 6). Cold stress imposed a rapid down-regulation of active CKs (*trans*-zeatin, isopentenyladenine, *cis*-zeatin, dihydrozeatin, and the corresponding ribosides) in the leaves of G3116; and slightly later in DV92. A profound decrease of active CKs was observed in the meristematic tissues (crowns). The decrease was more profound and longer in the winter line. A mild maximum was observed in DV92 roots after 21 days of cold. At the same time, a moderate maximum of active CKs was found in the DV92 crowns. A maximum of active CKs was found in G3116 crowns after 21 days also. The roots of G3116 exhibited a maximum after 42 days. At this period, high up-regulation of *cis*-zeatin and its riboside in leaves was observed.

The levels of precursors of the active CKs (CK phosphates) followed similar profiles as those of the active CKs (Suppl. Fig. 3). Cold exposure was associated with their transient down-regulation. Their elevation was found in DV92 crowns after 7–31 days. G3116 crowns exhibited maximum of CK phosphates at 21 days, only mild decrease being observed later on. The level of CK O-glucosides, which are due to the reversible character of O-glucosylation considered as storage forms, was relatively low, and only more abundant in crowns. Irreversible glucosylation at the N7 or N9 position of the purine ring was increased under cold in both lines. However, by far the most abundant CK metabolites were derivatives of *cis*-zeatin. The level of inactive CK metabolites gradually decreased during cold treatment, being very low after 31 and 42 days.

Down-regulation of the level of the endogenous auxin indole-3-acetic acid (IAA) was found during the early response to cold treatment in crowns (Fig. 7), with a transient maximum at 21 and 42 days in the DV92 and G3116 lines, respectively. Only minor changes were observed during stress progression in the roots. The levels of the IAA precursor indole-3-pyruvic acid (IpyA) were relatively high (Suppl. Fig. 4). The IpyA content was increased after 7 days of cold in the leaves of the winter line, and after 21 days in the spring one. In the roots, it was increased after 31 and 21 days in G3116 and DV92, respectively. In the crowns, a substantial elevation was observed after 31 and 42 days in G3116 and DV92, respectively. The levels of the precursors of the parallel biosynthetic routes (indole-3-acetamide and indole-3-acetonitrile) were close to the detection limits (results not shown). The most abundant deactivated IAA metabolite was IAA-aspartate. It was relatively low in leaves, but quite abundant in the roots, and highly abundant in the crowns; being higher in G3116 than in DV92. The second most abundant inactive IAA metabolite was oxo-IAA.

Upon cold application, jasmonic acid (JA) exhibited a rapid down-regulation in the roots of G3116, and in the leaves of DV92 (Fig. 8A). The response of DV92 roots and crowns of both lines was delayed by 1 day. JA levels remained stable or were elevated during the cold stress progression for 21–31 days. The JA levels in the roots and crowns were substantially higher than in the leaves. The levels of JA-Ile, the active JA metabolite which binds to the COI receptor, followed a similar pattern as did JA (Fig. 8B); with the exception of a prolonged down-regulation in G3116 roots during the alarm phase, and a faster JA-Ile decrease in G3116 crowns at the beginning of this stress phase.

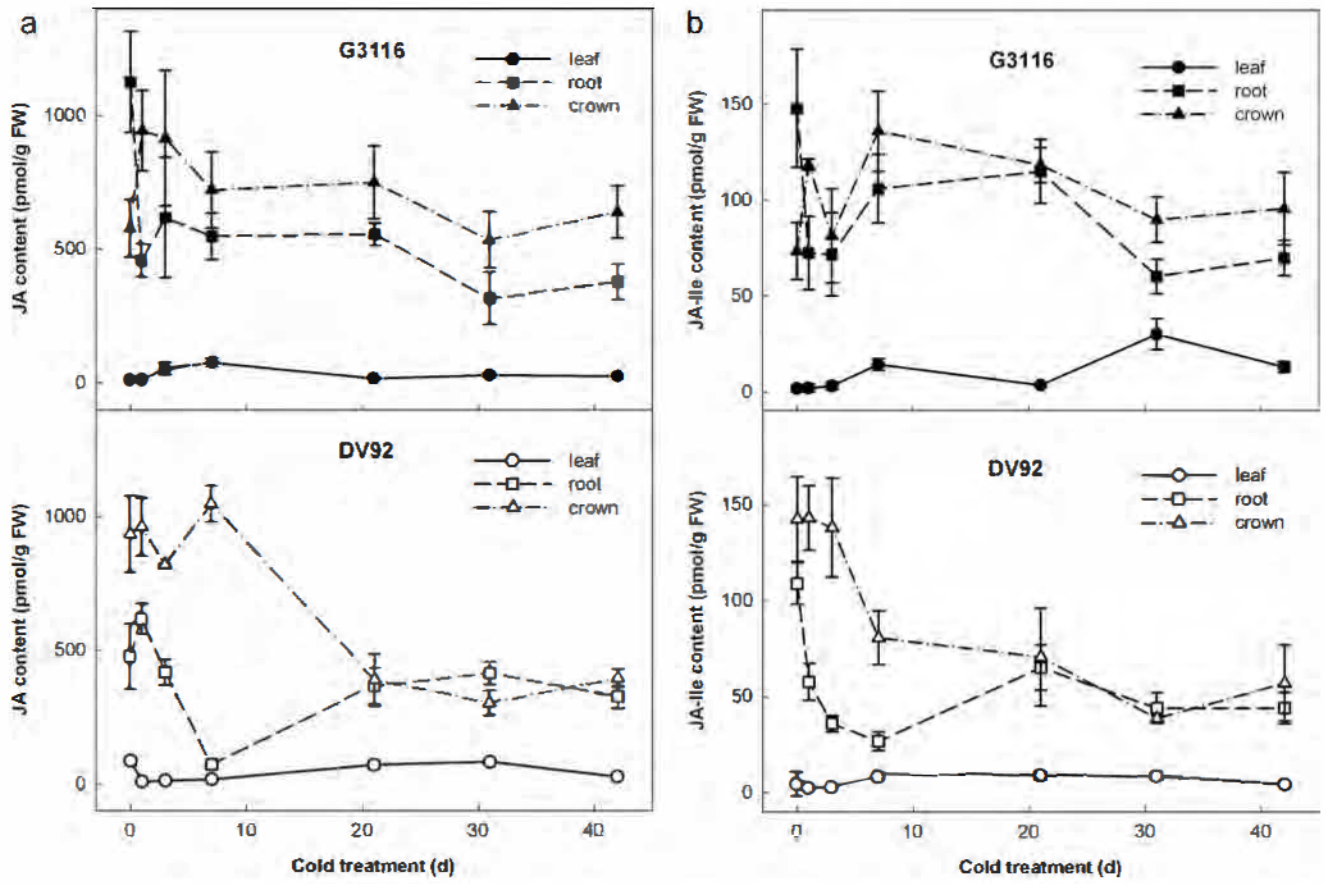

Fig. 8. Levels of jasmonic acid (JA) (A) and jasmonate isoleucine (JA-Ile) (B) in leaves, roots and crowns of two einkorn wheat lines (G3116 and DV92) during cold treatment (at 4 °C).

The aminocyclopropane carboxylic acid (ACC) content exhibited a rapid down-regulation in the crowns of both genotypes, and in the leaves and roots of the winter line (Fig. 9). G3116 roots showed a mild maximum after 3 days of cold stress. During pronged cold treatment (after 31 days), ACC increased in all organs of the winter line and in DV92 crowns.

The basal level of salicylic acid (SA) was quite similar in all organs (leaves, roots, and crowns), being slightly higher in DV92. Cold exposure resulted in SA decrease; relatively short in DV92 (1 day), and longer (3 days) in G3116 (Fig. 10). Only a mild increase above the basal level was observed until 21 days in both lines. Higher levels between day 7 and 21 were found in winter line leaves. After this period, a substantial increase was observed in the leaves of both lines, faster in G3116. A relatively minor increase was detected in the crowns.

3.6. Phenolic acids

SA belongs to the group of phenolic acids. These secondary metabolites include also derivatives of cinnamic and benzoic acids. Four cinnamic acid derivatives (*p*-coumaric, caffeic, ferulic, and sinapic acids) and four benzoic acid derivatives (*p*-hydroxybenzoic, protocatechuic, vanillic, and syringic acids) were ascertained in both lines (Table 1). One of the most abundant phenolic acids in the winter line was ferulic acid (FA). The FA level was rapidly and strongly promoted by cold stress in G3116 leaves, to a lesser extent also in the roots, and after 7 days in the crowns. In contrast, FA content was much lower in leaves of the spring line, and its up-regulation was postponed. The basal FA level in crowns was comparable in both lines; however, cold stress stimulation was more intensive in G3116. In leaves, G3116 exhibited low levels of sinapic acid (SiA), and its cold stimulation was only moderate. In contrast to G3116, the leaves of DV92 contained much higher levels of SiA than FA, and the cold stimulation of its content was fast. High up-regulation of SiA was mainly observed in the later stages of cold treatment (21–42 days). The levels of SiA in the roots were similar to FA, i.e., lower in the winter line than in the spring one. Caffeic acid levels increased in the leaves of both lines after prolonged cold; however, mild stimulation in the crowns was found only in G3116. Cold stress strongly promoted the levels of *p*-coumaric acid in G3116 in all organs (predominantly in the crowns). In both cultivars, *p*-hydroxybenzoic acid was substantially up-regulated by cold in the crowns (more in the spring one). Vanillic acid was stimulated in the leaves of both lines, and in the crowns of G3116. The highly abundant protocatechuic acid was suppressed by cold in both lines, especially in the crowns. Syringic acid was up-regulated in the crowns of both lines.

Vanillic, *p*-hydroxybenzoic, and *p*-coumaric acids may influence the auxin level by stimulating IAA oxidase activity, while the cinnamic acid derivatives (FA and SiA) may protect IAA from oxidative damage.

3.7. Multidimensional statistical analysis

All data obtained from the leaf samples in both genotypes (G3116 – W; DV92 – S), throughout the whole experiment, have been subjected to multidimensional statistical analyses in order to obtain complex information on the dynamics of *T. monococcum* cold response. The principal component analysis (PCA) has

Table 1A
The content of benzoic acid and cinnamic acid derivatives in leaves, roots and crowns of winter einkorn wheat line – G3116 during cold treatment (at 4 °C). Benzoic acid derivatives: PrA, protocatechuic acid; *p*-HBA, *p*-hydroxybenzoic acid; VA, vanillic acid; SyA, syringic acid. Cinnamic acid derivatives: *p*-CA, *p*-coumaric acid; CaA, caffeic acid; SiA, sinapic acid; FA, ferulic acid.

	PrA [nmol/g FW]	p-HBA [nmol/g FW]	VA [nmol/g FW]	CaA [nmol/g FW]	SyA [nmol/g FW]	p-CA [nmol/g FW]	SiA [nmol/g FW]	FA [nmol/g FW]
Leaves								
0 d	3.23 ± 0.24	1.22 ± 0.12	1.70 ± 0.13	1.10 ± 0.11	1.32 ± 0.03	1.01 ± 0.07	0.26 ± 0.02	1.79 ± 0.16
1 d	1.89 ± 0.26	1.06 ± 0.09	2.15 ± 0.26	1.23 ± 0.16	1.88 ± 0.20	2.44 ± 0.15	1.20 ± 0.21	3.79 ± 0.41
3 d	1.95 ± 0.13	0.80 ± 0.08	2.15 ± 0.19	0.81 ± 0.05	2.27 ± 0.07	1.69 ± 0.09	1.38 ± 0.07	4.10 ± 0.32
7 d	1.93 ± 0.18	1.56 ± 0.08	2.21 ± 0.33	2.73 ± 0.38	2.07 ± 0.26	2.66 ± 0.31	2.31 ± 0.28	5.46 ± 0.83
21 d	2.14 ± 0.27	0.90 ± 0.20	2.21 ± 0.28	2.13 ± 0.39	2.80 ± 0.51	2.75 ± 0.53	2.25 ± 0.34	7.4 ± 1.27
31 d	1.73 ± 0.14	0.91 ± 0.05	2.03 ± 0.13	1.56 ± 0.15	2.32 ± 0.08	2.20 ± 0.22	1.79 ± 0.07	8.6 ± 0.70
42 d	1.68 ± 0.16	0.45 ± 0.04	2.24 ± 0.37	1.45 ± 0.21	2.55 ± 0.26	1.55 ± 0.29	1.59 ± 0.15	7.42 ± 0.94
Roots								
0 d	0.99 ± 0.09	1.92 ± 0.17	0.50 ± 0.05	0.56 ± 0.05	0.16 ± 0.01	1.21 ± 0.11	0.31 ± 0.03	0.59 ± 0.05
1 d	1.90 ± 0.25	0.73 ± 0.10	0.39 ± 0.05	0.69 ± 0.09	0.20 ± 0.03	1.51 ± 0.20	0.38 ± 0.05	0.91 ± 0.14
3 d	3.39 ± 0.27	1.62 ± 0.13	0.96 ± 0.08	0.76 ± 0.06	0.37 ± 0.03	2.01 ± 0.16	0.33 ± 0.02	0.74 ± 0.06
7 d	2.39 ± 0.33	2.17 ± 0.31	0.52 ± 0.07	0.53 ± 0.08	0.28 ± 0.04	2.19 ± 0.31	0.30 ± 0.04	1.84 ± 0.26
21 d	1.76 ± 0.33	1.66 ± 0.29	0.61 ± 0.11	0.52 ± 0.1	0.58 ± 0.11	4.05 ± 0.74	0.28 ± 0.05	1.67 ± 0.29
31 d	2.09 ± 0.20	1.80 ± 0.17	0.45 ± 0.04	0.53 ± 0.05	0.32 ± 0.03	3.89 ± 0.37	0.31 ± 0.03	1.43 ± 0.14
42 d	1.43 ± 0.17	2.23 ± 0.27	0.43 ± 0.05	0.54 ± 0.06	0.22 ± 0.03	4.19 ± 0.51	0.26 ± 0.03	1.05 ± 0.13
Crowns								
0 d	2.94 ± 0.26	1.78 ± 0.18	0.80 ± 0.09	0.79 ± 0.09	0.69 ± 0.08	3.36 ± 0.44	1.39 ± 0.15	1.82 ± 0.18
1 d	0.22 ± 0.05	2.25 ± 0.13	0.48 ± 0.07	1.11 ± 0.14	0.90 ± 0.06	6.63 ± 0.58	1.33 ± 0.19	1.46 ± 0.19
3 d	0.31 ± 0.06	2.41 ± 0.44	1.30 ± 0.24	1.22 ± 0.22	2.85 ± 0.52	5.98 ± 1.10	2.54 ± 0.46	1.53 ± 0.28
7 d	0.30 ± 0.04	2.66 ± 0.38	1.41 ± 0.20	1.61 ± 0.23	2.22 ± 0.32	6.55 ± 0.94	2.77 ± 0.40	3.46 ± 0.50
21 d	1.90 ± 0.15	1.40 ± 0.11	1.28 ± 0.10	0.86 ± 0.07	1.27 ± 0.11	5.36 ± 0.43	1.55 ± 0.12	4.73 ± 0.38
31 d	2.72 ± 0.36	2.20 ± 0.29	1.74 ± 0.23	1.21 ± 0.16	1.76 ± 0.23	6.80 ± 0.90	1.61 ± 0.21	1.94 ± 0.26
42 d	2.02 ± 0.19	2.60 ± 0.24	1.81 ± 0.17	0.59 ± 0.05	2.28 ± 0.21	6.58 ± 0.60	1.00 ± 0.05	4.37 ± 0.40

distinguished three major groups of samples (experimental variants) (Fig. 11): 1/the group containing samples from optimal conditions (S 0, W 0) and samples from the early stages of cold treatment (S 1, W 1, W 3); 2/the group containing full cold-acclimated samples (S 21, W7, W 21, W 31, W 42); and 3/the group containing samples of the spring DV92 line (S) from the later stages of cold treatment (S 3, S 31, S 42). Comparison of the individual organs studied (leaves, crowns, and roots), separately and as a whole, in one PCA analysis (Suppl. Fig. 5) revealed similar dynamics of the cold response between the organs (with some minor differences, e.g., a distinct differentiation between control samples and samples from the early phases of cold treatment (1 and 3 days of cold) in the crown samples).

The cluster analysis of all data obtained from leaf samples has revealed four major patterns of response to a long-term cold treatment (Fig. 12). Cluster 1 includes WSD, LT50, OP, PrA, and ABA and its metabolites (PA, 9OH-ABA), which all exhibit a typical shock response to cold treatment – a strong induction at 1 day of cold, followed by a subsequent decrease to levels similar to the controls. Cluster 2 includes IAA, JA, GA_4, GA_{19}, some phenolic acids such as p-CA and SyA, and CK N-glucosides, as well as other inactive CK metabolites; generally, these substances revealed enhanced levels

Table 1B
The content of benzoic acid and cinnamic acid derivatives in leaves, roots and crowns of spring einkorn wheat line – DV92 during cold treatment (at 4 °C). Benzoic acid derivatives: PrA, protocatechuic acid; *p*-HBA, *p*-hydroxybenzoic acid; VA, vanillic acid; SyA, syringic acid. Cinnamic acid derivatives: *p*-CA, *p*-coumaric acid; CaA, caffeic acid; SiA, sinapic acid; FA, ferulic acid.

	PrA [nmol/g FW]	p-HB [nmol/g FW]	VA [nmol/g FW]	CaA [nmol/g FW]	SyA [nmol/g FW]	p-CA [nmol/g FW]	SiA [nmol/g FW]	FA [nmol/g FW]
Leaves								
0 d	3.42 ± 0.20	1.78 ± 0.31	2.60 ± 0.23	1.40 ± 0.18	2.51 ± 0.16	0.60 ± 0.06	1.43 ± 0.23	0.45 ± 0.05
1 d	2.20 ± 0.09	2.05 ± 0.13	3.06 ± 0.08	1.00 ± 0.18	2.19 ± 0.21	0.35 ± 0.02	2.3 ± 0.11	0.25 ± 0.02
3 d	2.24 ± 0.35	1.40 ± 0.24	3.31 ± 0.51	2.53 ± 0.25	2.11 ± 0.21	1.53 ± 0.12	4.72 ± 0.58	0.28 ± 0.03
7 d	1.80 ± 0.20	1.60 ± 0.13	3.51 ± 0.50	2.57 ± 0.18	2.14 ± 0.26	1.32 ± 0.09	5.18 ± 0.68	0.27 ± 0.05
21 d	1.83 ± 0.07	1.80 ± 0.17	2.72 ± 0.20	3.80 ± 0.37	1.56 ± 0.14	1.63 ± 0.07	6.57 ± 0.71	1.36 ± 0.11
31 d	2.61 ± 0.37	1.90 ± 0.28	2.61 ± 0.25	2.09 ± 0.31	2.00 ± 0.31	0.84 ± 0.08	9.09 ± 1.16	1.2 ± 0.11
42 d	1.61 ± 0.18	2.40 ± 0.19	2.64 ± 0.30	1.35 ± 0.07	2.15 ± 0.24	0.96 ± 0.07	10.21 ± 1.21	0.87 ± 0.09
Roots								
0 d	1.59 ± 0.21	1.79 ± 0.22	0.57 ± 0.08	0.55 ± 0.07	0.78 ± 0.01	0.40 ± 0.05	1.27 ± 0.17	1.29 ± 0.17
1 d	2.34 ± 0.19	1.07 ± 0.08	0.48 ± 0.04	0.67 ± 0.05	0.81 ± 0.06	0.36 ± 0.03	1.03 ± 0.08	1.52 ± 0.12
3 d	1.48 ± 0.21	1.58 ± 0.23	0.97 ± 0.14	0.74 ± 0.11	0.66 ± 0.09	0.36 ± 0.05	2.49 ± 0.36	1.97 ± 0.28
7 d	1.56 ± 0.29	1.16 ± 0.21	0.66 ± 0.12	0.56 ± 0.10	0.55 ± 0.10	0.38 ± 0.06	2.42 ± 0.44	2.94 ± 0.54
21 d	2.04 ± 0.20	1.74 ± 0.17	0.75 ± 0.07	0.56 ± 0.05	0.74 ± 0.07	0.32 ± 0.03	1.68 ± 0.16	3.5 ± 0.34
31 d	1.80 ± 0.22	2.78 ± 0.34	0.27 ± 0.03	0.51 ± 0.06	0.68 ± 0.08	0.29 ± 0.04	1.23 ± 0.15	2.34 ± 0.29
42 d	1.65 ± 0.21	1.29 ± 0.2	0.82 ± 0.07	0.59 ± 0.08	0.98 ± 0.12	0.29 ± 0.05	1.11 ± 0.12	2.84 ± 0.21
Crowns								
0 d	3.18 ± 0.41	2.42 ± 0.18	2.44 ± 0.27	1.04 ± 0.2	1.7 ± 0.17	0.41 ± 0.04	1.29 ± 0.12	1.18 ± 0.11
1 d	1.7 ± 0.31	3.25 ± 0.6	2.71 ± 0.5	0.74 ± 0.14	1.83 ± 0.34	1.44 ± 0.27	1.27 ± 0.23	1.85 ± 0.34
3 d	1.68 ± 0.24	3.16 ± 0.45	4 ± 0.57	0.81 ± 0.12	4.29 ± 0.61	1.15 ± 0.17	2.09 ± 0.3	1.02 ± 0.15
7 d	1.64 ± 0.13	3.1 ± 0.25	3.91 ± 0.31	0.57 ± 0.05	4.75 ± 0.38	1.12 ± 0.09	1.58 ± 0.13	1.37 ± 0.11
21 d	1.54 ± 0.2	4.22 ± 0.56	3.24 ± 0.43	0.78 ± 0.1	2.35 ± 0.31	1.52 ± 0.2	3.62 ± 0.48	2.41 ± 0.32
31 d	2.85 ± 0.26	5.2 ± 0.47	3.58 ± 0.33	1.59 ± 0.15	1.89 ± 0.17	1.45 ± 0.13	3.14 ± 0.29	1.03 ± 0.09
42 d	2.02 ± 0.18	2.49 ± 0.23	3.51 ± 0.32	1.26 ± 0.12	2.28 ± 0.21	1.05 ± 0.1	4.97 ± 0.46	1.87 ± 0.17

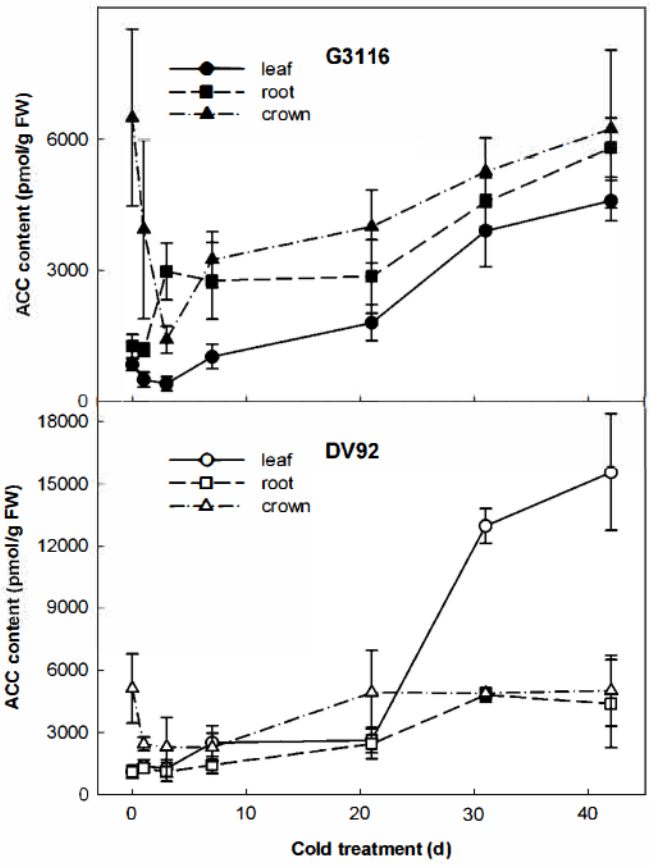

Fig. 9. Levels of the ethylene precursor aminocyclopropane carboxylic acid (ACC) in leaves, roots and crowns of two einkorn wheat lines (G3116 and DV92) during cold treatment (at 4 °C).

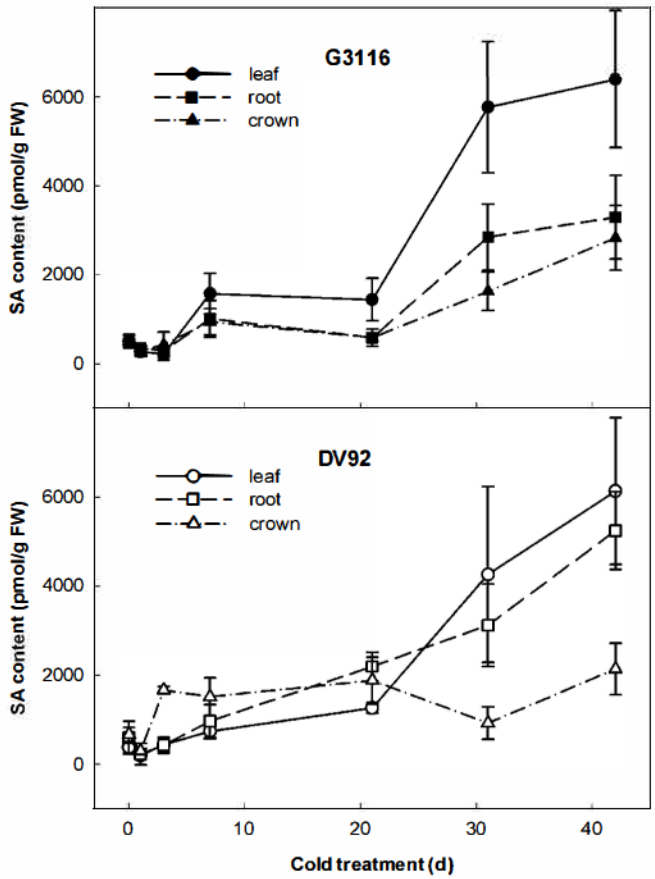

Fig. 10. Levels of salicylic acid (SA) in leaves, roots and crowns of two einkorn wheat lines (G3116 and DV92) during cold treatment (at 4 °C).

in the spring (DV92) line, with respect to the winter (G3116) line, at 0 and 1 days of cold. Thereafter, their levels decreased in both lines, followed by an increase in long-term cold acclimated plants (after 21 or 31 days of cold acclimation). Cluster 3 includes substances (e.g., ACC, ABA-GE and other ABA inactive metabolites, some phenolic acids such as VA, SiA, as well as active CKs and reversibly inactivated CK O-glucosides) which revealed an increased level in the spring (DV92) line, with respect to the winter (G3116) line, during basically the entire cold treatment; and finally, at the end of the experiment (42 days of cold), these substances exhibited a relative increase in their levels in both lines. Cluster 4 encompasses members (dehydrins, negative values of LT50, negative values of osmotic potential, SA, sum of phenolic acids and others) revealing the typical dynamics described for a cold acclimation response – an increase with the duration of the cold treatment, with emerging differences between the lines – the winter (G3116) line revealing an enhanced level, with respect to the spring (DV92) line, at the later stages of cold treatment. The cluster analysis of the individual organs (leaves, crowns, and roots) (Suppl. Fig. 6) revealed more similar dynamics of the cold response in the leaves and crowns compared to the roots. Generally, crowns and leaves also revealed higher level of the metabolites than roots. Some differences were also found between leaves and crowns – e.g., PA and 9OH-ABA (both in cluster 2), and some phenolic acids (e.g., FA and VA) revealed opposite accumulation between spring and winter line.

4. Discussion

In cereals, the complex mechanism of the cold stress response involves both cold acclimation and vernalization. The aim of the present study was to compare the dynamics of the cold response in the einkorn wheat lines DV92 and G3116 (which differ in their vernalization requirement, yet having relatively small differences in FrT) with the recently characterized response of hexaploid wheat

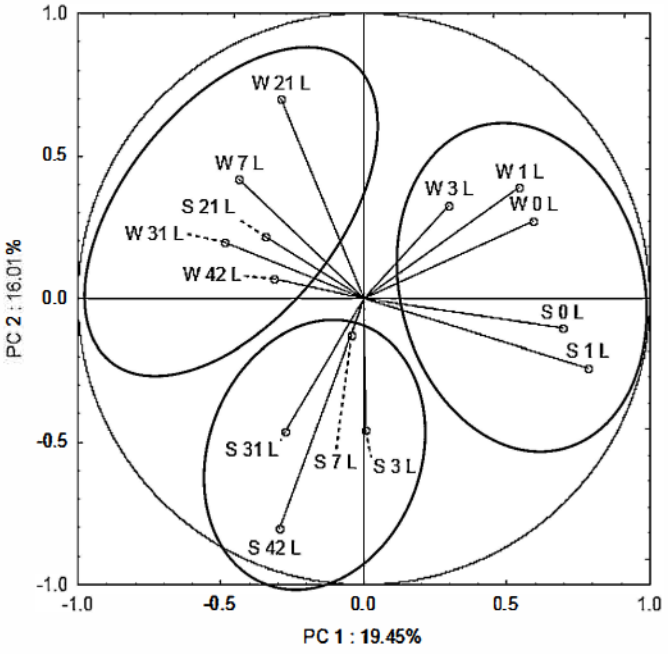

Fig. 11. Principal component analysis (PCA) of samples (S = DV92 line; W = G3116 line; 0, 1, 3, 7, 21, 31, and 42 days of cold treatment (4 °C)) in leaf (L) tissues.

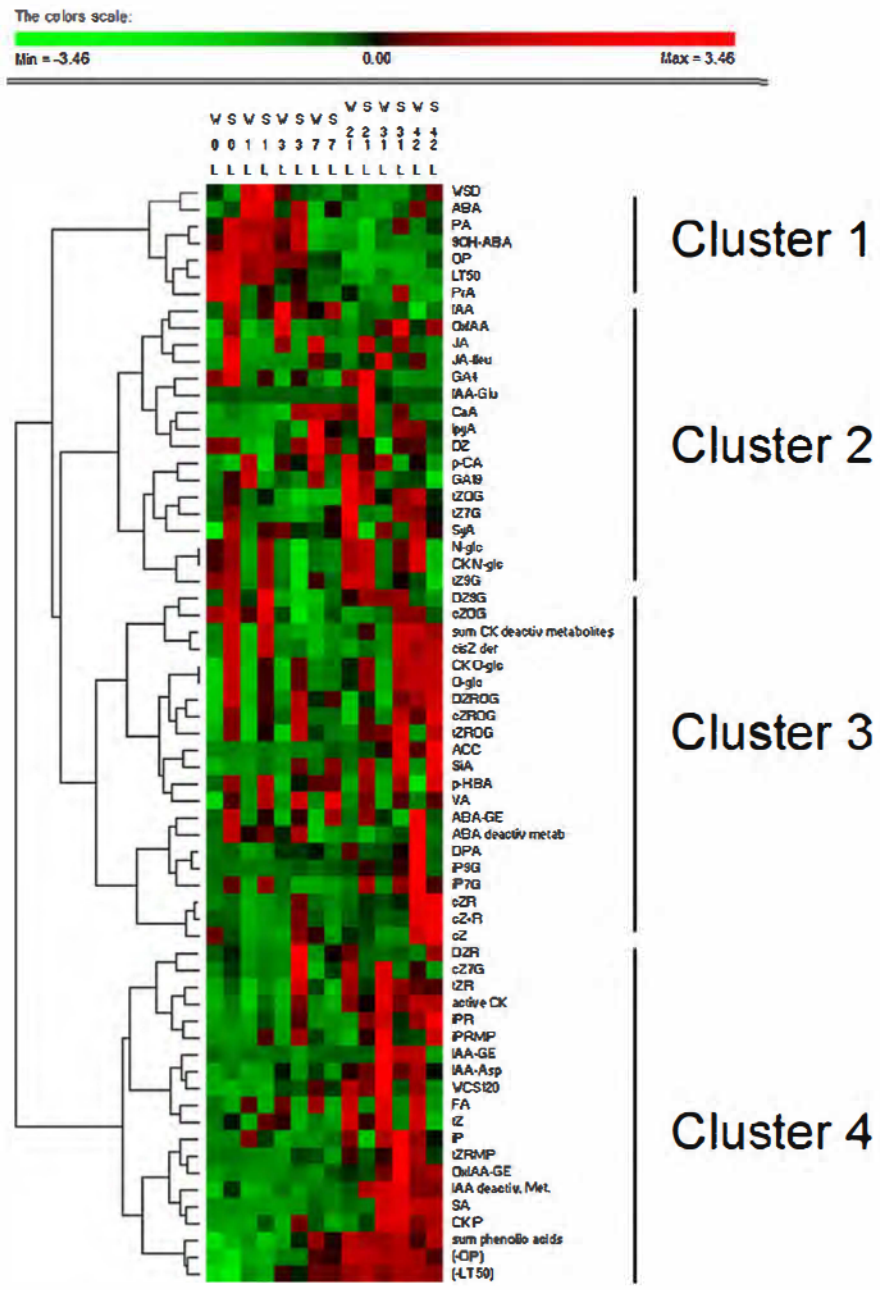

Fig. 12. Cluster analysis of all characteristics determined in the experiment in the individual samples (DV92 line: S 0, S 1, S 3, S 7, S 21, S 31, S 42; G3116 line: W 0, W 1, W 3, W 7, W 21, W 31, W 42 see Fig. 11) in leaf (L) tissues. Abbreviations: *Abscisic acid*: ABA, abscisic acid; ABA-GE, ABA-glucose ester; PA, phaseic acid; DPA, dihydrophaseic acid; NeoPA, neophaseic acid; 7OH-ABA, 7-hydroxy-ABA; 9OH-ABA, 9-hydroxy-ABA; ACC, 1-aminocyclopropane-1-carboxylic acid; *Auxins*: IAA, indole-3-acetic acid; IAA-GE, IAA-glucose ester; IAA-Ala, IAA-alanine; IAA-Asp, IAA-aspartate; IAA-Leu, IAA-leucine; IAA-Trp, IAA-tryptophane; IAA-Val, IAA-valine; OxIAA, oxo-IAA; OxIAA-GE, oxo-IAA-glucose ester; IAM, indole-3-acetamide; IAN, indole-3-acetonitrile; *Cytokinins*: active CKs, active cytokinins; CK N-glc, cytokinin N-glucosides; CK O-glc, cytokinin O-glucosides; CK P, cytokinin phosphates; *cZ*, *cis*-zeatin; *cZR*, *cis*-zeatin riboside; *cZ9G*, *cis*-zeatin-9-glucoside; *cZOG*, *cis*-zeatin-O-glucoside; *cZROG*, *cis*-zeatin riboside-O-glucoside; cisZder, sum of *cis*-zeatin derivatives; DZ, dihydrozeatin; DZR, dihydrozeatin riboside; DZ7G, dihydrozeatin-7-glucoside; DZ9G, dihydrozeatin-9-glucoside; DZOG, dihydrozeatin-O-glucoside; DZROG, dihydrozeatin riboside -O-glucoside; DZRMP, dihydrozeatin riboside monophosphate; iP, isopentenyladenine; iPR, isopentenyladenosine; iP7G, isopentenyladenine-7-glucoside; iP9G, isopentenyladenine-9-glucoside; iPRMP, isopentenyladenosine monophosphate; tZ, *trans*-zeatin; tZR, *trans*-zeatin riboside; tZ7G, *trans*-zeatin-7-glucoside; tZ9G, *trans*-zeatin-9-glucoside; tZOG, *trans*-zeatin-O-glucoside; tZROG, *trans*-zeatin riboside O-glucoside; tZRMP, *trans*-zeatin riboside monophosphate; GAn, gibberellin n, 4,19; Jasmonates: JA, jasmonic acid; JA-Ile, jasmonate isoleucine; SA, salicylic acid; *Phenolic acids*: CaA, caffeic acid; p-CA, p-coumaric acid; FA, ferulic acid; p-HBA, p-hydroxybenzoic acid; PrA, protocatechuic acid; SiA, sinapic acid; SyA, syringic acid; VA, vanillic acid; *Other characteristics*: LT50, lethal temperature of 50% of the sample; −LT50, a negative value of lethal temperature of 50% of the sample; OP, osmotic potential; −OP, a negative value of osmotic potential; WCS120, homologue of *Triticum aestivum* WCS120 protein; WSD, water saturation deficit;.

cultivars (Kosová et al., 2012), which exhibited both a large difference in their acquired FrT and in the vernalization requirement. The comparison of the einkorn and hexaploid genotypes enabled us to evaluate these processes separately.

The cold response was followed at the level of dehydrin relative accumulation, water characteristics (WSD, OP), LT50 values, phytohormone, and phenolic acid levels. The effects of a long-term cold treatment were compared in the crowns (which generally exhibited the highest hormone levels), as well as in the leaves and roots. The overall dynamics of the cold response was similar in the crowns and leaves. In the roots, the changes of the observed parameters and metabolites differed from the data obtained from the

upper parts of the plants. The dynamics of FrT and WCS120 accumulation revealed the typical response observed in cold-treated *Triticeae* (Fowler et al., 2001; Limin and Fowler, 2002; Vítámvás and Prášil, 2008); however, there were several differences from those responses observed in common wheat and barley in the lines discussed below. Based on the results of multidimensional statistical analysis (cluster analysis, PCA) and detailed tracking of the parameters, the following phases of cold response in einkorn wheat could be distinguished: optimal conditions (0 day of cold), alarm (1 day of cold), acclimation (3–7 days of cold), resistance (21–31 days of cold), and a subsequent decrease of FrT due to developmental processes (42 day of cold).

4.1. Optimal conditions (0 day)

Under optimal growth conditions (20 °C) both lines exhibited low basal FrT, indicated by a higher LT50, which coincided with very small (in the winter line) or undetectable (in the spring line) levels of WCS120. This relationship was also observed in common wheat and barley (Kosová et al., 2012). Generally, the levels of phytohormones were higher in the crowns than the roots and leaves. Significant differences were found between the tested lines in some parameters, e.g., CKs in crowns of the spring line were higher in comparison with the winter one. Under control conditions, relatively low levels of stress hormones SA and ACC were detected, which is in accordance with the intensive growth rate at optimal temperature (Vanková, 2010).

4.2. Alarm phase (1 day)

The initial phase of cold response can be characterized by a disturbance of the plant water relationships, caused by an imbalance between water uptake (caused by the decrease of root hydraulic conductivity) and water release (via transpiration) (Gusta et al., 2005). The immediate negative effects of cold treatment on the plant water uptake by the roots resulted in transient plant tissue dehydration in both lines, reflected by the dynamics of WSD. A sharp peak of WSD after cold application (Fig. 1B) was rapidly followed by the up-regulation of ABA (Fig. 4), which improved the water status via stomata closure, and stimulated the defence. Cluster analysis (Fig. 11) indicated the close correlation between both parameters. Responses of both winter genotypes (einkorn and hexaploid) were stronger and faster, not only in the regulation of ABA levels, but also in the stimulation of dehydrin content (Fig. 2B). High levels of ABA metabolites (especially dihydrophaseic and phaseic acids) in the alarm phase (Suppl. Fig. 2) may suggest enhanced biosynthesis, as well as tight control of the ABA levels. The ABA elevation at the beginning of the cold response is in accord with previous reports (Galiba et al., 1993; Janowiak et al., 2002).

Antagonistic interactions between ABA and other stress hormones were indicated by the coincidence of the ABA transient peak with the rapid down-regulation of SA and ACC, as well as the decrease of JA and JA-Ile content, which was slightly delayed in the crowns. Antagonism between ABA and SA has been demonstrated, especially in the biotic stress responses (Zabala et al., 2009; Okamoto et al., 2012). In *T. monococcum*, a decrease of the ethylene precursor ACC was detected in this response phase in the crowns of both genotypes, and also in the winter line in the leaves and roots. The down-regulation of ACC is in accordance with the reported negative effect of transcription factor EIN3 on the expression of CBF transcription factors and the negative regulators of CK signalling (ARR5, ARR7, and ARR15), which are stimulated by cold (Shi et al., 2012).

As growth suppression seems to be a necessary prerequisite for reallocation of energy sources to the synthesis of protective compounds, down-regulation of the content and/or of signal transduction of the hormones associated with the stimulation of cell division and growth represents a common response to different stresses. In *T. monococcum*, we found a down-regulation of gibberellin GA_4 in all organs of both lines (Fig. 5). Our analysis of *T. monococcum* lines revealed a predominant presence of GA_4, in contrast to hexaploid wheat where the prevalence of GA_1 was detected (Kosová et al., 2012). These data are in accordance with other reports on gibberellin functions in cold. The key transcription factors stimulated by cold, CBFs, up-regulate the expression of genes for the GA-inactivating enzymes (GA 2-oxidases) (Kurepin et al., 2013). The gibberellin decrease results in a lowered degradation of DELLA proteins (repressors of gibberellin signal transduction), which play an important role in cold tolerance (Achard et al., 2008).

Down-regulation of active gibberellins was accompanied by a decrease in the level of active CKs (Fig. 6). In leaves, the most profound CK decrease was observed in winter hexaploid wheat (Kosová et al., 2012); the change was milder in G3116, and delayed in DV92, while the spring hexaploid cultivar did not show a significant change. The observed decrease of active CK levels is in accord with the rapid cold stimulation of the type-A response regulators (ARR5, ARR6, and ARR7), which are negative regulators of CK signalling (Jeon et al., 2010). Recently, the functional CK signalling pathway (specifically ARR1, AHP2, AHP3, and AHP5) was reported as necessary for the induction of type-A RRs by cold (Jeon and Kim, 2013).

The levels of IAA also decreased in the alarm phase in all organs of both genotypes (Fig. 7). The response of *T. monococcum* was more profound in comparison with the hexaploid wheat cultivars, which showed a delay in the crowns. Cold induced suppression of the auxin response was reported in *Malus domestica* (Yuan et al., 2013). Within 12 h at 4 °C, auxin signal transduction was inhibited by more than 90% (after application of 10 μM IAA). The authors described the significant role of the GH3 family of conjugating enzymes in the regulation of the auxin levels. Alternatively, the regulation of auxin levels by IAA oxidation with p-coumaric acid, vanillic acid, or p-hydroxybenzoic acid has been reported (Volpert et al., 1994). The increase in the levels of these benzoic acid derivatives soon after cold application suggests their participation in the auxin decrease (Table 1). The role of individual phenolic acids seems to be organ- and genotype-specific.

4.3. Acclimation (3–7 days)

During this period, plants adapt to the unfavourable cultivation conditions, adjusting their metabolism. Osmotic potential decreased significantly in both lines, and a significant increase was observed between the acquired FrT level (LT50 values) and dehydrin accumulation, as was also shown in our previous reports on both common wheat (Kosová et al., 2012) and barley (Kosová et al., 2013). The detection of WCS120 homologues (Sarhan et al., 1997) has revealed the presence of four dehydrin proteins accumulating in cold-treated einkorn wheat plants (WCS200, WCS180, WCS120, WCS40). The band corresponding to the WCS66 protein, which is the second most abundant protein from the WCS120 family in cold-treated samples of common wheat, was missing. The absence of a WCS66 protein band was also observed in the leaves of the cold-treated common wheat winter cultivar Zdar (Vítámvás et al., unpublished).

Re-establishment of growth in this phase, even at a lower rate than under optimal conditions, was associated with a mild elevation of GA_4. The levels of active CKs rapidly increased, especially in the DV92 line. The response of the hexaploid lines was similar. These data correspond well to the inhibition of the activity of the main CK degrading enzyme cytokinin oxidase/dehydrogenase, found in the hexaploid lines in this stress response phase (Kosová et al., 2012).

The IAA levels generally exhibited a moderate increase during the acclimation phase. The increase of auxin level may be caused (at least partially) by IAA protection from oxidation by cinnamic acid derivatives (especially ferulic and sinapic acids), which were found elevated during the cold stress (Table 1). Protection of IAA from decarboxylation by ferulic acid was unequivocally demonstrated by De Klerk et al. (2011). In accordance with the study of Olenichenko et al. (2006) on *Triticum* sp., we found a relatively high increase (up to 170%) in the phenolic content upon cold treatment. However, it seems that the more important role of phenolic acids, up-regulated during the cold stress, may be their function as antioxidants – as oxygen radical scavengers (Brandolini et al., 2013). The oxidative stress, associated with hydrogen peroxide formation, has been found to be strongly promoted during cold treatment (Kocsy et al., 2001). According to Brandolini et al. (2013), einkorn wheat exhibits a higher antioxidant activity than hexaploid wheat. Olenichenko et al. (2006) suggested that apart from antioxidant activity, phenolics may also function upon cold stress as osmoprotectants.

At this stress response phase, ABA down-regulation was associated with gradual SA elevation. The ethylene precursor ACC started increasing in G3116. Stimulation of JA was postponed. The dynamics of stress hormones were similar in the einkorn and hexaploid cultivars.

4.4. Resistance phase (21–31 days)

During this response phase, the FrT of G3116 crowns reached its maximum (Fig. 2A), but remained relatively close to the LT50 in the DV92 line. The relatively low acquired FrT observed in the cold-treated G3116 winter line, compared with common wheat winter genotypes, can be explained by the presence of only the diploid A^m genome in einkorn wheat, compared with the presence of A^u, B, and D genomes in common wheat. Studies focused on genome distribution of FrT-related genes in common wheat have revealed a 6% over-representation of cold acclimation-related genes in the D genome, especially on chromosome 4D (Limin and Fowler, 1985; Qi et al., 2004; Monroy et al., 2007). The small difference in FrT in the leaves, observed between both lines, can be explained by the "pseudo-spring" genetic nature of the DV92 line, lacking a dominant *Vrn1* allele (i.e., possessing the same *Vrn1/Fr1* locus as the G3116 line). In fact, the DV92 line can be characterized as a facultative growth habit according to Von Zitzewitz et al. (2005), since it possesses a recessive combination of vrn1vrn2 alleles. The facultative genotypes can exhibit a relatively high FrT, similar to winter genotypes under short-day or neutral photoperiods, e.g., see study of Fowler et al. (2001) on facultative barley Dicktoo. The data obtained on the LT50s in our experiment are thus consistent with the facultative nature of the DV92 line.

The *T. monococcum* lines attained considerable differences in dehydrin levels at the end of this response phase (Fig. 2). DV92 stopped increasing dehydrin levels at 21 day of cold, while G3116 continued in dehydrin up-regulation until day 31. Similarly, the spring hexaploid cultivar Sandra reached the dehydrin maximum after 7 days of cold, while the winter wheat Samanta was able to increase the dehydrin levels until the end of the experiment (21 days, Kosová et al., 2012). Furthermore, our data also confirmed that dehydrin accumulation is in good correlation with the FrT levels. So, this relationship is already available in the ancestors of the current wheat varieties, regardless of the fact that hexaploid winter lines increased FrT during their breeding.

At the beginning of this response phase, DV92 exhibited a maximum of the active CKs (especially *cis*-zeatin and its riboside and *trans*-zeatin), which indicated the onset of the early stage of reproductive development (Tarkowska et al., 2012). The role of CKs in meristem reprogramming to the reproductive morphogenesis is discussed later in relationship to the fulfilment of the vernalization requirement in the G3116 winter line.

Low levels of ABA were accompanied by a gradual increase of SA and ACC during the cold stress progression, as well as relatively high levels of JA and JA-Ile in this phase. High levels of SA and ACC indicate their active role in the adaptation to cold stress conditions, related to the regulation of the redox state as described in Majlath et al. (2012) and Suzuki et al. (2012). The high content of JA and JA-Ile is in accordance with the reported stimulation of the expression of allene oxide synthase (the key JA synthetic gene) between days 2–49 of cold treatment, accompanied by an increase of the tolerance to the snow mould and other wheat pathogens (Gaudet et al., 2011). As JA and SA inhibit cell division and growth at the same checkpoints as ABA (Swiatek et al., 2002; Okamoto et al., 2012), enhanced JA and SA levels may contribute to the suppression of the growth rate under low temperatures.

4.5. Loss of high FrT (42 day)

Prolonged cultivation at 4 °C (5–6 weeks) was sufficient to fulfil the vernalization requirement of the G3116 winter line. Transition from the vegetative to the reproductive stage is indicated by the strong decrease of acquired FrT level, dehydrin accumulation, as well as by the changes in phytohormone content. According to a model presented by Dhillon et al. (2010), the fulfilment of vernalization associated with the upregulation of the expression of *VRN1* gene results in a downregulation of *VRN2* gene, cold-regulated *CBF* genes and the whole CBF regulon (*COR* genes). However, the relationships between *VRN1* gene expression and regulation of the expression of *VRN2* gene and several *COR* genes is more complex, involving photoperiod cues and other factors (Loukoianov et al., 2005; Trevaskis et al., 2007; Dhillon et al., 2010). The expression of the *VRN1* gene is known to have a significant morphogenetic impact on cereal shoot apex development, and it is considered a sign of plant transition to the reproductive stage (Preston and Kellogg, 2007).

The crucial role of CKs in flower induction and stimulation of reproductive development has been revealed in studies of CK dynamics under different photoperiod regimes (e.g., Macháčková et al., 1993). The inductive photoperiod (e.g., short days in the case of *Chenopodium rubrum*, or long days in the case of *Chenopodium murale*) was found to be associated with CK elevation in the apical parts (Macháčková et al., 1993). Enhanced levels of active CKs (especially of isopentenyladenosine and its phosphate) correlated well with the early phase of the transition to flowering upon photoperiod stimulus in *Arabidopsis thaliana* (Corbesier et al., 2003). Recently, CK dynamics were followed in the winter cultivar of *Brassica napus* during vernalization (Tarkowska et al., 2012). They found maxima of active CKs, especially of *cis*-zeatin riboside, at the onset of the reproductive state. In accordance with these results, our data on CK dynamics show significant differences between the DV92 and G3116 lines throughout the cold treatment. The former line, lacking a vernalization requirement, exhibited active CK maxima after 21 days. Cluster analysis showed a high level of *cis*-zeatin, *cis*-zeatin riboside, and CK O-glucosides. Later on, a significant CK decrease was found. This result is in agreement with Tarkowska et al. (2012), who found a CK maximum at the onset of reproductive development, followed by a decrease in CK levels. A comparison of DV92 and G3116 proves that CK up-regulation is associated with the onset of the vegetative – reproductive transition. The dynamics of these phytohormones exhibits an analogy to the dynamics of *VRN1* gene expression described in a set of common wheat reciprocal substitution lines between the winter cultivar Norstar and the spring cultivar Manitou, with interchanged *Vrn1/Fr1* loci (Danyluk et al., 2003). Our data demonstrate involvement of CKs in

the developmental transition to the reproductive stage in einkorn wheat.

5. Conclusions

The characterization of the cold responses of einkorn wheat DV92 spring line and G3116 winter line and their comparison with common wheat revealed the characteristic features of plant behaviours under low temperatures.

(i) All genotypes responded to cold shock by an imbalance of the water relationship, reflected by an increase in WSD, which resulted in elevation of ABA, and subsequently in a re-establishment of a new homeostasis (homeostasis between water uptake and water release via transpiration, due to regulation of the stomata).
(ii) Cold shock imposed growth suppression, which was associated with the decrease of gibberellins, cytokinins, and auxin. The speed of the response was negatively correlated with the frost tolerance of the genotype.
(iii) During acclimation to the stress conditions, plant frost tolerance increased, mainly due to the accumulation of protective compounds, especially dehydrin proteins and phenolic acids. The maximal dehydrin level was proportional to the genotype frost tolerance. Plant growth was re-established in the diminished rate, which was accompanied by a moderate elevation of gibberellins, cytokinins, and auxin. ABA decrease coincided with the up-regulation of other stress hormones (salicylic acid, jasmonic acid and aminocyclopropane carboxylic acid), which might diminish the growth rate and increase the tolerance to potential biotic stresses.
(iv) The developmental changes associated with transition from the vegetative to the reproductive stage were associated with a decrease of dehydrin levels as well as a decrease of frost tolerance. The cytokinin maximum was found at the onset of flower induction in the crowns. This transition was observed after 21 days in the DV92 spring line, and after fulfilment of the vernalization requirement in winter line G3116 (possibly with elevation of the expression of the *VRN1* gene).
(v) Comparison of the individual parameters has generally revealed similar patterns in the dynamics of cold response of different organs (leaves, crowns and roots); although some minor differences were found. For example, the decrease of cytokinin levels in the crowns, while an increase in the leaves and roots at the later stages of cold treatment (vernalization fulfilment) were detected.
(vi) In spite of many similarities, some differences were also found in the cold response between einkorn wheat and common wheat species. Einkorn wheat winter line G3116 exhibited a relatively low level of acquired FrT and relative dehydrin accumulation when compared to the winter cultivar Samanta. Moreover, a significant decrease in acquired FrT has been found in the winter line G3116, coinciding with vernalization fulfilment. The observed decrease in acquired FrT indicated the less robust mechanisms of FrT acquisition and maintenance in einkorn wheat than in common wheat, as well as a higher sensitivity of FrT in einkorn wheat to developmental changes (vernalization) compared to common wheat.

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Appendix A. Supplementary data

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