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Dynamics of Endogenous Phytohormones during Desiccation and Recovery of the Resurrection Plant Species *Haberlea rhodopensis*

Dimitar L. Djilianov · Petre I. Dobrev · Daniela P. Moyankova · Radomira Vankova · Dessislava Ts. Georgieva · Silvia Gajdošová · Václav Motyka

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Abstract Drought is one of the most significant threats to world agriculture and hampers the supply of food and energy. The mechanisms of drought responses can be studied using resurrection plants that are able to survive extreme dehydration. As plant hormones function in an intensive cross-talk, playing important regulatory roles in the perception and response to unfavorable environments, the dynamics of phytohormones was followed in the resurrection plant *Haberlea rhodopensis* Friv. during desiccation and subsequent recovery. Analysis of both leaves and roots revealed that jasmonic acid, along with and even earlier than abscisic acid, serves as a signal triggering the response of the resurrection plants to desiccation. The steady high levels of salicylic acid could be considered an integral part of the specific set of parameters that prime *H. rhodopensis* desiccation tolerance. The dynamic changes of cytokinins and auxins suggest that these hormones actively participate in the dehydration response and development of desiccation tolerance in the resurrection plants. Our data contribute to the elucidation of a global complex picture of the resurrection plant's ability to withstand desiccation, which might be successfully utilized in crop improvement.

Keywords Abscisic acid · Auxin · Cytokinin · Desiccation tolerance · *Haberlea rhodopensis* · Jasmonic acid · Phytohormones · Resurrection plant · Salicylic acid

D. L. Djilianov and P. I. Dobrev contributed equally to this work and should be considered as first authors.

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D. L. Djilianov (✉) · D. P. Moyankova · D. Ts. Georgieva
Abiotic Stress Group, AgroBioInstitute, Agricultural Academy, 8 Dragan Tzankov Blvd, 1164 Sofia, Bulgaria
e-mail: d_djilianov@abi.bg

P. I. Dobrev · R. Vankova · S. Gajdošová · V. Motyka (✉)
Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Rozvojová 263, 16502 Prague 6, Czech Republic
e-mail: motyka@ueb.cas.cz

Present Address:
S. Gajdošová
Institute of Biology and Ecology, Pavol Jozef Šafárik University, Mánesova 23, 040 01 Košice, Slovak Republic

Introduction

At present, environmental changes hamper the world's food and energy supply. Abiotic stresses are responsible for desertification of large areas of the global surface and a predominant part of crop losses, which results in food and energy shortages. Drought is among the most significant threats to world agriculture and motivates plant scientists to reveal the mechanisms of plant adaptation to unfavorable environments.

A small group of plant species, the so-called resurrection plants, are able to survive very harsh dehydration. This makes them excellent model systems to study drought responses. They live in different habitats and belong to different botanical families. Their common feature is the ability of their vegetative tissues to withstand long periods of full desiccation and to recover very rapidly upon rewatering (Ingram and Bartels 1996; Oliver and others 2000; Toldi and others 2009).

Plant hormones have been recognized as major players in the regulation of growth, development, and responses to unfavorable environments (Zeevaart and Creelman 1988;

Bray 1997; Creelman and Mullet 1997; Hare and others 1997; Woodward and Bartel 2005; Wasternack 2007; Iqbal and others 2011; Peleg and Blumwald 2011; Ha and others 2012). Research on endogenous plant hormone regulation in resurrection plants is relatively scarce and has focused mainly on analysis of abscisic acid (ABA), the key hormone in dehydration responses (Bartels and Hussain 2011). Data on other phytohormones are very limited or even missing, although recent molecular studies show indirectly their potential involvement in desiccation tolerance (Rodriguez and others 2010).

Haberlea rhodopensis Friv. is a Balkan endemic resurrection species belonging to the family Gesneriaceae. The plants grow on limestone rocks and shady slopes at various altitudes. Physiological, biochemical, and molecular aspects of *H. rhodopensis* desiccation tolerance have been studied extensively in recent years (Djilianov and others 2011 and references therein; Georgieva and others 2012). The aim of this study was to follow the dynamics of endogenous plant hormones—ABA, jasmonic acid (JA), salicylic acid (SA), cytokinins (CKs), indole-3-acetic acid (IAA), and their metabolites—during desiccation and rehydration of *H. rhodopensis* plants to reveal their potential role in stress signal perception and plant tolerance to desiccation.

Materials and Methods

Plant Material

The in vitro propagation system of *H. rhodopensis* was used as described earlier (Djilianov and others 2005; Petrova and others 2010). Briefly, the culture was initiated from fresh, fully developed young leaves. Culture initiation, direct organogenesis, and culture transfers were performed using basic woody plant medium (WPM) (Lloyd and McCown 1980). To obtain optimal plant growth and rooting, the last transfer of the plantlets was done in tubes with liquid medium and paper bridges.

The in vitro plants were cultured in a plant growth room at a 16/8-h light/dark photoperiod, 22 °C, 75 µmol m^{-2} s^{-1} light intensity, and about 50 % air humidity. Well-developed and rooted 1.5-2-month-old plants were used.

Dehydration and Rehydration Procedure

Drought stress and recovery procedures were performed as described earlier (Djilianov and others 2011). Well-developed plantlets were taken out from culture vessels and left to dry to the full air-dry stage in a culture room at controlled conditions (22–25 °C and 50 % relative humidity in the shade). After 1 week (168 h), rehydration was carried out on cotton beds by watering the dried plantlets under the same conditions.

Relative Water Content (RWC)

Relative water content was measured in detached leaves at various time points of drying and rehydration according to Barr and Weatherley (1962):

$$\text{RWC } \% = [(\text{FW} - \text{DW})/(\text{FTW} - \text{DW})] \times 100,$$

where FW is fresh weight, DW is dry weight (measured after drying for 48 h at 80 °C), and FTW is full turgid weight (measured after the leaves were left in distilled water for 24 h at 22 °C in the dark).

Sampling for Hormone Analyses

Plant samples were taken at various time points of dehydration or rehydration and immediately frozen in liquid nitrogen. They were then freeze-dried, divided into leaves and roots, and stored at −20 °C until the hormone analyses. Six biological replicates were collected at every time point. The experiment was repeated three times.

Phytohormone Analysis

Samples were purified and analyzed essentially as described in Dobrev and Kamínek (2002) and Dobrev and Vankova (2012). Samples of 20 mg dry weight were homogenized with a ball mill (MM301, Retsch, with 5-mm-diameter zirconium balls) and extracted in cold (−20 °C) extraction buffer consisting of methanol/water/formic acid (15/4/1 v/v/v, 0.5 ml/sample). To account for sample losses and for quantification by isotope dilution, the following stable isotope-labeled internal standards (10 pmol/sample) were added: $^{13}C_6$-IAA (Cambridge Isotope Laboratories); $^{2}H_4$-SA (Sigma-Aldrich); and $^{2}H_3$-PA (phaseic acid) (NRC-PBI), $^{2}H_6$-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) (Table 1; for abbreviations of CKs see Kamínek and others 2000). Extract was applied to a SPE-C18 column (SepPak-C18, Waters). Flowthrough was evaporated in a vacuum concentrator (Alpha RVC, Christ). Sample residue was redissolved into 1 ml of 0.1 M formic acid and applied to a mixed mode reversed phase–cation exchange SPE column (Oasis-MCX, Waters). Two hormone fractions were sequentially eluted: (1) fraction A, eluted with methanol containing hormones of acidic and neutral character (auxins, ABA, SA, JA); and (2) fraction B, eluted with 0.35 M NH_4OH in 60 % methanol containing the hormones of basic character (cytokinins). Fractions were

evaporated to dryness in a vacuum concentrator and dissolved into 30 µl of 10 % methanol. An aliquot (10 µl) from each fraction was separately analyzed on a high-performance liquid chromatograph (HPLC) (Ultimate 3000, Dionex) coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer (3200 Q TRAP, Applied Biosystems), which was set in selected reaction monitoring mode. Chromatographic conditions for fraction A included a HPLC column Luna C18(2) (100 × 2 mm, 3 µm, Phenomenex), a flow rate of 0.25 ml/min, and a linear gradient of solvent A (5 mM ammonium formate, pH 3 in water) and solvent B (5 mM ammonium formate, pH 3, in acetonitrile) from 10 to 50 % B for 15 min. Chromatographic conditions for fraction B included a HPLC column Luna C18(2) (150 × 2 mm, 3 µm, Phenomenex), a flow rate of 0.25 ml/min, and a linear gradient of solvent A (5 mM ammonium acetate, pH 4 in water) and solvent B (5 mM ammonium acetate, pH 4, in methanol) from 10 to 40 % B for 20 min. The mass spectrometer was run in electrospray ionization mode, negative for fraction A and positive for fraction B. Ion source parameters included ion source voltage of −4,000 V (negative mode) or +4,500 V (positive mode), nebulizer gas at 50 psi, heater gas at 60 psi, curtain gas at 20 psi, and heater gas temperature of 500 °C. Compound-specific parameters are listed in (Supplementary Table 1). Quantification of hormones was done using the isotope dilution method with multilevel calibration curves ($r^2 > 0.99$). Data processing was carried out with Analyst 1.5 software (Applied Biosystems). Data are presented as mean ± standard error.

Results

RWC Dynamics During Desiccation and Recovery of *Haberlea rhodopensis*

At the start of desiccation, the RWC of *H. rhodopensis* plantlets was about 93 % (Fig. 1). During the first 2 h of air-drying, the RWC of the plantlets decreased significantly to almost half the value at the start (55 %). In the next few hours, desiccation resulted in further drastic decrease in RWC to about 25 % at 6 h and about 15 % at 18 h. The plantlets were practically fully desiccated (about 13 %) after 24 h and remained at the same level under further air-drying.

Rehydration experiments were performed with fully air-dried plantlets. The recovery started immediately at the start of rehydration. RWC increased considerably, reaching about 56 % as early as 1 h after rewatering (data not shown). This RWC level did not change within the next hour (Fig. 1), but increased to about 67 % 6 h after rewatering. At 24 h, the recovery was complete and control levels of RWC were reached.

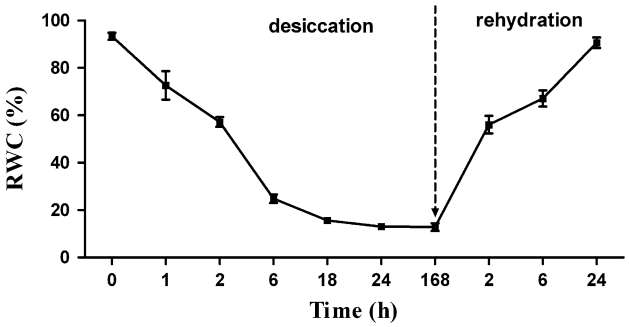

Fig. 1 Dynamics of relative water content (RWC) of *Haberlea rhodopensis* during desiccation and rehydration. RWC was measured in detached leaves at various time points of dehydration (0, 1, 2, 6, 18, 24, and 168 h) and rehydration. Recovery (2, 6, and 24 h) was performed with samples dried for 168 h

Endogenous Content of Phytohormones

Considerable differences in the dynamics of endogenous hormone contents between roots and leaves were observed.

Stress Hormones

Leaves

At normal conditions, *H. rhodopensis* leaves showed very low levels of JA and ABA, whereas the levels of SA were relatively high (Fig. 2a, c). Almost immediately after the start of desiccation (1 h), JA increased up to 25 times, whereas ABA and SA remained at levels similar to control conditions. When RWC decreased approximately twice in comparison with control conditions (2 h of air-drying), JA increased further and reached a sharp peak, almost 2,000 times higher than control levels. At this point, SA remained at a relatively steady level and ABA content increased approximately six times. At about 25 % RWC (6 h desiccation), ABA reached its maximum, with values 100-fold over the basal level, SA increased moderately (twice), whereas the level of JA started declining. During the late stages of drying and at the full dry state (18–168 h), both ABA and JA declined, whereas SA reached its maximum. Nevertheless, the overall increase of SA was only three times that of control levels. A steady decline for all stress hormones was observed during recovery. It was particularly remarkable in the case of SA, which reached control levels.

The active JA metabolite, JA-isoleucine (JA-ILE), increased as drying progressed, reaching the maximum at the full desiccation state (Fig. 2a, c). The most abundant ABA metabolite, dihydrophaseic acid (DPA), returned to the control level after an initial decline (1 h air-drying) and exhibited elevation only at maximal desiccation. Concentrations of another ABA derivative, phaseic acid (PA),

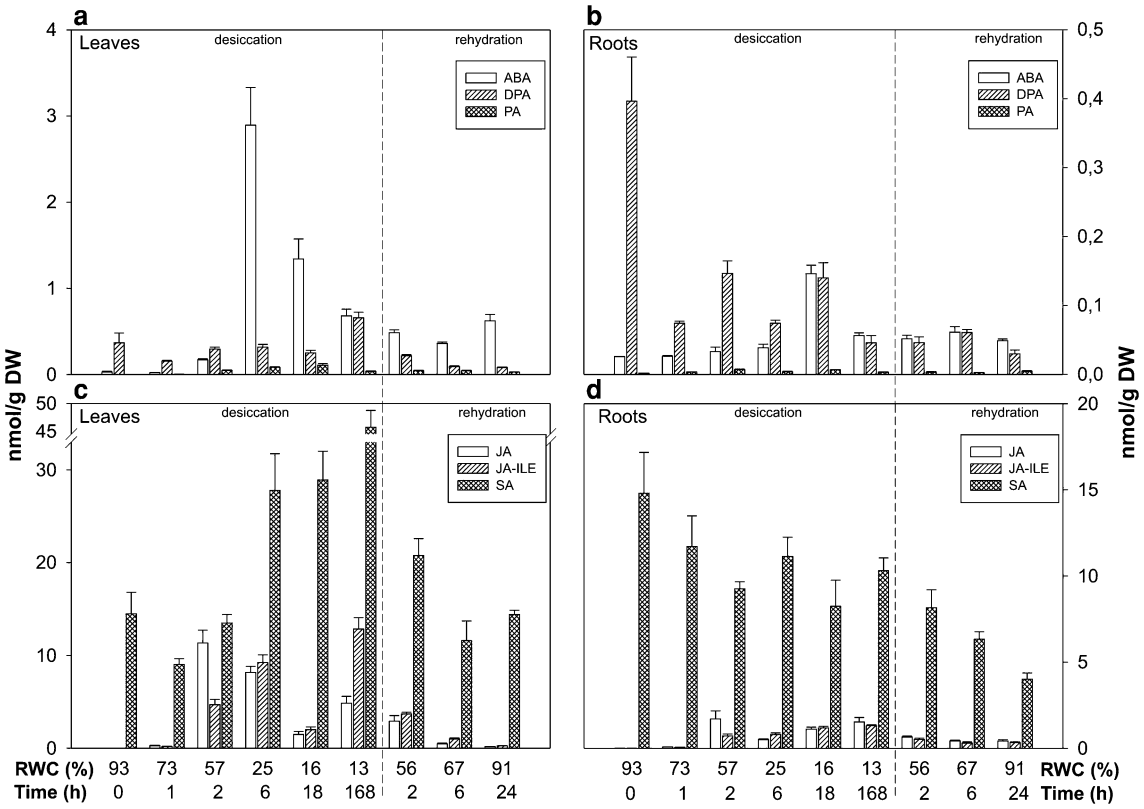

Fig. 2 Endogenous content of abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA), and their respective metabolites during desiccation and rehydration of *Haberlea rhodopensis* leaves and roots. Dynamics of ABA and its derivatives dihydrophaseic acid (DPA) and phaseic acid (PA) in leaves (**a**) and roots (**b**). Dynamics of JA, JA-isoleucine (JA-ILE), and SA in leaves (**c**) and roots (**d**)

were rather low, increasing in both leaves and roots during desiccation and diminishing during recovery.

Roots

The ABA content remained at a steady level during the predominant period of desiccation (Fig. 2b). At about 15 % RWC (18 h), ABA increased about six times. At the full dry state and during recovery, the ABA level was retained in the root tissue without major changes. Accumulation of the inactive ABA metabolite DPA was highest in control plants.

As in leaves, JA content in *H. rhodopensis* roots underwent the most dynamic changes and reached the highest levels at about 58 % RWC at 2 h of air-drying (Fig. 2d). The contents remained high until the end of drying. Rewatering of dried plantlets led to decreased JA content, but the levels remained higher than controls.

JA-ILE increased with the onset of drying, reaching a peak at the full dry state. It dropped in the course of recovery but remained higher than in controls.

The highest level of SA was found under control conditions (Fig. 2d).

Growth Hormones

Substantial differences in total isoprenoid CK distributions were found in *H. rhodopensis* leaves and roots (Fig. 3, Supplementary Fig. 1). Under control conditions, levels of N^6-(2-isopentenyl)adenine (iP) and *trans*-zeatin (*trans*Z) types of CKs in roots were about twice as high as in leaves, whereas the opposite was true for *cis*-zeatin (*cis*Z) and its derivatives. In both leaves and roots, iP-type CKs predominated, followed by *trans*Z > *cis*Z > dihydrozeatin (DHZ) types. The prevailing CK metabolites were *N*-glucosides (in particular, *N*7-glucosides), accounting for about 72 and 80 % of the total isoprenoid CK pool in leaves and roots, respectively.

Leaves

Levels of total isoprenoid CKs in leaves rose progressively, reaching a peak after 6 h of desiccation, especially as a result of accumulation of inactive (or weakly active) CK forms *N*- and *O*-glucosides (Fig. 3, Supplementary Fig. 1). Afterward, the amount of total CKs declined gradually during desiccation. All major CK groups (iP, *trans*Z, and

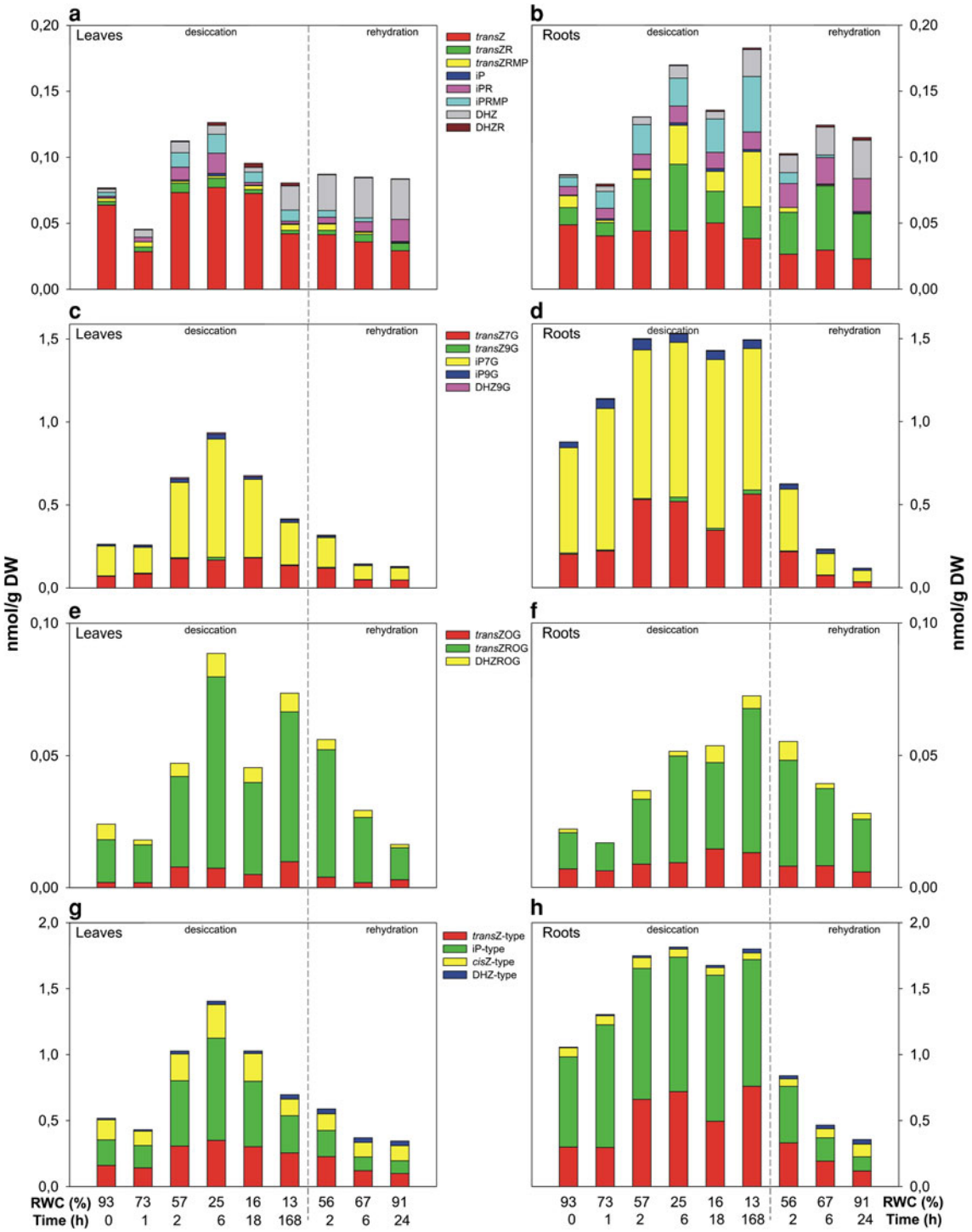

Fig. 3 Dynamics of endogenous content of cytokinins (CKs) during desiccation and rehydration of *Haberlea rhodopensis* leaves (**a**, **c**, **e**, **g**) and roots (**b**, **d**, **f**, **h**). The detected CKs are divided according to the conjugation status/physiological functioning to bioactive forms and their biosynthetic precursors CK phosphates (**a**, **b**), irreversible CK-N-glucosides (**c**, **d**), reversible CK-O-glucosides (**e**, **f**), and according to the chemical structure of *trans*-zeatin (*trans*Z)-, N^6-(2-isopentenyl)adenine (iP)-, *cis*-zeatin (*cis*Z)-, and dihydrozeatin (DHZ)-types (**g**, **h**)

*cis*Z types) showed similar peak-like dynamics, with a sharp maximum at 25 % RWC (6 h), whereas the less abundant DHZ types differed, reaching a relatively high level at the end of the drying phase as a consequence of considerably enhanced concentrations of free-base DHZ. In contrast to other CK groups, the levels of *cis*Z types

dropped considerably after 1 h of desiccation (almost by one third below the control level). Interestingly, a relatively high proportion of *cis*Z-9-riboside-O-glucoside (*cis*ZROG) was typical for leaves of desiccating plantlets (in contrast to roots) (Supplementary Fig. 1).

The level of bioactive CKs, especially *trans*Z, iP, and DHZ-9-riboside (DHZR), decreased after 1 h of desiccation, exhibiting a transient peak later on (together with iP-9-riboside, iPR) (Supplementary Fig. 1). The rehydration period was accompanied by the decline of inactive (or weakly active) CK forms *N*- and *O*-glucosides (ca. 39 and 53 % below the control level, respectively, 6 and 24 h after rewatering), and by the elevation of bioactive CKs, especially iPR and DHZ, at later stages also of iP. A typical feature for the recovery period was a steep increase in *cis*-zeatin-9-riboside (*cis*ZR) concentration 24 h after rewatering (about 40 times higher compared to the control).

The dynamics of other plant growth- and division-promoting hormones (that is, auxins) was also followed. Changes in the levels of the most commonly occurring auxin, indole-3-acetic acid (IAA), and its amino acid conjugate, IAA-aspartate (IAA-Asp), were monitored (Fig. 4). In leaves, the contents of IAA-Asp exceeded remarkably those of IAA, at normal conditions as well as during the whole desiccation and recovery process (ca. 4–17 times). The IAA and IAA-Asp concentrations followed similar tendencies, showing distinct minima almost immediately after the start of desiccation (1 h) and reaching maxima at about 25–16 % RWC (6–18 h). Rewatering of dried plantlets resulted in an approximately twofold increase in IAA and IAA-Asp contents after 2 h, followed by their decline to ~80 % of the control levels after 6 h. Enhanced amounts of IAA and IAA-Asp (1.2 and 3.4 times, respectively, compared to the control) were found at the end of recovery (24 h after rewatering) in leaves.

Roots

An increase in total isoprenoid CK contents was observed in the roots of desiccated plants, with maxima in a relatively broad range (57–13 % RWC corresponding to 2–168 h of desiccation) (Fig. 3, Supplementary Fig. 1).

The iP-type CKs accumulated in roots right after the beginning of drying. The main iP metabolites, iP-*N*-glucosides (iP7G and iP9G), reached steady levels as early as after 2 h and did not change considerably up to 168 h. On the other hand, the bioactive forms iP and its riboside (iP-9-riboside, iPR), together with iP-9-riboside-5′-monophosphate (iPRMP), accumulated almost linearly throughout the whole desiccation phase. Other bioactive CKs differed in their responses to drying, whereas DHZR exhibited a minor peak after 1 h of desiccation, a transient maximum of *trans*Z-9-riboside (*trans*ZR) was observed later on (at 25 % RWC at 6 h), and *cis*Z contents decreased during the whole dehydration phase. Interestingly, glucosyl conjugates of *trans*Z exhibited two distinct maxima in desiccated roots (6 and 168 h), especially due to *trans*Z-*N*-glucosides (*trans*Z7G and *trans*Z9G), *trans*Z-9-riboside-O-glucoside (*trans*ZROG), and *trans*Z-9-riboside-5′-monophosphate (*trans*ZRMP).

Although iP- and *trans*Z-type CKs showed decreasing tendencies during recovery, *cis*Z and DHZ types exhibited opposite trends (Fig. 3, Supplementary Fig. 1). In general, rehydration led to the decrease of inactive or weakly active CK forms *N*- and *O*-glucosides, with the exception of *trans*ZROG. Bioactive bases exhibited a transient decrease at the very beginning of rehydration and then started increasing considerably (except for *trans*Z), and all bioactive ribosides showed elevation during recovery. An especially high increase was recorded for iPR, DHZR, and *cis*ZR 24 h after rewatering (ca. 4, 5, and 53 times, respectively, compared to the control).

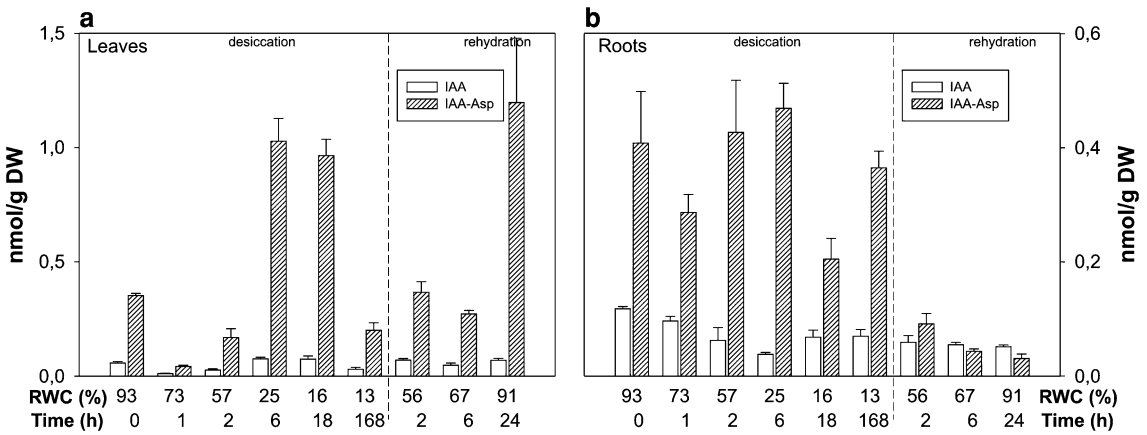

Fig. 4 Dynamics of endogenous content of auxins indole-3-acetic acid (IAA) and IAA-aspartate (IAA-Asp) during desiccation and rehydration of *Haberlea rhodopensis* leaves (**a**) and roots (**b**)

In roots of both unstressed and desiccated plantlets, the levels of IAA-Asp were considerably higher than those of IAA (ca. 3–12 times) (Fig. 4). This proportion changed following rewatering; IAA-Asp exceeded IAA at the early stage (1.5 times after 2 h), whereas it was less abundant than IAA in the course and at the end of recovery (1.3 times after 6 h and 1.7 times after 24 h). During desiccation, IAA contents decreased to only one third of that of the control at 25 % RWC (6 h) and then slightly increased; however, it did not reach the control level. In contrast, IAA-Asp reached a peak after 6 h of desiccation and then dropped (18 h). Both IAA and IAA-Asp concentrations declined during recovery.

Discussion

The air-drying of developed and grown in-vitro plantlets had been proven a useful system for elucidation of biochemical and genetic aspects of desiccation tolerance in *H. rhodopensis* (Djilianov and others 2011; Georgieva and others 2012). Plants of uniform age, grown under controlled conditions, represent a highly reproducible system with a fast and fairly consistent stress/recovery response, which allows the study, in detail, of the progression of dehydration and rehydration. The patterns of RWC changes showed that *H. rhodopensis* plantlets dried out quickly, reaching a fully dry state (about 12–13 %) after 24 h. They demonstrated their resurrection potential when, after 1 week at the dry state, all plants were able to recover upon rewatering (Fig. 1).

Our experimental system, based on air-drying of in vitro *H. rhodopensis* plantlets, enabled us to examine the dynamics of hormonal changes accompanying various stages of drying and recovery.

It is already widely accepted that JA plays a signal role in the plant response to both biotic and abiotic stress (Creelman and Mullet 1995; Hu and others 2009; Yun-xia and others 2010; Ismail and others 2012). Our data show that JA is a good candidate for the first stress signal in the cascade of events that occur in *H. rhodopensis*, finally resulting in desiccation tolerance. Almost immediately after the start of air-drying (1 h), JA content increased more than 25 and 10 times in leaves and roots, respectively (Fig. 2). The highest values of JA (elevated about 1,100 and 240 times, respectively) were reached 2 h after stress initiation, which coincided with ~60 % RWC. A fast and significant increase of JA content has been well documented after plant wounding (Albrecht and others 1993; Wasternack and others 2006; Wasternack 2007; Glauser and others 2008). The function of JA in the development of desiccation tolerance may involve regulation of the expression of multiple genes in ascorbic acid and glutathione biosynthesis and ROS detoxification (Xiang and Oliver 1998; Sasaki-Sekimoto and others 2005; Brosché and Kangasjärvi 2012). Very high JA accumulation, which we observed in the first hours of desiccation, could be considered one of the triggers of glutathione accumulation shown in our previous studies, when plant RWC decreased below 35 % (Djilianov and others 2011). Interestingly, and to our knowledge for the first time in resurrection plants, we report that the first reaction of *H. rhodopensis* to desiccation with respect to hormone dynamics is an early increase of JA.

ABA plays a key role in the response to dehydration, in the case of abiotic stresses (such as drought, cold, or salinity), and in the developmental stages (for example, seed desiccation or dormancy). Identification of ABA receptors (PYR/PYL/RCAR) and elucidation of their mode of action revealed mechanisms by which ABA stimulates ion flux to close stomata and affects gene expression (Cutler and others 2010). ABA functions have been well documented in various plant systems (Djilianov and others 1994; Oliver and others 2000; Djilianov and others 2003; Le and McQueen-Mason 2006). This is probably the reason that so far ABA has been the predominantly studied hormone in drought stress responses, including the group of resurrection plants (Bartels and Hussain 2011). In most cases, endogenous ABA levels were measured under normal conditions and after full desiccation. The published data are strongly species- and experimental design-dependent. ABA content was found to increase several times under the full dry state in leaves of *Borya nitida* and *Myrothamnus flabellifolia* (Gaff and Loveys 1984) and *Selaginella tamariscina* (Wang and others 2010); in the leaves and calli of *Craterostigma plantagineum* (Bartels and others 1990); in *Riccia fluitans* (Hellwege and others 1996), *Exormotheca holstii* (Hellwege and others 1994), *Craterostigma plantagineum*, *Myrothamnus flabellifolia*, and *Xerophyta humilis*; and in the roots of the aquatic resurrection species *Chamaegigas intrepidus* (Schiller and others 1997). Exogenous ABA applications additionally confirmed the role of this hormone in the desiccation tolerance of resurrection plants (Bartels and others 1990; Werner and others 1991; Reynolds and Bewley 1993; Bartels and Salamini 2001; Ghasempour and others 2001).

Only limited data have been published about ABA dynamics in the time course of desiccation and recovery of resurrection plants. In *Funaria hygrometrica* (Werner and others 1991), the hormone content was determined at several time points during the drying process. ABA content of slowly dried mosses increased about six times, reaching the maximum when the RWC of leaves reached the lowest level and then remained constant. In *Sporobolus stapfianus*, ABA reached the highest level in leaves at about 15 % RWC and then started to decrease at the full dry state (Gaff

and Loveys 1992). On the other hand, the peak in roots appeared earlier, at about 45 % RWC. Perhaps, the only exception was the study of *Craterostigma wilmsii*, where higher ABA levels were found at about 80 % RWC compared to lower levels at 20 % RWC (Vicre and others 2004).

In our experimental system, the increase in ABA content in leaves started when the RWC fell to values twice lower than normal. At this stage, the increase of the hormone was about sixfold. The maximum ABA content (100 times higher than that in control) was reached after 6 h of desiccation, when RWC was about 25 %. At the full dry stage and under rehydration, ABA concentration decreased steadily.

In contrast to leaves, ABA in roots remained relatively unchanged until the full dry state, when it increased about sixfold. No major changes occurred at recovery (Fig. 2).

There are numerous molecular and proteomic studies that tried to elucidate the role of ABA in triggering the desiccation tolerance of various resurrection plants (reviewed in Le and McQueen-Mason 2006; Bartels and Hussain 2011; Cushman and Oliver 2011; Tuba and Lichtenthaler 2011). As early transcripts and newly formed proteins precede or coincide in most cases with the maximal accumulation of ABA, both ABA-independent and ABA-dependent components of desiccation tolerance have been considered (Bartels and others 1990; Piatkowski and others 1990; Bartels and others 1992; Gaff and Loveys 1992; Ingram and Bartels 1996; Blomstedt and others 1998).

Using the same experimental system, we showed in another study that *H. rhodopensis* plants are able to survive rapid desiccation due to a coordinated increase of soluble sugars, total phenol content, and total glutathione at the late stages of dehydration (Djilianov and others 2011). In accordance with other reports, the ABA maximum appears rather late to trigger all protective mechanisms. In *H. rhodopensis*, the peak is at about 35 % RWC, a "point of no return" for nonresurrection plants that are not able to recover when their RWC decreases below this level (Djilianov and others 2011).

The dynamics of phaseic acid, in general, accompanied that of ABA during desiccation of *H. rhodopensis*. This is in agreement with the data for drought stress response in sunflower (Bano and others 1994) and barley (Seiler and others 2011), where free and conjugated forms of PA and ABA were found to increase during drought stress. To our knowledge, there are no data for PA dynamics in other resurrection plant species, so it is difficult to draw any clear conclusions on this hormone.

SA could be regarded as a compound with dual characteristics: a plant hormone and a phenolic acid (Hayat and others 2010). Although there is substantial evidence for the important role that SA plays in plant response to biotic and abiotic stress (for review see Vlot and others 2009), so far SA has not been evaluated in resurrection plants.

In *H. rhodopensis*, SA was the most abundant stress hormone (Fig. 2). There are differences in the dynamics of SA accumulation during the desiccation-recovery process in leaves and roots. In leaves, SA showed more or less insignificant fluctuations around the control levels during the first stages of drying. At 25 % RWC (6 h desiccation), the level increased twice, reaching the maximum at the full dry state (three times higher than the initial value). During recovery, SA decreased to normal values. In roots, the SA amounts decreased slowly but steadily. The pattern of SA accumulation resembles that of other compounds, particularly total phenols, involved in *H. rhodopensis* tolerance to oxidative stress during desiccation (Djilianov and others 2011). In this respect, it is tempting to assume that similar to other systems (for example, Yang and others 2004), the role of SA as an antioxidant is of paramount importance in our case.

The complete picture of the function of individual hormones in the desiccation tolerance of resurrection plants is still missing. Our data indicate that all stress hormones participate in the drought response of *H. rhodopensis.* Its desiccation tolerance appears to be strongly influenced by the earliest and very high accumulation of JA, the several-fold increase of ABA, also at relatively early stages of stress, and the steady high levels of SA during the whole process of desiccation. The increase of ABA and especially the preceding increase of JA coincide with the accumulation of early upregulated transcripts (Georgieva and others 2012), some of which match those found in other plant systems under ABA-dependent and/or ABA-independent (JA-dependent) control.

During desiccation, a rapid downregulation of bioactive CKs (especially *trans*Z) occurred in *H. rhodopensis* leaves, followed by their elevation until RWC 25 % (6 h desiccation) (Fig. 3, Supplementary Fig. 1). This profile is in accordance with the dynamics of *trans*Z and its riboside, the only two CKs analyzed in leaves of *Craterostigma wilmsii* (Vicre and others 2004). These authors also found CK downregulation at the initial phase of desiccation, followed by elevation at low RWC. In contrast to *Craterostigma wilmsii*, *H. rhodopensis* exhibited a shorter period of bioactive CK downregulation and earlier stimulation of their content.

In roots, after a slight and transient decrease, bioactive CK levels increased during the whole desiccation period (especially at low water content, with maxima at 25 and 13 % RWC) (Fig. 3). CK dynamics in our resurrection plant differed substantially from that of nonresurrection plant species, for example, tobacco, which was reported to exhibit downregulation of bioactive CKs during drought stress (especially in shoots) (Havlová and others 2008).

Rehydration coincided with elevation of bioactive CKs, especially iPR and DHZ, and iP at later stages (Supplementary Fig. 1). Surprisingly, the level of *trans*Z, the most physiologically active CK in the stimulation of cell division, was decreased after rehydration. A gradual decrease of *trans*Z during rehydration was reported also in *Craterostigma wilmsii* (Vicre and others 2004). Rehydration in *H. rhodopensis* was associated with a tremendous increase of *cis*ZR. Recently, the CK activity of this compound and its corresponding base (*cis*Z) was demonstrated (Gajdošová and others 2011). This may indicate a biological relevance of *cis*Z-type CKs, which is still debated, for example, in relation to abiotic stress responses (Vyroubalová and others 2009; Dobrá and others 2010), including drought (Havlová and others 2008) and cold (Tarkowska and others 2012). The lower biological activity of *cis*Z-type CKs compared with that of *trans*Z types might allow for multiple CK functions simultaneously with reduced growth. This feature may contribute to the dehydration tolerance of *H. rhodopensis*, similar to, for example, plants constitutively overexpressing the CK oxidase/dehydrogenase gene, which have a diminished growth rate as well as low contents of *trans*Z-type CKs in particular (Werner and others 2003; Nishiyama and others 2011).

The dehydration response of *H. rhodopensis* was, in accordance with that of other species such as, for example, maize (Vyroubalová and others 2009) and tobacco (Havlová and others 2008), associated with accumulation of deactivated CK forms, CK glucosides, which represent the vast majority of the *H. rhodopensis* isoprenoid CK pool. In contrast, a relatively strong decline in CK glucoside levels in both leaves and roots was found after rehydration. CK-*N*-glucosylation was relatively more abundant in roots compared to leaves during desiccation and rehydration, whereas the levels of leaf CK-*O*-glucosides mostly exceeded those in roots, indicating tissue-specific mechanisms of CK deactivation.

Analysis of a wide range of CK derivatives revealed that the most prevalent CKs in *H. rhodopensis* are of the iP-type, especially iP7G, in both leaves and roots. The higher content of *trans*Z-type CKs in roots in comparison with shoots corresponds very well with the "root-born" character of these CKs (Hirose and others 2008). Very interesting is the relatively high occurrence of *cis*Z-type CKs in *H. rhodopensis* leaves, which might contribute to extreme drought tolerance of this species.

The dynamics of auxin content during dehydration and rehydration differed between *H. rhodopensis* leaves and roots. The active auxin IAA and its inactive amino acid conjugate IAA-Asp followed similar trends in leaves, reaching a maximum at the late stages of desiccation (RWC 25–16 %) (Fig. 4). This is in a very good agreement with the results in *C. wilmsii*, where the IAA maximum was found at RWC 20 % (Vicre and others 2004). The elevation of auxin is in accordance with the increased IAA levels observed under drought stress in desiccation-sensitive tobacco plants (Havlová and others 2008; Dobrá and others 2010). In roots, the profiles of IAA and IAA-Asp were not parallel during the course of desiccation. The minimum of IAA and the simultaneous maximum of IAA-Asp contents were found at 25 % RWC. The decline in IAA concentration in roots during the desiccation process of *H. rhodopensis* is in contrast with an increase of IAA levels observed under drought stress in maize roots (Ribaut and Pilet 1994; Xin and others 1997).

To summarize, to our knowledge, for the first time the dynamics of the majority of phytohormones was followed in a resurrection plant during desiccation and subsequent recovery. Analysis of *H. rhodopensis* leaves and roots showed that JA, along and even earlier than ABA, serves as a signal that triggers the response of resurrection plants to desiccation. The steady high levels of SA may represent one of the parameters that prime *H. rhodopensis* desiccation tolerance. The dynamic changes of CKs and auxins suggest that these hormones actively participate in the dehydration response and development of desiccation tolerance in the resurrection plants. The detailed elucidation of their function in these processes, however, is still missing.

Our data provide a complex picture of plant hormone interplay that results in an extreme desiccation tolerance of resurrection plants, mechanisms of which may be successfully utilized for crop improvement.

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