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RESEARCH ARTICLE

Molecular mechanisms of desiccation tolerance in the resurrection glacial relic *Haberlea rhodopensis*

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Abstract *Haberlea rhodopensis* is a resurrection plant with remarkable tolerance to desiccation. *Haberlea* exposed to drought stress, desiccation, and subsequent rehydration showed no signs of damage or severe oxidative stress compared to untreated control plants. Transcriptome analysis by next-generation sequencing revealed a drought-induced reprogramming, which redirected resources from growth towards cell protection. Repression of photosynthetic and growth-related genes during water deficiency was concomitant with induction of transcription factors (members of the NAC, NF-YA, MADS box, HSF, GRAS, and WRKY families) presumably acting as master switches of the genetic reprogramming, as well as with an upregulation of genes related to sugar metabolism, signaling, and genes encoding early light-inducible (ELIP), late embryogenesis abundant (LEA), and heat shock (HSP) proteins. At the same time, genes encoding other LEA, HSP, and stress protective proteins were constitutively expressed at high levels even in unstressed controls. Genes normally involved in tolerance to salinity, chilling, and pathogens were also highly induced, suggesting a possible cross-tolerance against a number of abiotic and biotic stress factors. A notable percentage of the genes highly regulated in dehydration and subsequent rehydration were novel, with no sequence homology to genes from other plant genomes. Additionally, an extensive antioxidant gene network was identified with several gene families possessing a greater number of antioxidant genes than most other species with sequenced genomes. Two of the transcripts most abundant during all conditions encoded catalases and five more catalases were induced in water-deficient samples. Using the pharmacological inhibitor 3-aminotriazole (AT) to compromise catalase activity resulted in increased sensitivity to desiccation. Metabolome analysis by GC or LC–MS revealed accumulation of sucrose, verbascose, spermidine, and γ-aminobutyric acid during drought, as well

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as particular secondary metabolites accumulating during rehydration. This observation, together with the complex antioxidant system and the constitutive expression of stress protective genes suggests that both constitutive and inducible mechanisms contribute to the extreme desiccation tolerance of *H. rhodopensis*.

Keywords Antioxidant genes · Catalase · Desiccation tolerance · Drought stress · Metabolome analysis · Resurrection plants

Abbreviations

AT	3-Aminotriazole
ELIP	Early light-inducible genes/proteins
GABA	γ-Aminobutyric acid
HSP	Heat shock genes/proteins
LEA	Late embryogenesis abundant genes/proteins
NGS	Next-generation sequencing
RFO	Raffinose family oligosaccharides
ROS	Reactive oxygen species
RWC	Relative water content

Introduction

Drought, the most common abiotic stress factor for land plants, may lead to cell and tissue damage, impaired development, reduced productivity and crop yield, and in extreme cases, even plant death [1]. Sensitivity to drought stress varies greatly among species. Some species are extremely sensitive to water deprivation and show signs of damage after only 10 % water loss, while others are more tolerant to drought; tolerance variation may also exist between different cultivars of the same species [1–3]. Resurrection plants display a remarkable ability to survive extreme drought or desiccation (<5 % relative water content, RWC); they can remain in a seemingly inactive physiological state for extended periods of time and resume vegetative growth rapidly upon rehydration. Such extreme loss of water is normally tolerated only by seeds (except "recalcitrant" seeds), pollen grains, and the majority of bryophytes and lichens [2, 3]. In contrast, most other plants are sensitive to desiccation of their vegetative tissues [4].

The mechanism of desiccation tolerance is common in bryophytes and lichens, less common in pteridophytes, rare in angiosperms, and absent from gymnosperms [5, 6]. The complex plant tissues require mechanisms that prevent desiccation-induced cell and tissue damage in the first place; notably, the recovery from desiccation can be fast [7, 8]. The small group of angiosperm resurrection plants (about 300 species) displays remarkable habitat and geographic diversity [5]. They are found in dry and desert areas, in more temperate areas with sufficient rain but short periods of drought or/and cold winters, and even in the tropical rainforests of Africa, where humidity is normally high [5, 9, 10]. Most of the resurrection species are herbaceous plants, but there are also shrubs and trees [4].

Desiccation-sensitive and resurrection plants may share similar mechanisms of drought perception and dehydration responses such as the induction of late embryogenesis abundant (LEA) and heat shock (HS) genes, or the adjustment of carbohydrate metabolism; however, the final physiological result varies between species, and tolerance to desiccation is only observed in the resurrection plants [11]. This difference implies the existence of additional, unique protective mechanisms in the resurrection plants, a notion corroborated by the identification of the unusual eight-carbon sugar octulose, the *CDT1* gene in *Craterostigma plantagineum,* and the phenolic antioxidant 3,4,5-tri-*O*-galloylquinic acid in *Myrothamnus flabellifolia* [12–15]. Furthermore, there is increasing evidence that some of the drought-protective mechanisms inducible in most plants by mild drought are constitutively active in resurrection plants [16, 17].

Haberlea rhodopensis is a resurrection species and glacial relic endemic in the mountains of the Balkan Peninsula in southeastern Europe [18]. *H. rhodopensis* can survive long periods of desiccation (for up to 2 years, T. Gechev, unpublished data) and quickly resume normal growth within hours of re-watering. Fast recovery from desiccation is possible because *H. rhodopensis*, like other homoiochlorophyllous resurrection plants, retains its chlorophyll and photosynthetic machinery during desiccation. The potential risk of this strategy is photooxidative damage caused by the remaining chlorophyll, necessitating the development of efficient strategies to protect against the resulting oxidative stress [19]. In addition to drought, *H. rhodopensis* can also tolerate chilling and the sub-zero temperatures which often occur during harsh winters. This phenomenon is observed in a few other resurrection species such as the mosses *Physcomitrella patens* and *Ceratodon purpureus* [20, 21].

Here, we have applied an integrated physiological, transcriptome, and metabolome analysis to comprehend the intricate response of *H. rhodopensis* to drought and desiccation followed by subsequent rehydration. The physiological analysis confirmed the resurrection nature of *H. rhodopensis*, including unusual features associated with drought tolerance such as active photosynthesis during mild drought and the retention of chlorophyll during desiccation. The transcriptome analysis unveiled diverse mechanisms employed to counteract desiccation stress and highlighted new genes with potential roles in drought tolerance. Moreover, the metabolome analysis supported the gene expression studies and additionally revealed massive accumulation of particular metabolites

during water deficit, which could, in concert with the genetic reprogramming, act to provide stress tolerance.

Materials and methods

Plant material, growth conditions, stress treatment, and evaluation of stress tolerance

H. rhodopensis collected from the Rhodope Mountains was grown in a climate-controlled room on soil taken from its natural habitat at 21 °C, 20 μE m^{-2} s^{-1} light intensity, 16 h/8 h photoperiod, and 65 % relative humidity. This was the natural light intensity below the trees where *H. rhodopensis* was collected, so that this light intensity was maintained throughout the experiments.

Well-watered *H. rhodopensis* plants grown on soil at the above conditions (time point 1, T1) were used as unstressed controls. For the drought stress treatment, water supply was withheld and samples for the drought (time point 2, T2) time point and the desiccation time point (time point 3, T3) were taken after 4–20 days, when the relative water content (RWC) reached 42 and 4 %, respectively. Relative water content (RWC) was measured using the formula $\text{RWC}\ (\%) = [(\text{FM} - \text{DM})/(\text{TM} - \text{DM})] \times 100$, where FM, DM, and TM are the fresh, dry, and turgid masses of the leaves, respectively. TM was measured after immersing the leaves in H_2O for 24 h and DM was determined after drying the leaves at 80 °C for 48 h. Rehydration (time point 4, T4) was achieved by resuming water supply and samples were taken 4 days after re-watering, when plants regained their normal appearance and RWC was similar to unstressed controls. In all cases, rosette leaves were used as samples. For the AT treatment, plants were sprayed either with water (controls) or with 0.1 mM 3-aminotriazole (AT), a concentration that led to no visible symptoms, and the plants were subjected to drought, desiccation, and rehydration in the same manner as described above.

Electrolyte leakage was assessed by measuring the increase in conductivity with a HI 8733 conductivity meter (Hanna Instruments, Woonsocket, RI, USA) as previously described [22]. *Haberlea* leaves from the four time points (T1–T4) were briefly washed with ultrapure water (conductivity below 1 μS). The leaves were then incubated in milli-Q water for 10 min, and the conductivity of the resultant solution was measured and expressed in mS. Chlorophyll was isolated by 80 % acetone extraction and its content was measured photometrically as reported [23]. Oxidative stress was assessed by measuring malondialdehyde levels with thiobarbituric acid as previously described [24]. Variable fluorescence (F_v/F_m) was measured with a pulse amplitude modulated fluorimeter MINI-PAM (Walz, Effeltrich, Germany).

RNA isolation, next-generation sequencing (NGS), and quantitative RT-PCR

Four total-RNA samples were prepared for mRNA sequencing using the TriZOL method of RNA isolation followed by DNAse treatment [25]. The concentration of the samples was analyzed with a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The quality and integrity of the RNA samples were analyzed on an RNA 6000 Lab-on-a-Chip using the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Sample quality met the requirements for sample preparation.

Illumina mRNA-Seq Sample Prep Kits were used to process the samples. Sample preparation was performed according to the Illumina protocol "Preparing Samples for Sequencing of mRNA" (1004898 Rev. D). Briefly, mRNA was isolated from total RNA using poly-dT-oligo-attached magnetic beads. After fragmentation of the mRNA, cDNA synthesis was performed. The cDNA was used for ligation of the sequencing adapters and subsequent PCR amplification. The quality and yield after sample preparation was measured with a DNA 1000 Lab-on-a-Chip. The size of the resulting products was consistent with the expected size of approximately 200 bp.

Clustering and DNA sequencing using the Genome Analyzer IIx was performed according to manufacturer's protocols at ServiceXS (Leiden, The Netherlands). A total of 8 pmol of DNA was used. Two sequencing reads of 76 cycles each using the Read 1 sequencing and Read 2 sequencing primers were performed with the flow cell.

Quantitative real-time PCR (qRT-PCR) analysis was performed using an ABI PRISM 7900 HT PCR instrument (Applied Biosystems, Darmstadt, Germany). Primers for the qRT-PCR analysis were designed using Primer3 software. The following genes/contigs and corresponding primer pairs (forward and reverse) were used for the analysis: histone (Contig_003178, primers 5′-CACAAGTT GGTGTCCTCGAAT and 5′-GAAGCTGCTGAGGCC TATCTT); ATP synthase (Contig_004642, 5′-GTTTTTCC TACACCAGCACCA and 5′-AGCTCCTTCATTTGTT GAGCA); light harvesting complex (Contig_007433, 5′-ACCTGCTGTGGATCACCTCTA and 5′-AGCCTCC AAGCTACCTCAAAG); stachyose synthase (Contig_ 043609, 5′-ACGCTCTGGAACTT CTCCTTC and 5′-CT ACAAAAGCCCGAAGGAATC); non-annotated gene (Contig_000225, 5′-CTTGCCGTACATTTGGGTCTA and 5′-TGGCTGGACTGATGAT AGGTT); hypothetical protein (Contig_004112, 5′-CTACAATGGCGACGAAG AAAG and 5′-GGTTTCGCTAAA TTCCTCGTT); protein phosphatase (Contig_093482, 5′-CCATAAGTTGGTGG GGAAGAT and 5′-GCCTTATTCACCTCCTCATCC); MADS-box family gene (Contig_093532, 5′-CAGGAA

GATGCTTGTCTCAGG and 5′-TGCGTTGCTTGTGAA ATAAGA); heat shock protein (Contig_095867, 5′-TCG GCGAGAAGAAATAATTGA and 5′-ATACAATGCCC GTTTCATTTG), xyloglucan endotransglycosylase (Contig_006774, 5′-TTGGTTTGATCCTTCTGCTG and 5′-A CCCTG ATGGGTACTTGATCC); cold-induced glucosyl transferase (Contig_017641, 5′-TGAGAAGCTGAGT TGTA TGGAGG and 5′-AAGCTACCATCGGCACA CC); temperature-induced lipocalin (Contig_031036, 5′-CAACC AAAGCATACTGATAATCCTC and 5′-TT ACAAAGCTGATGCAAACAGC). All reactions contained 10 µl of SYBR Green Master Mix (Applied Biosystems), 25 ng of cDNA, and 200 nM of each gene-specific primer in a final volume of 20 µl. The qRT-PCR reactions were executed using the following program: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Bioinformatics analysis

Quality-filtered sequence tags were used for de novo assembly into contigs using the short-read assembler Velvet v1.0.15 [26]. The assembly was optimized for the number of contigs and total coverage of the DNA sequenced. The assembly resulted in 96,353 contigs with an assembly size of 405,855,716 bases, an average contig length of 421 bases, and a maximum length of 6,886 bases. The reads were mapped onto the reference sequence obtained from the assembly that was generated using the splice junction mapper for RNA-Seq reads Tophat v1.3.0 [27]. The alignments are reported in BAM format, which can be imported into most genomics software tools. The count of the number of reads per contig was then performed using the HTSeq-count program (http://www-huber.embl.de/users/anders/HTSeq/doc/count.html).

To analyze count data from RNA-Seq and test for differential expression, we used the DESeq package [28]. DESeq uses a model based on the negative binomial distribution to test for differential expression between two experimental conditions. Differentially expressed contigs were then annotated using the Blast2Go annotation tool [29].

Metabolome analysis

Metabolites were determined by gas chromatography coupled with mass spectrometry (GC–MS, primary metabolites), liquid chromatography coupled with mass spectrometry (LC–MS, secondary metabolites and some sugars), and by photometric assays (ascorbate). Plant material taken for the four time points was immediately frozen in liquid nitrogen and ground to a fine powder. Six biological replicates were used for all metabolome assays.

The metabolite profiling for primary metabolites was conducted using an established GC–MS protocol as described [30]. Chromatograms and mass spectra were evaluated by Chroma TOF® 4.2 (Leco, St. Joseph, MI) and TagFinder 4.0 [31] for quantification and annotation of the peaks using the MPI Golm Metabolome Database (GMD, http://gmd.mpimp-golm.mpg.de/, [32]). The amounts of metabolites were determined as relative metabolite abundances, calculated by normalization of signal intensity to that of ribitol, which was added as an internal standard and then by dry weight of the material.

Secondary metabolite analysis by LC–MS was performed as described [33]. All data were processed using Xcalibur 2.1 software (Thermo Fisher Scientific, Waltham, MA, USA). The obtained data matrix of peak area was normalized using the internal standard (isovitexin, CAS: 29702-25-8). Metabolite identification and annotation were performed using metabolite databases [34] and literature survey of *H. rhodopensis* [35] and Gesneriaceae species [36]. Peak annotation of detected compounds is shown in Supplemental Table 5 with information on "Recommendations for Reporting Metabolite Data" described in [37]. The VANTED software was used to generate a metabolic map from the relative metabolic levels [38].

For the targeted LC–MS analysis of sugars, analytical-grade water and solvents used for chromatography were purchased from Fisher Scientific (Loughborough, UK). The carbohydrate standards fructose, glucose, galactinol, raffinose, stachyose, and verbascose were purchased from Sigma-Aldrich (Poole, UK); sucrose was purchased from BDH (Poole, UK). Standard stock solutions were prepared in water at a concentration of 1.0 mg ml^{-1} and stored at −20 °C prior to use. Calibration standards were prepared from these stock solutions by dilution with water. The frozen plant material was reconstituted in 150 µl of water followed by centrifugation at 10,000 × *g* at ambient temperature for 20 min. The supernatant was then analyzed by liquid chromatography ion trap mass spectrometry. Due to the high concentrations of the target analytes sucrose and raffinose in these samples, a further 50-fold dilution in water of an aliquot of the supernatant was performed to prevent column overloading for these analytes. LC–MS analyses were performed using a Thermo Finnigan LCQ deca XP plus ion trap mass spectrometer, fitted with a Thermo Finnigan orthogonal electrospray interface, coupled with a surveyor HPLC system (Thermo Finnigan, San Jose, CA, USA). Chromatographic separation was carried out using a Thermo Scientific Hypercarb™ PGC column (5 µm, 100 × 4.6 mm; Thermo Finnigan, Runcorn, UK) at a flow rate of 600 µl min^{-1} as described [39].

The plant extract sample injection volume was 10 µl, while the standard calibrant injection volume was 20 µl. The binary mobile phase system consisted of (A) water modified with 0.1 % (v/v) of formic acid and (B) acetonitrile modified with 0.1 % (v/v) of formic acid. The gradient elution profile, modified from [39], was as follows: $t = 0$–5 min, 1–4 % B, $t = 5$–7 min, 4–25 % B, held at 25 % B for 3 min, $t = 10$–12 min, 25–50 % B, held at 50 % B for 4 min, $t = 16$–18 min, 50–1 % B, maintained for 10 min.

Results

H. rhodopensis is a resurrection plant that does not experience severe oxidative stress and cell damage during water deficiency

Well-watered mature *H. rhodopensis* plants grown on soil in optimal conditions (time point 1, T1) were exposed to drought stress by withholding water supply (T2, 4 days without watering, 42 % RWC), severe desiccation

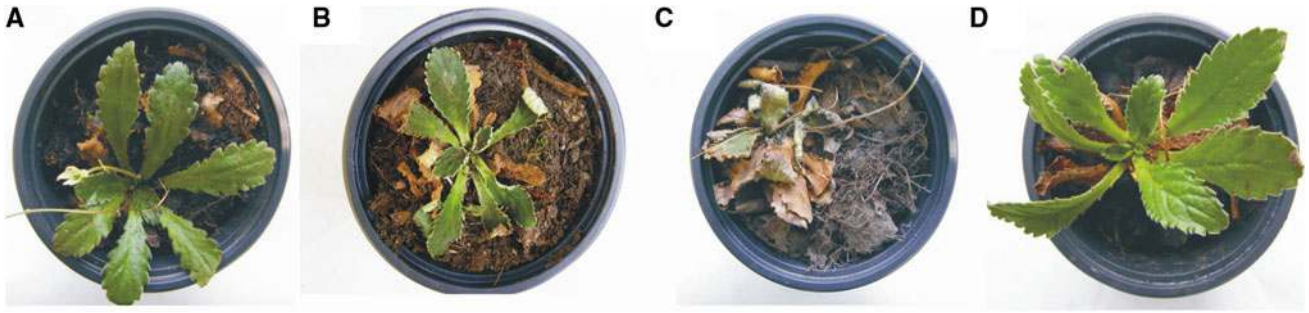

Fig. 1 *Haberlea rhodopensis* under normal conditions (**a**), drought stress (**b**), desiccation (**c**), and after rehydration (**d**). Drought stress (**b**) was achieved by withholding the water supply for 4 days until RWC reached 50 %. Desiccated plant (**c**) obtained after 20 days without water had 5 % RWC; rehydrated plant (**d**) pictured 4 days after resuming water supply had the same RWC as control plants (**a**)

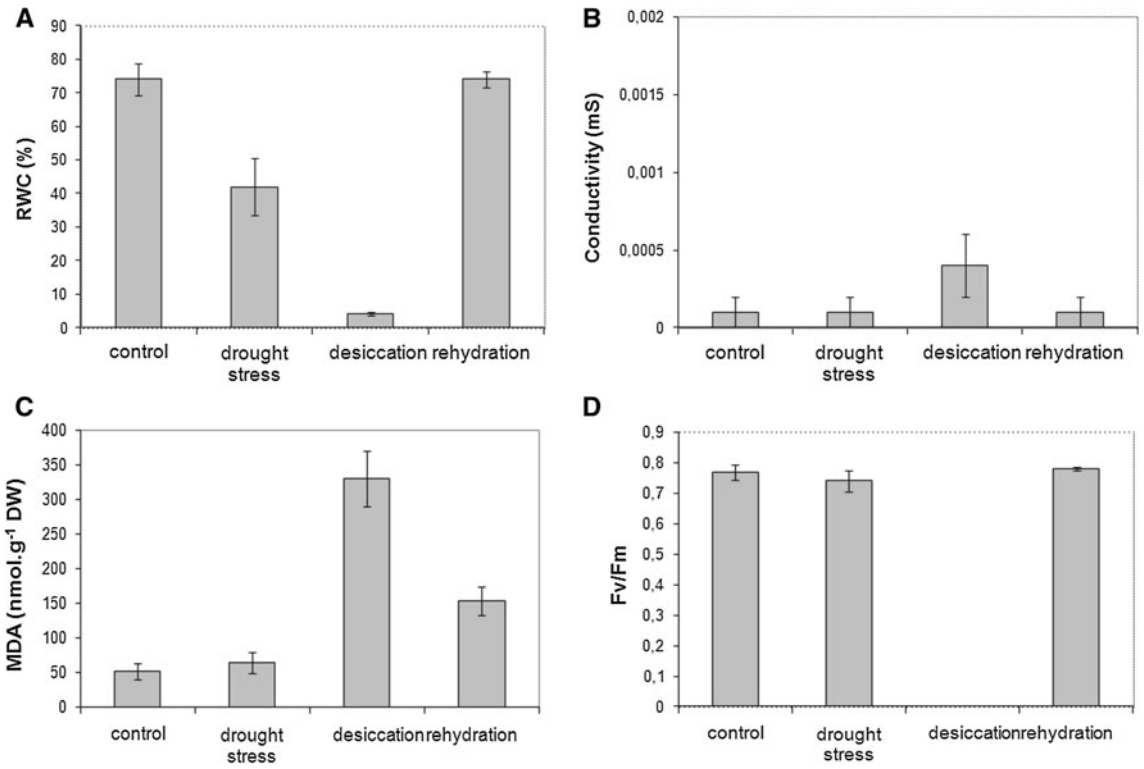

Fig. 2 *Haberlea rhodopensis* is a genuine resurrection plant. Well-watered, fully hydrated unstressed control plants were subjected to drought stress, desiccation, and subsequent rehydration. **a** Relative water content (RWC, %); **b** conductivity (mS), a measure of electrolyte leakage and cell damage; **c** malondialdehyde (MDA) level, a measure of lipid peroxidation and oxidative stress; **d** chlorophyll fluorescence (Fv/Fm). Data are means from three biological replicates ± SD

(T3, 20 days without water, 4 % RWC), and subsequent rehydration (T4, regaining the normal RWC 4 days after watering of the severely desiccated plants) (Figs. 1, 2a). Wilting, observed during drought, was followed by shrinkage and rolling of the leaves during desiccation (Fig. 1b, c). After rehydration, the desiccated dry, "stone-like" plants completely restored their appearance and were even difficult to distinguish from the well-watered control plants (Fig. 1a, d). Measuring ion conductivity, an indicator of membrane damage and cell death, revealed no increase in electrolyte leakage during drought stress and only a relative small increase during desiccation, consistent with the retained vitality of the plants during mild and severe water deficiency (Fig. 2b). Malondialdehyde levels were unchanged during mild drought and increased six-fold during desiccation (Fig. 2c). Chlorophyll content was reduced slightly during the initial drought and to about 40 % of its initial value during desiccation, but returned to normal levels on re-watering (data not shown). At the same time, F_v/F_m measurements of chlorophyll fluorescence showed no changes during drought, but complete absence of signal in the desiccated plants which fully returned to normal after rehydration (Fig. 2d). All this demonstrates that neither drought nor severe desiccation leads to permanent damage or cell death.

Transcriptome analysis of *H. rhodopensis* exposed to water-limiting conditions

To gain further insight into the molecular mechanisms of desiccation tolerance in *Haberlea*, transcriptome analysis of gene expression during normal watering, drought, desiccation, and rehydration was performed using the Illumina next-generation sequencing technology. This approach facilitates transcriptome characterization of plants with unsequenced genomes, revealing both highly regulated genes and previously unidentified genes that may have no sequence homology to genes in other species. 96353 expressed sequence contigs (ESC) were identified. Their sequences and expression patterns in fully hydrated, drought-stressed, desiccated, and rehydrated plants are given in Supplemental Table 1 and on our Web site (http://nature.uni-plovdiv.bg/uploads/Gechev Supplemental/). The expression pattern of 12 selected genes (listed in Materials and methods) was verified by qRT-PCR (Supplemental Table 2). Comparative analysis with other genomes showed most BLAST hits with genes from *Vitis vinifera*, *Populus trichocarpa*, and *Ricinus communis* (26, 9, and 9 %, respectively) and only a small fraction of all transcripts showed similarities to genes from popular model species such as *Arabidopsis thaliana* and *Oryza sativa* (Fig. 3). The species distribution was very similar to the recently published *C. plantagineum* transcriptome [19]. Of the top hits, 41 % had no sequence similarity to other genomes. As short-sequence contigs were omitted from the analysis (some of the unannotated genes were as long as several thousands of base pairs), most of the sequences with no homologies to sequences from other species may represent unique genes and are sources for further gene discovery. Cluster analysis of the expressed sequences indicated two distinct groups, the first one comprising the fully hydrated unstressed controls (T1) plus the rehydrated

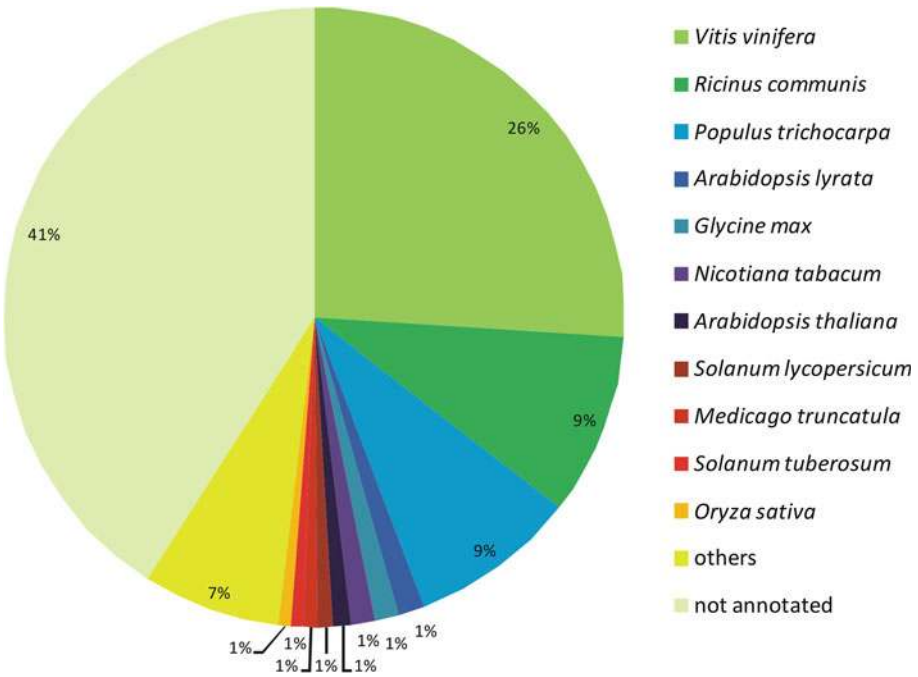

Fig. 3 Top species distribution of *Haberlea rhodopensis* genes obtained by BLAST analysis (Blast2Go annotation tool). Of the transcripts, 44 % are most similar to transcripts from *V. vinifera*, *R. communis*, and *P. trichocarpa*. 41 % of the transcripts did not match any species and may be a source for gene discovery

Table 1 Genes highly regulated by drought/desiccation and/or rehydration

Contig number	Control	Drought	Desiccation	Rehydration	Gene description
Genes induced by drought and/or desiccation					
Contig_094317	23	**15659**	21839	25	NA
Contig_043609	55	10223	11430	191	stachyose synthase
Contig_093552	27	15464	37333	82	early light-inducible protein
Contig_095116	1	5494	13361	7	early light-inducible protein
Contig_003402	295	5013	3547	339	sucrose synthase
Contig_093893	12	5840	10878	25	NA
Contig_093482	0	1154	6775	3	protein phosphatase
Contig_053200	145	4396	2858	400	late embryogenesis abundant protein
Contig_038160	291	4306	3664	547	late embryogenesis abundant protein
Contig_028014	117	3741	3290	400	multidrug resistance-associated protein 6
Contig_058229	84	2464	4454	109	galactinol synthase
Contig_025372	158	2318	3140	227	beta-amylase 1
Contig_038977	133	2045	3839	158	senescence-related protein
Contig_052581	177	1976	2677	97	glutamine synthetase
Contig_060539	77	1867	1212	74	NADP-malic enzyme
Contig_059111	30	833	4014	39	NAC transcription factor
Contig_052321	22	630	1922	19	MYB transcription factor MYB69
Contig_093512	0	593	2159	1	tumor-related protein, putative [*Medicago truncatula*]
Contig_042845	34	569	1134	21	myo-inositol oxygenase
Contig_093532	0	417	176	0	MADS-box protein 1
Contig_012998	11	36	87	8	phospho*enol*pyruvate carboxylase
Contig_013275	52	1529	1502	0	NA
Contig_033019	103	1200	2563	107	zinc finger (C3HC4-type ring finger) family protein
Contig_038666	220	1869	5333	171	CBL-interacting serine threonine-protein kinase
Contig_045681	98	716	2318	95	unknown [*Populus trichocarpa* x *Populus deltoides*]
Contig_082434	37	342	1057	40	multidrug resistance-associated protein 6
Contig_046660	43	323	2195	58	hypothetical protein [*Vitis vinifera*]
Contig_063909	33	291	1044	36	WRKY transcription factor
Contig_043157	27	258	1910	35	unnamed protein product [*Vitis vinifera*]
Contig_089452	11	102	1109	3	cytochrome P450
Contig_087323	9	243	67	24	late embryogenesis abundant protein group 9 protein

(T4) samples, and a second cluster consisting of the water-deficient T2 and T3 samples (Supplemental Fig. 1).

Several of the most abundant transcripts in the unstressed controls and the rehydrated samples were related to photosynthesis (chlorophyll *a/b* binding protein, light harvesting complex Lhcb1, rubisco small subunit) (Supplemental Table 3). Not surprisingly, the most profound transcript among them was *Lhcb1*, which encodes the most abundant membrane protein in the biosphere. Transcripts of *Lhcb1* and the other photosynthesis-related genes were highly represented under normal conditions and rehydration, but repressed during drought and desiccation. This was consistent with the physiological data on chlorophyll content (see above) and was in line with the observation that photosynthesis-related genes were generally repressed during various types of stress [19, 22]. However, phosphoenolpyruvate carboxylase (PEPC) transcripts related to C4/CAM-type photosynthetic metabolism were substantially induced in the water-deficient samples, possibly indicating a drought-induced switch from C3 to C4-type photosynthesis and possible CAM idling (Table 1). Two of the other most abundant transcripts in unstressed, fully hydrated plants but also in all other conditions, were from catalase-encoding genes. Although their expression was

Table 1 continued

Contig number	Control	Drought	Desiccation	Rehydration	Gene description
Contig_074392	255	2612	1853	389	cytosolic NADP-malic enzyme
Contig_090143	3	1078	19	5	gag-pol polyprotein
Contig_044473	33	1026	262	60	sulfate bicarbonate oxalate exchanger and transporter sat-1
Contig_028521	499	3148	4173	621	sucrose transport
Contig_038875	117	639	1692	105	NAC transcription factor
Contig_031036	142	823	1839	205	temperature-induced lipocalin
Contig_004849	92	568	1616	54	embryo-abundant protein
Contig_025689	51	387	1350	97	nitrate transporter nrt1-5
Contig_012006	50	278	1063	84	NAC transcription factor
Contig_094787	1	7	379	165	Af364864_1 pathogenesis-related protein 5-
Contig_055618	42	224	6171	28	polyubiquitin
Contig_052759	20	131	916	16	beta-amylase pct-bmyi
Contig_055059	4	26	67	7	phospho*enol*pyruvate carboxylase
Contig_094500	0	7	661	0	NA
Contig_043057	161	536	1664	131	zinc finger
Contig_025899	104	484	1770	107	translation initiation factor-related
Contig_042552	89	334	1608	65	WRKY-type transcription factor
Contig_053039	23	74	270	34	phospho*enol*pyruvate carboxylase
Contig_093528	0	328	0	0	NA
Contig_092914	2	33	6	4	late embryogenesis-like protein
Contig_092107	2	32	0	5	ATP binding protein
Contig_094915	0	28	0	0	calmodulin binding protein
Contig_094037	0	26	0	1	hypothetical protein [*Vitis vinifera*]
Contig_095865	0	0	946	0	NA
Contig_095867	0	0	946	0	12 kDa heat shock protein (glucose and lipid-regulated protein)
Contig_095913	0	0	946	0	NADPH oxidase
Contig_091094	0	0	228	0	YSIRK type signal peptide
Genes induced by drought/desiccation and/or rehydration					
Contig_088217	3	2345	3061	579	ecp63 protein
Contig_090887	4	1577	2152	318	late embryogenesis protein
Contig_060209	7	1540	1884	527	NA
Contig_045763	10	570	1199	57	zinc-binding dehydrogenase family protein
Contig_070602	0	410	1077	13	NAC transcription factor
Contig_093486	0	366	347	13	disease resistance response protein 1
Contig_094227	0	61	72	95	NA
Contig_091076	0	9	32	14	emb3006 (embryo defective 3006)
Contig_094787	1	7	379	165	pathogenesis-related protein
Contig_094674	0	12	8	159	hypothetical protein VITISV_016971 [*Vitis vinifera*]
Contig_095749	0	4	3	113	hypothetical protein [*Vitis vinifera*]
Contig_095609	0	2	0	96	NA

Table 1 continued

Contig number	Control	Drought	Desiccation	Rehydration	Gene description
Contig_095810	0	0	0	173	NA
Contig_096338	0	0	0	113	conserved hypothetical protein [*Penicillium marneffei* ATCC 18224]
Contig_096343	0	0	0	89	auxin efflux carrier component
Genes repressed by drought and/or desiccation					
Contig_033166	10356	855	367	8802	NA
Contig_069048	5461	256	208	3988	tumor-related protein
Contig_029537	9643	247	197	13918	chlorophyll a-b binding protein chloroplastic precursor
Contig_000739	5453	226	354	7576	carbonic anhydrase
Contig_000610	5056	85	135	8103	lipoxygenase [*Camellia sinensis*]
Contig_006774	1387	0	1	1668	xyloglucan endotransglucosylase hydrolase
Contig_007308	823	0	0	449	glycogen starch alpha-glucan phosphorylase
Contig_031558	655	0	0	504	zinc finger
Contig_010110	1142	0	0	1105	proline-rich protein
Contig_011488	689	0	0	856	leucine-rich repeat family protein
Contig_037649	8360	256	299	19290	NA
Contig_043070	13144	2385	2725	19583	NA
Genes repressed by drought/desiccation and/or rehydration					
Contig_000225	3290	0	1	970	NA
Contig_015420	529	0	0	16	hypothetical protein [*Vitis vinifera*]
Contig_015850	601	0	1	36	class VII chitinase precursor
Contig_001193	187	0	0	2	NA
Contig_004027	540	0	0	71	unnamed protein product [*Vitis vinifera*]
Contig_036853	71	4	10	9	somatic embryogenesis receptor-like kinase 1
Contig_027729	88	14	0	0	major facilitator transporter
Contig_000013	316	2	3	117	unnamed protein product [*Vitis vinifera*]
Contig_021735	678	1	0	329	pectate lyase
Contig_024518	89	3	0	0	4FE-4S iron-sulfur binding protein
Contig_024700	166	2	0	0	NA
Contig_017570	155	1	5	0	NA
Contig_026915	52	0	0	0	hypothetical protein LOC100278933 [*Zea mays*]
Contig_014112	61	0	0	0	hypothetical protein DDB_G0280017 [*Dictyostelium discoideum* AX4]
Contig_011525	117	0	2	0	unnamed protein product [*Vitis vinifera*]

The table lists the most induced/repressed genes in drought-stressed, desiccated, and rehydrated plants relative to the unstressed controls. Expression values are the numbers of transcripts per sample per contig/gene. *Pink, red,* and *dark red colors* indicate from two to fivefold, from five to tenfold, and more than tenfold induction of the respective genes. *Pale green, green*, and *dark green* indicate from two to fivefold, from five to tenfold, and more than tenfold gene repression. The full list of the most regulated genes during drought, desiccation, and rehydration is given in Supplemental Table 4

reduced during water deficiency, their transcript levels were still very high compared with those of other genes and this presumably contributes to the lack of electrolyte leakage and protection against oxidative stress in water-deficient samples.

The genes most strongly affected under water deficiency included those encoding transcription factors and regulators, proteins involved in cell wall metabolism (xyloglucan endotransglucosylases, pectate lyases), proteins involved in antioxidant metabolism (ascorbate peroxidases), heat-shock proteins, and genes encoding components of signaling cascades (Table 1; Supplemental Table 4).

Among the up-regulated genes by water deficiency, the strongest induction was observed for a gene encoding a

putative protein phosphatase/hydrolase (Table 1). It was virtually undetectable in unstressed controls and rehydrated samples, but its expression rose sharply in drought-stressed plants and reaches extremely high levels in desiccated plants. Other genes encoding signaling compounds were also highly up-regulated by drought and desiccation: a receptor-like protein kinase, several other protein kinases and phosphatases, including a CBL-interacting serine/threonine-protein kinase, a Ca^{2+} antiporter cation exchanger, and a phospholipase D (Table 1; Supplemental Table 4). Transcripts for most of these genes were absent in unstressed and rehydrated plants but highly induced in drought-stressed and especially desiccated plants.

A number of transcription factor-encoding genes were highly induced in both drought-stressed and desiccated plants. These included MYB, NAC, WRKY, and GRAS family members, a heat shock transcription factor, and several other transcription factors and co-regulators. Furthermore, genes encoding DREB2, NF-YA, and MADS-box transcription factors were exclusively expressed in water-deficient plants (Table 1). These transcription factors and regulators may be responsible for the massive global transcriptional reprogramming during drought and the switch from normal growth to energy-saving, stress-protective programs.

Many abiotic and biotic stress-related genes were also highly induced by drought and desiccation (Table 1). Besides the numerous heat shock genes, these also included genes encoding cold stress-related proteins, temperature-induced lipocalins, early response to dehydration, multi-drug resistance-associated, and several pathogenesis and disease-related genes. Additionally, transcripts related to ubiquitin/proteasome-mediated protein degradation and auxin signaling were also up-regulated by water deficiency, highlighting a possible involvement of these proteins in the stress response. Several cysteine proteinases, metacaspases, and other proteases were induced as well, substantiating the notion that cleavages of specific target proteins contribute to the events that accompany desiccation and subsequent rehydration.

Several of the most abundant transcripts in drought-stressed and desiccated samples were related to carbohydrate metabolism. In particular, sucrose metabolism appears to be affected, substantiated by the massive induction of several sucrose synthases, sucrose-6-phosphate synthases, a sucrose transporter, and a sucrose responsive element-binding protein (Table 1). Several genes encoding galactinol synthases and a stachyose synthase were also massively induced by water deficiency, consistent with enhanced biosynthesis and accumulation of stachyose and other raffinose-family oligosaccharides (RFOs) during drought and desiccation (Table 1). This correlates with the induction of β-amylase in drought-stressed and especially in desiccated plants, which indicates that at least some of the carbon needed for RFOs synthesis and for energy expenditure derives from the breakdown of starch. The role of sugar metabolism for the adaptation of *H. rhodopensis* to drought and desiccation was further substantiated by the presence of two transketolases in the list of the most abundant transcripts (Supplemental Table 3). Conversion of sugars and sugar derivatives is very important not only for the synthesis of osmoprotectants but also for cell-wall remodeling, which is essential for making the wall more flexible and countering the mechanical stress plant cells experience during desiccation [6]. A more flexible cell wall not only prevents breaking of plasmodesmata bridges during plasmolysis but also allows leaves to fold or roll, thereby reducing the surface exposed to solar radiation. As *H. rhodopensis* retains most of it is chlorophyll during drought and desiccation, leaf folding is likely a vital adaptation to minimize the production of reactive oxygen species (ROS). The need for such protection is evident from the dramatic regulation of two genes encoding early light-inducible proteins (ELIPs, Table 1; Supplemental Table 4). These genes were almost not expressed in well-watered and rehydrated plants, but their levels rise very sharply in plants experiencing drought and desiccation. Early light-inducible proteins are believed to associate with chlorophyll and prevent ROS-induced photooxidative damage [40].

The transcriptome analysis revealed an extensive antioxidant gene network with many gene families having more members in *H. rhodopensis* than in other species with sequenced genomes (Supplemental Table 5). In particular, *H. rhodopensis* contained more superoxide dismutases, catalases, monodehydroascorbate reductases, and glutathione reductases than *V. vinifera* and the model species *A. thaliana*. Drought/desiccation regulated several superoxide dismutases and ascorbate peroxidases as well as induced a number of monodehydroascorbate reductases, glutathione reductases, glutathione peroxidases, and cys-peroxiredoxins (Supplemental Table 5).

A YSIRK signaling peptide, an alcohol oxidase, a laccase, several genes encoding heat shock proteins, and many other genes with unknown functions were exclusively expressed in the desiccated samples (Supplemental Table 4). Transcripts most abundant in the rehydrated plants belong to genes encoding an auxin efflux carrier, several hypothetical proteins, and many unannotated genes. Genes of unknown function or/and with no sequence similarity to genes from other organisms were also well represented in all other conditions (Supplemental Table 4). As short sequences were excluded from homology searches, it is unlikely that the lack of homologies is due to insufficient length alignment (some of the unknown

Table 2 Expression of catalase genes in well-watered unstressed control, drought-stressed, desiccated, and rehydrated *Haberlea rhodopensis* plants

Gene	Description	Expression			
		Control	Drought	Desiccation	Rehydration
Catalase1	Heat-induced catalase, catalase 1	4	17	29	7
Catalase2	Catalase 2	10586	4523	5743	12151
Catalase3	Catalase 3	10560	3455	3713	13125
Catalase4	Cytosolic probable ctt1-catalase	2	63	250	9
Catalase5	Heat-induced catalase	0	0	51	0
Catalase6	Peroxisomal catalase	8	11	39	5
Catalase7	Catalase peroxidase hpi	10	7	9	9
Catalase8	Catalase peroxidase 2	0	9	26	1

Expression values are the numbers of transcripts per sample per contig/gene. *Red/green colors* indicate more than twofold induction or repression, respectively, of the genes compared to unstressed controls

H. rhodopensis sequences were more than 3,000 bp long). In particular, the desiccated and the rehydrated transcriptomes were abundant in unannotated genes, representing potential new baits for gene discovery.

Catalase is required for drought and desiccation tolerance

Analysis of catalase sequences, BLAST searches, and analysis of the expression profiles of the different catalase genes indicated that *Haberlea rhodopensis* has at least eight catalase genes, which is more than in *Arabidopsis* (three genes) and most other species with sequenced genomes [41] (Supplemental Table 5). The initial NGS data and sequence comparison revealed 18 ESCs but after filtering out the short reads and further computational analysis aided by BLAST and detailed study of the expression patterns in the four rehydrating conditions, the number of catalase genes was eventually shortened to eight. Catalase1 has similarity to heat-induced catalases from fungi (most similar to *Mycosphaerella graminicola*). It is very divergent from plant catalases, especially from the catalases in Arabidopsis. It was expressed at low levels in well-watered controls but is rapidly upregulated on drought and especially desiccation, and subsides in rehydrated samples, suggesting a protective role during water deficiency (Table 2). Catalase2 and 3 have similarities to Arabidopsis catalases and are the most abundantly expressed in all conditions. BLAST searches reveal that catalase4 and catalase6 are similar to a catalase from *Picea glauca*. Both catalases had very similar expression patterns, with strong upregulation during drought and desiccation. Sequence comparison between the two ESCs revealed that they are indeed similar, but not identical (data not shown). Catalase5 was exclusively expressed on desiccation, which suggests that extra protection against H_2O_2 accumulation during severe water deficiency is brought about by switching on additional antioxidant genes. Catalase7 was the only one which expression was not noted to change. Catalase8, similar in sequence to bacteria and fungi, was almost absent from well-watered and rehydrated samples, but notably expressed during drought and especially desiccation.

As increased production of ROS, and in particular hydrogen peroxide, is always observed during drought, we hypothesized that catalase is important for preventing oxidative stress and cell death during drought and especially desiccation. To test this hypothesis, we utilized the catalase-specific pharmacological inhibitor 3-aminotriazole (AT) [43]. At high concentrations, AT can lead to severe oxidative stress and subsequent cell death in a programmed way, while at low doses AT only partially inhibits catalase without inducing any visible phenotype or symptoms of damage [42, 43]. We sprayed rosette leaves of

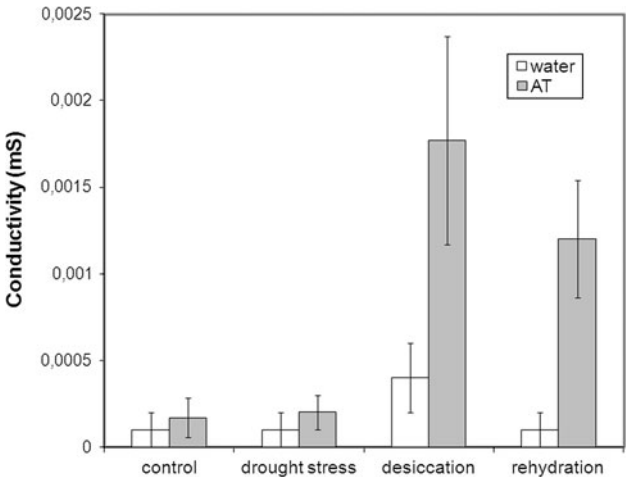

Fig. 4 *Haberlea rhodopensis* with inhibited catalase activity displays increased sensitivity to desiccation. Well-watered controls, drought-stressed, desiccated, and rehydrated plants were sprayed either with water (*white columns*) or the catalase inhibitor 3-aminotriazole (AT, *gray columns*), and electrolyte leakage (a measure for cell damage) expressed as conductivity (mS) was determined. Data are means of three biological replicates ± SD

H. rhodopensis plants with 0.1 mM AT, a concentration that led to no visible symptoms, and subjected these plants to drought, desiccation, and rehydration in the same manner as water-sprayed controls. As AT-treated plants exhibited much more electrolyte leakage during desiccation and rehydration than the controls, we concluded that AT compromised the recovery of *H. rhodopensis* from drought (Fig. 4). In accordance with this, the majority of the plants never recovered completely and died 1 month after the AT treatment.

Metabolome analysis of *H. rhodopensis* exposed to drought stress, desiccation, and subsequent rehydration

To gain further insight into the intricate molecular mechanisms of desiccation tolerance, metabolome analysis of control, drought-stressed, desiccated, and rehydrated samples was performed by GC and LC–MS analysis. A total of 144 primary and secondary metabolites were detected across the four treatment conditions (Supplemental Table 5). Principal component analysis of primary metabolites present in plants grown under the four conditions showed two separate clusters: one with the drought and desiccation time points and another with control and rehydration time points (Supplemental Fig. 2). This observation suggests that despite the metabolite changes during drought and desiccation, metabolic activities return to normal after rehydration. This notion is supported by the physiological data and transcriptome analysis of *H. rhodopensis* (Supplemental Fig. 1).

Most of the metabolites decreased in abundance in drought-stressed samples. Water deficiency drastically affected sugar metabolism. Accumulation of several sugars, including maltose, sucrose, and verbascose, was observed during drought and especially during desiccation (Fig. 5). The increase in maltose during drought and desiccation was concomitant with the induction of a β-amylase gene (Table 1). In addition, two metabolites, tentatively assigned as sugars on the basis of their molecular masses and their mass spectrometric fragmentation behavior, accumulated during water-limiting conditions, and rehydration, respectively (unknowns 3 and 6 from Supplemental Table 6). On the other hand, levels of glucose and galactinol decreased during drought stress and desiccation, in line with the massive accumulation of sucrose, but increased in the rehydrated plants. Levels of organic acids, pyruvate, and citrate were also elevated during rehydration.

The results of targeted LC–MS analysis of carbohydrates are entirely consistent with the results of the GC–MS analyses. In addition, the targeted LC–MS analysis of sugars demonstrated elevated levels of stachyose and verbascose in desiccated plants (Fig. 5), indicating increased synthesis of complex RFOs. This observation is consistent with decreased levels of galactinol, the precursor for RFOs (Fig. 5). The metabolite data are also consistent with the induction of galactinol synthases and stachyose synthase in drought-stressed and desiccated plants (Table 1).

Drought and desiccation also induced many changes in amino acid abundance. The amino acids derived from shikimate, phenylalanine, and tyrosine accumulated under water-limited conditions, suggesting activation of the shikimate pathway (Fig. 5). Non-proteinogenic amino acids, i.e., β-alanine and γ-aminobutyric acid (GABA), and the polyamine spermidine also accumulated under dehydration conditions. On the other hand, the amino acids derived from glutamate and aspartate, namely glutamate, glutamine, proline, pyroglutamate, aspartate, threonine, and asparagine, decreased on water limitation. Since these amino acids are closely related to nitrogen assimilation, these results suggest inactivation of the nitrogen assimilatory pathway. Serine dramatically decreased in dehydrated and rehydrated samples. We also observed an accumulation of threonate under water-limiting conditions. The increase in threonate, a known breakdown product of ascorbate [44], suggests degradation of ascorbate. Indeed, both ascorbate and dehydroascorbate levels decreased in drought-stressed and desiccated plants (Supplemental Table 5).

LC–MS analysis of secondary metabolites identified 72 peaks (Supplemental Table 6; Supplemental Fig. 3). Phenolic compounds are common and widespread plant secondary metabolites related to the response to biotic and abiotic stresses. However, the exact chemical compositions of secondary metabolites in *Haberlea rhodopensis* are poorly known. Although most of the secondary metabolites decreased in abundance during drought and desiccation, a few did not change, and some increased on rehydration (Supplemental Fig. 3). In particular, the major phenylpropanoid myconoside and the anthocyanin Cy-3Glc-Ara decreased in water-deficient samples, but recovered upon rehydration, while the flavonoids hispidulin-8-C-Glc-2-O-syr, hispidulin-8-C-Glc-6-O-acetyl, and hispidulin 8-C-Gal had even higher levels in rehydrated samples than in unstressed controls (Supplemental Table 6). Interestingly, several metabolites decreased only during drought, recovered during desiccation, and increased substantially in the rehydrated plants. Two unidentified metabolites did not decrease under water-limiting conditions and increased 3-fold in the rehydrated samples compared with unstressed controls.

Discussion

Desiccation tolerance is a complex phenomenon, understanding of which requires an integrated systems biology

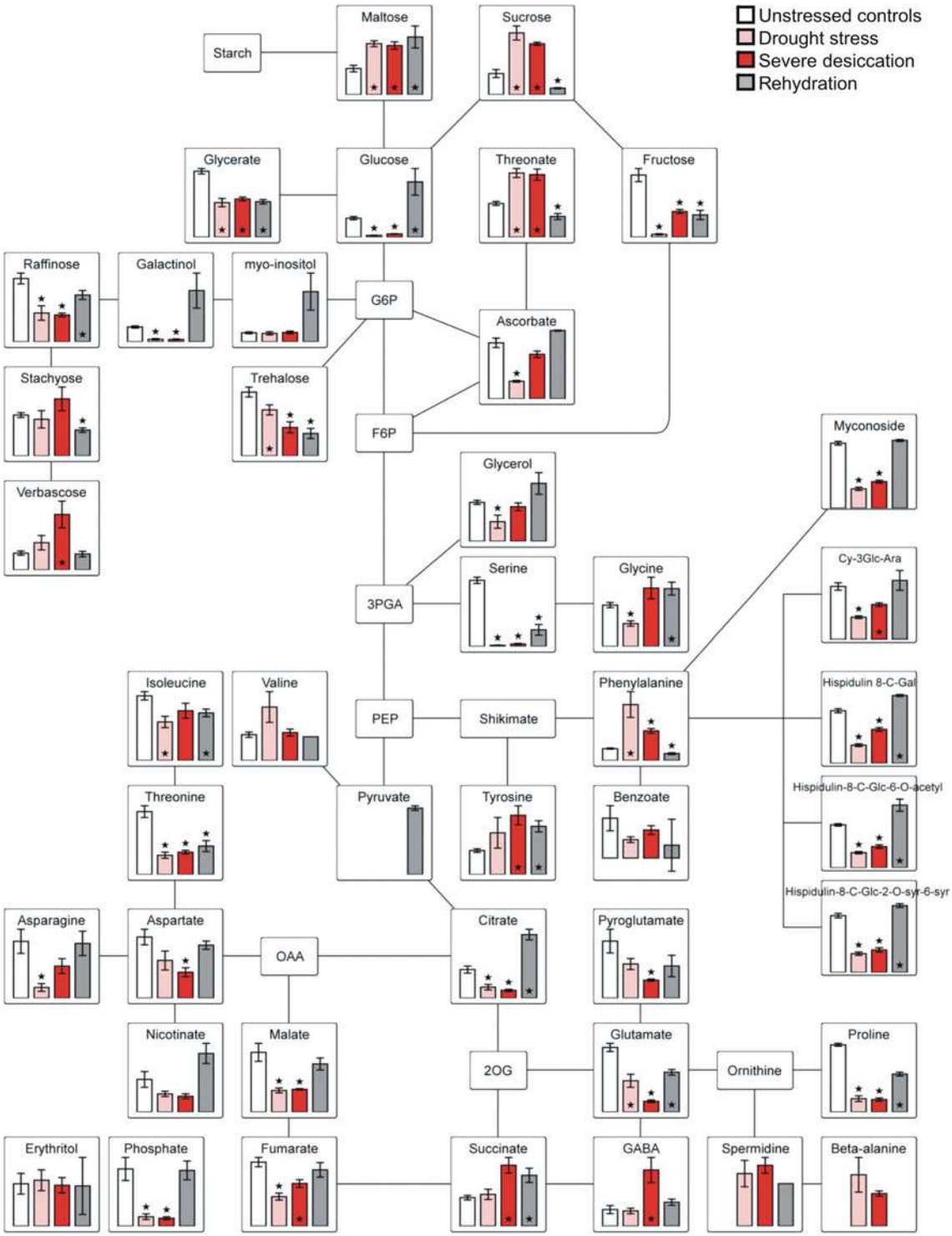

Fig. 5 Relative abundance of metabolites in *H. rhodopensis* during dehydration and following rehydration. The relative metabolite levels are normalized by dry weight of the samples and depicted on a metabolic map using the VANTED software [38]. The map is drawn according to the metabolic network in *A. thaliana*. The *white, pale red, red, and gray bars* represent the four conditions: unstressed controls (RWC 80 %), drought stress (4 days without watering, RWC 50 %), severe desiccation (20 days without watering, RWC 5 %), and rehydration (4 days after re-watering to desiccated plants, RWC as in unstressed controls). The values are the mean ± SEM of 3–6 biological replicates. A *star* indicates the value showed significant difference from that of the unstressed controls in the *t* test by VANTED

approach. The combined analysis of physiological, transcriptome, and metabolome data provides valuable insights into the molecular mechanisms involved in drought and desiccation tolerance of *Haberlea rhodopensis*. The lack of cell damage and severe oxidative stress during desiccation shows that *H. rhodopensis* is a genuine resurrection species. Energy metabolism and growth programs are co-ordinately regulated with induction of stress defense mechanisms in order to provide protection against water deficiency.

Signaling events and global transcriptional reprogramming during drought and desiccation in *Haberlea rhodopensis* lead to cessation of growth, inhibition of photosynthesis, and induction of diverse protective mechanisms

Protein phosphorylation and dephosphorylation are essential signaling events leading to acquisition of drought/desiccation tolerance, and changes in the phosphorylated status of a number of proteins have been shown for *C. plantagineum* [45]. The most drought- and desiccation-upregulated gene in *H. rhodopensis* encoded a protein phosphatase, indicating that phosphorylation events are also an essential part of drought stress signaling in this resurrection species. Some of the other highly regulated genes encoding protein kinases, serine-threonine protein phosphatases, a Ca^{2+} antiporter cation exchanger, and a YSIRK-type signal peptide could also be involved in the signaling events leading to dehydration tolerance.

GABA is a stress-associated metabolite that accumulates during desiccation in *Haberlea* (Fig. 5). It is elevated in response to many stresses in a number of species and is believed to have signaling functions. In addition to its role in abiotic and biotic stresses, GABA seems to control growth by negatively regulating cell elongation [46]. The *Arabidopsis pop2* mutant overaccumulates GABA, the result of which is growth inhibition of the primary root, pollen tube defects, and downregulation of cell wall-related genes [47]. Growth cessation is also observed in *Haberlea* during drought and desiccation, which is in line with the morphological observations and the downregulation of many cell wall-related genes, including several expansins and xyloglucan endotransglucosylases. In *Arabidopsis*, these genes participate in cell expansion and growth by loosening the cell wall [48]. Likewise, downregulation of cell wall growth and photosynthesis-related genes were observed during a recent transcriptomic analysis of two near-isogenic rice lines with different levels of drought tolerance [49]. This growth inhibition allows redirection of resources towards synthesis of stress-protective compounds.

The massive transcriptional reprogramming, resulting in growth cessation and induction of diverse protective mechanisms, is likely to be governed by transcription factors and transcriptional co-regulators. Some of them could act as master switches of growth, development, and stress-related genetic programs. For example, members of the GRAS family of transcription factors regulate many developmental processes and stress responses in *A. thaliana*, including salt and drought tolerance [50, 51]. MADS-box transcription factors are better known for their involvement in developmental processes and floral organ identity formation but in *Haberlea* the drought-induced MADS-box transcription factor may be unique to this species or have functions distinct from those of their homologues in other species. Functional characterization of these transcription factors and identification of their downstream target genes would further decipher their role in desiccation tolerance.

Photosynthesis of *H. rhodopensis* under water-deficient conditions

Downregulation of photosynthesis-related genes observed in *H. rhodopensis* as well as in *Craterostigma plantagineum* [19] is a common response to desiccation. This may also serve to minimize the well-documented excessive production of ROS during drought and desiccation. However, *Haberlea* retains most of its chlorophyll and preserves its thylakoid structure upon tissue dehydration, features characteristic of the so-called homoiochlorophyllous plants [4, 52]. The photosynthetic systems in such plants are reversibly inactivated, but not destroyed, enabling them to recover quickly after rehydration [53]. Although high F_v/F_m values do not necessarily mean proportionately high photosynthesis, as was shown in the moss *Polytrichum formosum* [54], an unaltered F_v/F_m value during drought indicates photosynthetic activity, while a zero F_v/F_m value indicates the opposite. A temporary shift from C3 to CAM metabolism during initial drought stress may increase water use efficiency and decrease photorespiratory H_2O_2 production, a notion supported by the sharp decrease in serine, a direct product of photorespiration. Although the decrease in photorespiratory serine could in part be due to the general decrease in RUBISCO, the possible C3-to-CAM shift is further supported by the induction of genes encoding phosphoenolpyruvate carboxylase (PEPC), the key enzyme for C4-/CAM-type photosynthesis. Drought stress can induce CAM metabolism in C3/CAM-intermediate plants [55]. This strategy is particularly important under severe drought stress, when stomata remain closed and CO_2 is recycled through respiratory release followed by assimilation, a phenomenon known as 'CAM idling' [56]. Although no carbon is being gained during CAM idling, this could be an important ecophysiological adaptation to maintain photosystem stability and survive prolonged periods without water.

The strategy of retaining chlorophyll and photosynthetic machinery is potentially dangerous as excessive ROS may be produced upon illumination by the remaining chlorophyll. *H. rhodopensis* and other homoiochlorophyllous plants therefore must have evolved strategies to minimize ROS production and alleviate possible damage inflicted by oxidative stress. An obvious morphological adaptation is leaf folding and rolling, which minimizes the illuminated surface (Fig. 1). Such leaf rolling and folding is observed in other resurrection species such as *Craterostigma wilmsii, C. plantagineum*, and the woody shrub *Myrothamnus flabellifolia* [6, 19]. This acts in concert with biochemical and molecular mechanisms such as the accumulation of anthocyanins and other polyphenols to protect against UV light and other radiation [57]. Further protection against chlorophyll-induced photodamage may be provided by accumulation of early light-inducible proteins (ELIPs), as is proposed for the 22-kDa ELIP dsp22 in *C. plantagineum* [58]. Suppression of thylakoid ELIP accumulation in the *chaos* mutant of *Arabidopsis thaliana* results in leaf bleaching and extensive photooxidative damage under high light while overexpression of ELIPs in the *chaos* mutant background prevents this damage [41]. Indeed, transcriptome analysis of *H. rhodopensis* shows that the two most abundantly expressed genes during drought and desiccation encode ELIPs (Table 1) and these are also two of the genes most regulated by water deficiency. Further drought stress protection is observed in the resurrection plant *Paraboea rufescens* by activation of cyclic electron flow and stimulation of non-photochemical quenching in both, low and high light levels [59].

Antioxidant defense mechanisms protect against drought-induced oxidative stress

Maintaining a strong antioxidant system is essential for preventing drought-induced oxidative stress and alleviating the consequences of oxidative damage. Among the antioxidant enzymes, catalase has been shown to function as a cellular sink for H_2O_2 and to be of paramount importance for protection against oxidative stress [60]. As H_2O_2 is diffusible and can cross biological membranes through peroxiporins [61], peroxisomal calatases can decompose excess H_2O_2 produced in other compartments. *H. rhodopensis* has more catalases than *A. thaliana* and most other species with sequenced genomes; it also has more genes encoding superoxide dismutases, monodehydroascorbate reductases, and glutathione reductases, supporting the notion of a potent and redundant antioxidant gene network. The higher number of catalase genes together with the poor recovery from desiccation of AT-treated plants collectively implies a role for catalases in drought and desiccation tolerance in *Haberlea rhodopensis*. Other antioxidant genes might also be involved in stress protection, as indicated by the induction of monodehydroascorbate reductases, glutathione reductases, glutathione peroxidases, cys-peroxiredoxins, and the regulation of several superoxide dismutases and ascorbate peroxidases. Full-length isolation of catalases and other selected antioxidant genes followed by detailed studies of gene expression and enzyme activities during drought and other abiotic stresses could decipher the role of the *H. rhodopensis* antioxidant system in abiotic stress protection. The correlation between an active antioxidant system and desiccation tolerance was also noted in a recent comparative study between *C. plantagineum* and *L. brevidens*, two resurrection species, and their relative *L. subracemosa*, a desiccation-sensitive species. Antioxidant genes encoding Cu/Zn superoxide dismutases, catalases, ascorbate peroxidases, and monodehydroascorbate reductases were upregulated or constitutively expressed during desiccation in the tolerant *C. plantagineum* and *L. brevidens*, while the same genes were down-regulated in the sensitive *L. subracemosa* [62]. Additionally, metabolite analysis suggested the consumption of ascorbate and activation of the shikimate pathway in dehydrated plants. Ascorbate is a well-known antioxidant and the shikimate pathway is closely related to the biosynthesis of antioxidants such as phenylpropanoids and caffeic acid esters. These results suggest important roles of antioxidant metabolites for the protection of the cells from oxidation.

Secondary metabolites and stress defense

Although most of the secondary metabolites decrease during early drought stress in *Haberlea*, a few of them increase. The decrease in most of the secondary metabolites may reflect the metabolic shift from normal growth to cell defense, when all energy and metabolic resources are directed towards the synthesis of a small number of protective molecules such as particular sugars and selected secondary metabolites. The polyamine spermidine is not detectable under normal conditions but accumulates massively during drought and desiccation. Accumulation of polyamines correlates with drought tolerance and in *C. plantagineum* the levels of polyamines were reported to be higher than those reported in *Arabidopsis* after drought treatment [63].

Sugar metabolism and desiccation tolerance

In addition to oxidative stress, severe drought and desiccation impose a number of other stresses, most notably metabolic and mechanical. Carbohydrate metabolism plays a central role in the protective mechanisms, as evidenced by the massive accumulation of sucrose during drought and

especially desiccation. The sucrose levels in desiccated samples could enable protection by osmotic adjustment, water replacement and vitrification. The main source of carbon for sucrose synthesis seems to be starch, as both the transcriptomic (induction of β-amylase) and the metabolomic (accumulation of maltose, concomitant with sucrose increase) data indicate extensive starch breakdown during drought and desiccation. This is in contrast to *C. plantagineum*, where the main carbon source for sucrose synthesis in leaves appears to be octulose [64]. Glucose and fructose are also rapidly converted to sucrose, as their levels sharply decline on water stress. A reduction in the abundance of glucose and other monosaccharides is also observed in other resurrection species and is believed to be linked to the increased production of sucrose and the decreased respiration and associated ROS production [65].

Raffinose-family oligosaccharides (RFOs) such as raffinose and stachyose, also accumulate during desiccation in many angiosperm resurrection plants and may have prominent roles protecting the cells by water replacement and vitrification [66]. Raffinose is synthesized by raffinose synthase from galactinol and sucrose [67]. It has recently been shown that both galactinol and raffinose can protect from paraquat-induced oxidative damage [67]. Interestingly, both galactinol synthases and raffinose synthases are induced by oxidative stress and this could be mediated by heat shock transcription factors in *Haberlea*. The two enzymes are concomitantly induced together with the heat shock transcription factor HsfA2 in wild-type *Arabidopsis* and furthermore, transgenic *Arabidopsis* overexpressing HsfA2 shows enhanced activities of the two enzymes as well as elevated levels of galactinol and raffinose [67]. The activation of many heat shock transcription factors in *H. rhodopensis* by drought stress is further supported by the induction of heat shock protein genes. Raffinose family oligosaccharides have also been speculated to be possible sources of carbon for sucrose synthesis [66]. The strong induction of stachyose synthase in water-deficient samples implies involvement of RFOs in the acquisition of tolerance against drought stress and desiccation, a notion supported by the accumulation of the RFO member verbascose. Recent studies of *Arabidopsis* have suggested that a combination of different sugars may constitute a highly redundant cryoprotective system [68, 69]. Given the importance of water homeostasis in frost tolerance [70], the accumulation of a large variety of sugars is likely to contribute to establishing a robust system to cope with osmotic stress, including desiccation.

The transcriptomic data also suggest changes in the biochemical composition of the cell wall, as several genes encoding xyloglucan endotransglucosylases, pectin esterases and pectate lyases are switched off during drought and desiccation and then switched on again upon rehydration. The notion of cell wall remodeling is further substantiated by the expression of a laccase gene presumably involved in lignin biosynthesis, which is exclusively expressed in desiccated samples. While the exact alterations in the cell wall remain undefined, the importance of cell wall remodeling and reversible changes in cell wall architecture in counteracting the mechanical stress that follows from dehydration has been shown for a number of species [6]. Biochemical changes in the plant cell wall polysaccharides and proteins take place, making the cell wall more flexible [71], thus enabling the morphological rolling and reversible folding of the leaves (Fig. 1) [72, 73]. These changes could be more specific for particular species and could involve incorporation of specific proteins in the cell wall as observed for the glycine-rich protein BhGRP1 in *Boea hygrometrica* [74], substitution of glucose residues with galactose residues in xyloglucan as observed in *Craterostigma wilmsii* [75], activation of expansins resulting in a more extendable cell wall in *C. plantagineum* [71], or arabinose-rich pectin polymers in *Myrothamnus flabellifolia* [72].

Induction of LEA and heat shock proteins may prevent protein denaturation and unfavorable molecular interactions

Several LEA and HSP genes are highly induced by drought and desiccation. Hydrophilins and LEA proteins in particular are ubiquitous proteins rapidly synthesized during desiccation in vegetative tissues and seeds of both desiccation-sensitive and resurrection plants [76, 77]. Many LEA genes express the most abundant transcripts in slow-dried and rehydrated (obtained from both rapid and slow drying) *Tortula ruralis*, a resurrection moss that can survive extreme desiccation [78, 79]. LEA proteins prevent unfavorable molecular interactions or biochemical activities, and macromolecular denaturation, acting to provide a water hydration 'shell' to target proteins and other macromolecules [80]. Several functional studies confirm a role for LEA proteins and dehydrins in drought and osmotic stress tolerance. A knockout mutant of the dehydrin gene *PpBHNA* in the moss *Physcomitrella patens* is impaired in its recovery from salt and osmotic stress treatment [81]. Transgenic tobacco plants expressing two foreign LEA genes, *BhLEA1* and *BhLEA2* from the dicot *Boea hygrometrica*, showed higher relative water content, increased PSII activity, decreased electrolyte leakage, and increased peroxidase and superoxide dismutase activities during drought stress [82]. In addition, novel types of hydrophilic proteins are expressed during desiccation in resurrection plants, such as CpEdi-9 in *C. plantagineum* [83]. The small heat shock proteins (HSPs) have similar properties but some of them also act as molecular

chaperones for other proteins, thus preventing proteins from aggregating and denaturing [6]. Both LEAs and HSPs are not normally expressed at high levels in hydrated vegetative tissues; however, they are found in seeds and in tissues experiencing water deficiency. Of note, we found some of these genes to be expressed in well-watered control samples in *Haberlea rhodopensis*, which is an exception and suggests that the transcriptome and proteome of this species may be primed to some extent for desiccation tolerance.

Drought-induced genes involved in acquired tolerance to heat shock, salinity, low temperature, and pathogen attack could provide cross-protection against a variety of abiotic and biotic stresses

The upregulation of heat shock transcription factors and many HSP genes by water deficiency indicates a possible cross-tolerance against high temperature stress. The 20-fold induced CBL-interacting serine/threonine-protein kinase has high similarity with the *Arabidopsis* CALCINEURIN B-LIKE10/SOS3-LIKE CALCIUM BINDING PROTEIN8 (SCABP8), a Ca^{2+}-interacting protein kinase that mediates salt stress tolerance in shoots via the salt overly sensitive (SOS) pathway [84, 85]. Salt induces CBL10 phosphorylation by SOS2, which stabilizes the CBL10-SOS2 interaction and enhances the activity of the Na^+/H^+ antiporter at the membrane [84]. Furthermore, the induction of many disease and pathogen-related genes and several genes encoding low temperature-induced proteins, lipocalins, and other chilling stress-associated proteins indicates tolerance against other abiotic and biotic stresses. Plant lipocalins are small ligand-binding proteins localized at the plasma membrane or in chloroplasts and their expression levels generally correlate with the plant's capacity to develop freezing tolerance [86]. Chloroplast-localized lipocalins, induced by various abiotic stresses, may protect against singlet oxygen, produced under excess light [87]. In *Thellungiella halophila*, ELIP, LEA, alcohol dehydrogenase, and sucrose synthases are among the top ten genes most induced by low temperature stress (Yang Ping Lee, Max-Planck Institute of Molecular Plant Physiology, Potsdam-Golm, Germany, pers. comm.). Homologues of all these genes are highly induced by drought in *Haberlea*. In nature, *H. rhodopensis* survives long harsh winters and subzero temperatures, preceded by periods of drought. We found that desiccated *H. rhodopensis* plants can withstand −20 °C (data not shown). These findings indicate that the freezing tolerance of *H. rhodopensis* observed in nature could be facilitated by dehydration and activation of a stress-protective genetic program.

Comparison of drought/desiccation- and rehydration-induced molecular responses in *Haberlea rhodopensis* with the transcriptomes/metabolomes of *Craterostigma plantagineum* and *Sporobolus stapfianus*

Induction of many LEAs, sucrose synthase, pathogenesis-related and other stress-related genes by dehydration are common features of both *H. rhodopensis* and *C. plantagineum* transcriptomes. On the other hand, the induction of ELIPs, galactinol synthases, stachyose synthase, several signaling compounds, and many transcription factors observed in *H. rhodopensis* is not that evident in *C. plantagineum*. In subsequent studies, special emphasis could be paid on the analysis of the transcription factors exclusively expressed in water-deficient *H. rhodopensis* as well as on the un-annotated *Haberlea* genes.

Comparative analysis of the fully hydrated (unstressed) and dehydrated metabolomes of *H. rhodopensis* and *S. stapfianus* revealed both similarities and differences. Unfortunately, however, the metabolome of rehydrated *S. stapfianus* was not determined [16], so that a comparison of that stage is currently not possible. The aromatic amino acids phenylalanine and tyrosine increased in both species, indicating the phenylpropanoid pathway as a common player in drought and desiccation responses. However, most of the other amino acids showed different accumulation patterns. Both species accumulated sucrose and stachyose. Unlike *Haberlea, Sporobolus* accumulated raffinose as well, but verbascose was not determined in this species. GABA synthesis appeared to be induced at the latest stages of desiccation in *Sporobolus*, in a manner similar to *Haberlea*, corroborating a role for this metabolite in drought signaling/protection. Both species accumulate threonate, a breakdown product of ascorbate, during dehydration. On the other hand, the secondary metabolites of *Haberlea* appear to be different and furthermore most of them are repressed by drought in *Haberlea*, in contrast to *S. stapfianus*.

Conclusions

Desiccation tolerance is an intricate, highly complex phenomenon that allows resurrection plants to survive extreme loss of cellular water. *H. rhodopensis* survives periods of drought that are unusually long, even for resurrection plants. The integrated transcriptome and metabolome approach reported here demonstrates that both inducible and constitutive mechanisms contribute to the desiccation tolerance in this unique glacial relic (Fig. 6). The constitutive expression of several dehydrins and HSPs as well as the high levels of sucrose, raffinose, GABA and other stress-related metabolites distinguishes *H. rhodopensis* from drought-sensitive species such as *Arabidopsis*

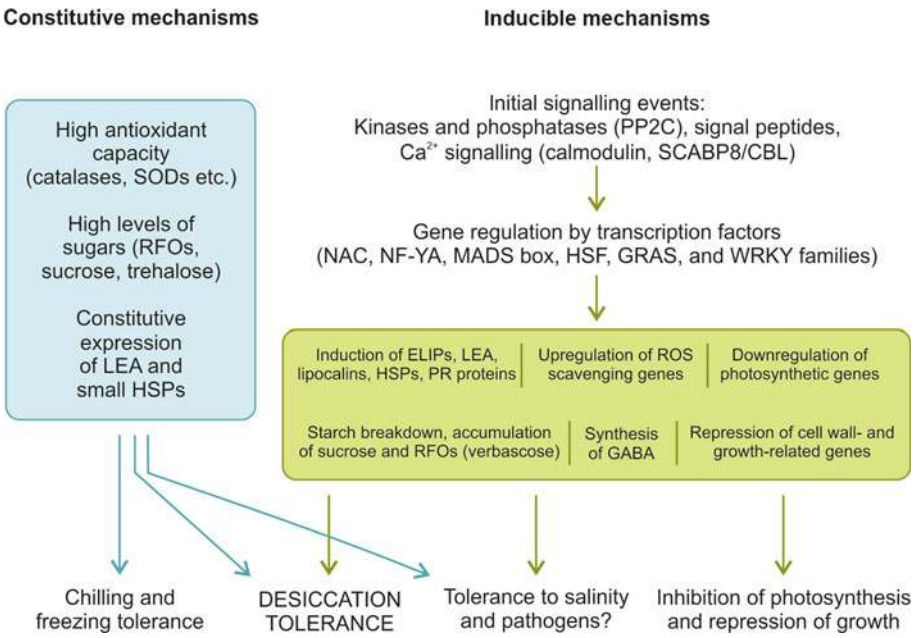

Fig. 6 A unifying model based on the physiological, transcriptomic, and metabolome data representing the collective actions of different mechanisms that contribute to the establishment of desiccation tolerance and other drought-related biological effects in *Haberlea rhodopensis*

thaliana and collectively indicate that the transcriptome and metabolome of *H. rhodopensis* are primed for drought-stress tolerance. Additionally, further stress protective mechanisms are rapidly induced upon sensing drought stress. These mechanisms include the upregulation of ROS-scavenging genes, further accumulation of sucrose and verbascose, and expression of new LEA and heat shock protein genes. This is concomitant with cell wall remodeling, growth cessation, and inhibition of photosynthesis but at the same time preservation of chlorophyll and the photosynthetic machinery. Complex morphological (leaf folding), biochemical, and molecular (massive induction of ELIPs, lipocalins) mechanisms further assist in preventing photooxidative damage. At a molecular level, these changes are regulated by massive transcriptional reprogramming executed by specific transcription factors and coregulators (NAC, NF-YA5, MADS box, HSF, GRAS, WRKY, and others). The highly regulated genes with unknown functions and no sequence homology to genes from other species are a potential source for gene discovery as they may contain novel players involved in drought stress tolerance. The fundamental knowledge gained from understanding the molecular mechanisms of desiccation tolerance in *H. rhodopensis* may lead to improving drought tolerance in important crop species.

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