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Global Transcriptome Analysis Reveals Acclimation-Primed Processes Involved in the Acquisition of Desiccation Tolerance in *Boea hygrometrica*

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Boea hygrometrica **resurrection plants require a period of acclimation by slow soil-drying in order to survive a subsequent period of rapid desiccation. The molecular basis of this observation was investigated by comparing gene expression profiles under different degrees of water deprivation. Transcripts were clustered according to the expression profiles in plants that were air-dried (rapid desiccation), soil-dried (gradual desiccation), rehydrated (acclimated) and air-dried after acclimation. Although phenotypically indistinguishable, it was shown by principal component analysis that the gene expression profiles in rehydrated, acclimated plants resemble those of desiccated plants more closely than those of hydrated acclimated plants. Enrichment analysis based on gene ontology was performed to deconvolute the processes that accompanied desiccation tolerance. Transcripts associated with autophagy and α-tocopherol accumulation were found to be activated in both air-dried, acclimated plants and soil-dried non-acclimated plants. Furthermore, transcripts associated with biosynthesis of ascorbic acid, cell wall catabolism, chaperone-assisted protein folding, respiration and macromolecule catabolism were activated and maintained during soil-drying and rehydration. Based on these findings, we hypothesize that activation of these processes leads to the establishment of an optimal physiological and cellular state that enables tolerance during rapid air-drying. Our study provides a novel insight into the transcriptional regulation of critical priming responses to enable survival following rapid dehydration in *B. hygrometrica*.**

Keywords: Acclimation • Autophagy • *Boea hygrometrica* • Desiccation tolerance • Gene expression • α-Tocopherol.

Abbreviations: DAB, diaminobenzidine; ER, endoplasmic reticulum; EST, expressed sequence tag; F_v/F_m, potential PSII photochemical efficiency; GEO, Gene Expression Omnibus; GO, Gene Ontology; JA, jasmonic acid; KEGG, Kyoto Encyclopedia of Genes and Genomes; MDC, monodansylcadaverine; NBT, nitro blue tetrazolium; PCA, principal component analysis; PI, propidium iodide; ROS, reactive oxygen species; RT-PCR, real-time PCR; RWC, relative water content.

Sequence data from this article can be found in the Short Read Archive database at NCBI under accession no. SRR1040631 (experiment accession No. SRX385264). The complete array data have been deposited in the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession No. GSE53058.

Introduction

Desiccation tolerance is the ability to survive the loss of almost all free water in cells when in equilibrium with moderately dry air and to resume normal function when rehydrated (Proctor and Pence 2002). This phenomenon is observed in seeds, some pollen grains and a small group of so-called resurrection plants (Sun and Leopold 1997). Studies have revealed complex activation of genes and accumulation of metabolites related to processes such as osmotic regulation and cell wall remodeling (Rodriguez et al. 2010, Oliver et al. 2011, Georgieva et al. 2012, Yobi et al. 2012, Gechev et al. 2013).

From an evolutionary perspective, desiccation tolerance is critically important as primitive terrestrial forms colonized dry land environments. In the plant kingdom, many bryophytes and a limited number of vascular plants possess such an adaptive trait (Gaff and Oliver 2013). The acquisition of systems for water conservation within vascular species led to the loss of desiccation tolerance in vegetative organs, and genes involved in desiccation tolerance were recruited for activation of drying responses in reproductive structures (e.g. spores, pollen and seed) under the control of plant developmental programs. Resurrection plants are hypothesized to have re-evolved vegetative desiccation tolerance based on inducible responses to environmental cues (Bartels and Salamini 2001, Gaff and Oliver 2013). Unlike bryophytes, most vascular species can

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survive desiccation only if the drying rate is gradual (Oliver and Bewley 1997). With the exception of *Chamaegigas intrepidus*, angiosperm resurrection plants require dehydration times of the order of days to enable functional recovery following rehydration (Heilmeier and Hartung 2011). Given that tolerance in vascular resurrection plants relies predominantly on cell protection during dehydration, an understanding of the sensitivity to the rate of water loss provides a valuable insight into mechanisms that are critical for desiccation tolerance.

Boea hygrometrica is a small perennial dicotyledonous plant and is widespread in East Asia, inhabiting shallow rock crevices, and survives winter in a desiccated state (Mitra et al. 2013). Of the angiosperm resurrection plants, *B. hygrometrica* is one of the more extensively studied model systems. Proteome, transcriptome and genomic approaches have identified protective mechanisms related to photosynthesis, cell wall folding and genome stability (Jiang et al. 2007, L. Wang et al. 2009, Zhao et al. 2014). *Boea hygrometrica* is propagated mainly via seeds, which is rare among other resurrection plant species within the Gesneriaceae, such as *Haberlea rhodopensis* and *Ramonda myconi* (Xavier Pico and Riba 2002, Bogacheva-Milkoteva et al. 2013). Unlike *H. rhodopensis* and *R. myconi*, both young and perennial *B. hygrometrica* plants are found in nature during the wet season (**Supplementary Fig. S1**). In contrast, young plants are seldom found in the natural populations during the dry season. Previously we found that plants collected from native habitats are predominantly able to tolerate air-drying under controlled conditions (Jiang et al. 2007). We hypothesize that plants acquire tolerance to rapid drying during preceding, gradual drought period(s) that frequently occur in nature. To test the conditions required for desiccation tolerance, *B. hygrometrica* seeds were collected in Beijing and cultivated under well-watered greenhouse conditions. These plants lost viability upon air-drying but recovered upon rehydration when subjected to gradual dehydration (>90% water loss after 12–14 d of soil-drying by withdrawing irrigation). Furthermore, the dehydration–rehydration treatment enabled plants to tolerate a subsequent period of rapid air-drying. Based on these observations, a global assessment of transcriptome changes was conducted under acclimated and non-acclimated conditions to identify biological processes that are critical to enable tolerance to rapid drying.

Results

Boea hygrometrica acquires tolerance to rapid drying after a cycle of acclimation

To investigate the conditions that enable *B. hygrometrica* to acquire air-drying tolerance, seedlings grown in well-watered greenhouse conditions were subjected to air-drying with or without a preceding period of gradual dehydration. Electrolyte leakage and PSII activity were monitored to assess plant viability, and the results are shown in **Fig. 1**. Gradual dehydration was achieved by withdrawing irrigation. Leaf wilting was initially observed [relative water content (RWC) reduced to ~80%], with leaf shrinkage appearing after 5 d, and finally leaf RWC dropped to <10% after 12–14 d. These plants were able to recover fully within 3 d after rehydration, as evidenced by restored vigor and leaf RWC, PSII activity (F_v/F_m) and low electrolyte leakage. Three-day-rehydrated plants were removed from soil and subjected to air-drying in parallel with hydrated, non-acclimated plants. Desiccation tolerance was only observed in acclimated plants (**Fig. 1**). Both acclimated and non-acclimated plants lost water and showed decreased PSII activity at similar rates. Leaf RWC was lowered to approximately 80% within 2 h (equivalent to soil-drying for 5 d) and to approximately 10% within 48 h (equivalent to soil-drying for 14 d). This finding provided a foundation for identifying critical time points in responses that enable tolerance to rapid drying.

Transcriptome sequencing and assembly

To enable transcriptome analysis, 454 pyrosequencing of a normalized cDNA pool comprised of samples representing critical time points during soil-drying and air-drying states was conducted (**Fig. 1**). A total of 411,788 sequences were generated [National Center for Biotechology Information (NCBI) Sequence Read Archive accession SRR1040631], ranging from 40 to 805 bp. The total sequence length was 159 Mb with average size of approximately 386 bp; the length distribution of reads is shown in **Supplementary Fig. S2a**. Sequences were processed and assembled into 38,290 unique sequences, including 21,989 isotigs ranging from 100 to 4,981 bp with N50 of 951 bp, and 16,301 singlets, of which 11,663 were longer than 400 bp (**Supplementary Fig. S2b**).

The isotigs and singlets were combined and clustered to generate unigenes that represented clusters of genes with >95% sequence identity. In total, 36,365 unigenes were obtained (**Supplementary Fig. S2c**), of which 24,230 (66.6%) unigenes were assigned gene descriptions on the basis of a BLASTX search (E-value <1e-5) against public databases. The proportion of annotated *B. hygrometrica* unigenes was determined, with matches detected to sequences from *Vitis vinifera*, castor bean (*Ricinus communis*), black cottonwood (*Populus trichocarpa*), *Glycine max* and *Medicago truncatula* (**Supplementary Fig. S2d**). The species distribution was similar to that reported for *Craterostigma plantagineum* (Rodriguez et al. 2010). In addition, 75.7% and 76.1% of the unigenes shared significant homology with sequences from other African and European resurrection plants, namely *Streptocarpus rexii* and *H. rhodopensis*, respectively (Chiara et al. 2013, Gechev et al. 2013).

To obtain an overview of gene function, the assembled unigenes were further compared with those for Arabidopsis proteins using BLASTX and categorized according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) databases. A total of 15.1% of the unigenes were annotated by KEGG databases and 43.7% by the GO categories for biological processes, cell components and molecular function (**Supplementary Fig. S2e**).

Transcript profiling of dehydrated and rehydrated plants

Hybridization with RNA from samples representing critical time points during soil-drying and air-drying of non-acclimated and

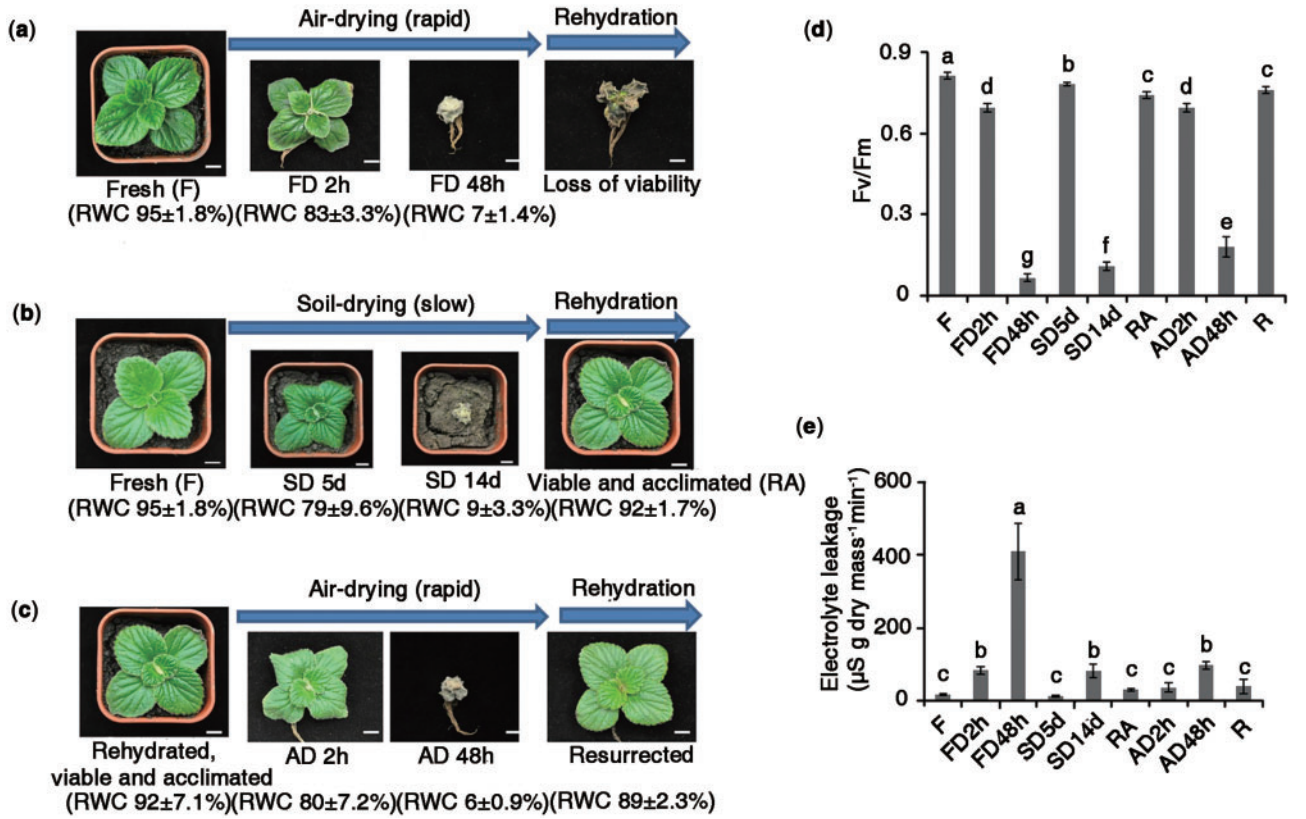

Fig. 1 Physiological analysis of untreated, dehydrated and rehydrated *B. hygrometrica* plants. (a) Fresh plants of *B. hygrometrica* lost viability when dehydrated by air-drying and failed to resurrect after rehydration. (b) Fresh plants of *B. hygrometrica* survived desiccation when irrigation of the pot was slowly withdrawn, recovered upon rehydration and became acclimated. (c) Rehydrated and acclimated plants tolerated air-drying and resurrected upon rehydration. Plants were sampled after air-drying for 2 and 48 h and after soil-drying for 5 and 14 d as indicated. Scale bar = 1 cm. The relative water content (RWC, %) of each sample is indicated under the corresponding plant image. (d, e), F_v/F_m (d) and electrolyte leakage (e) of *B. hygrometrica* shown in (a–c). The values are the mean of three independent experiments. A total of 3–5 plants per treatment in each were measured. The bars indicate the standard deviation. The different letters marked above the bars denote significant difference among the values following a one-way ANOVA test ($P < 0.05$).

acclimated plants [NCBI Gene Expression Omnibus (GEO) accession GSE53058] revealed 6,999 (58.4%) dehydration- and rehydration-responsive transcripts with at least 2-fold ($\log2 \geq 1$ or $\log2 \leq -1$) differences from the levels in well-watered plants. Among them, 1,937, 2,555 and 1,912 transcripts were induced, while 2,197, 2,504 and 2,341 transcripts were repressed in air-dried non-acclimated plants, in soil-dried non-acclimated plants and in air-dried acclimated plants, respectively (**Fig. 2a**). Dehydration-inducible expression patterns of positive controls, i.e. genes that were previously characterized to be dehydration inducible, were confirmed. These genes, including *BhGolS1*, *BhHsf1*, *BhLEA1* and *BhLEA2* (Liu et al. 2009, Z. Wang et al. 2009, Zhu et al. 2009), were up-regulated, not only during air-drying of acclimated plants but also during air-drying and soil-drying of non-acclimated plants. The expression patterns of these genes were further confirmed using quantitative real-time PCR (RT-PCR) (**Supplementary Fig. S3**).

In order to evaluate the transcript profiling data, principal component analysis (PCA) was applied to capture the overall variance among the samples in three dimensions (**Fig. 2b**). PCA illustrated an unexpected finding that the gene expression profiles in rehydrated, acclimated plants more closely resemble those of desiccated plants rather than hydrated, non-acclimated plants.

Enrichment analysis of GO and KEGG annotated expressed sequence tags (ESTs) between hydrated, non-acclimated and rehydrated, acclimated plants

Enrichment analysis according to GO annotation was used to compare biological processes that were differentially regulated between hydrated non-acclimated and rehydrated acclimated plants at the transcript level. In total 478 and 407 probes exhibited significantly higher and lower expression levels in rehydrated acclimated plants, respectively. Transcripts encoding peroxidases, cinnamyl alcohol dehydrogenase, pectin lyases, pectin esterases, dirigent proteins, chitinases, β-1,3-glucanase, α-galactosidase, endoglucanase and cellulase were induced in rehydrated acclimated plants, indicating cell wall loosening during rehydration. In agreement with this finding, cell wall catabolism was enriched among the up-regulated transcripts and cell wall organization was enriched among the down-regulated transcripts.

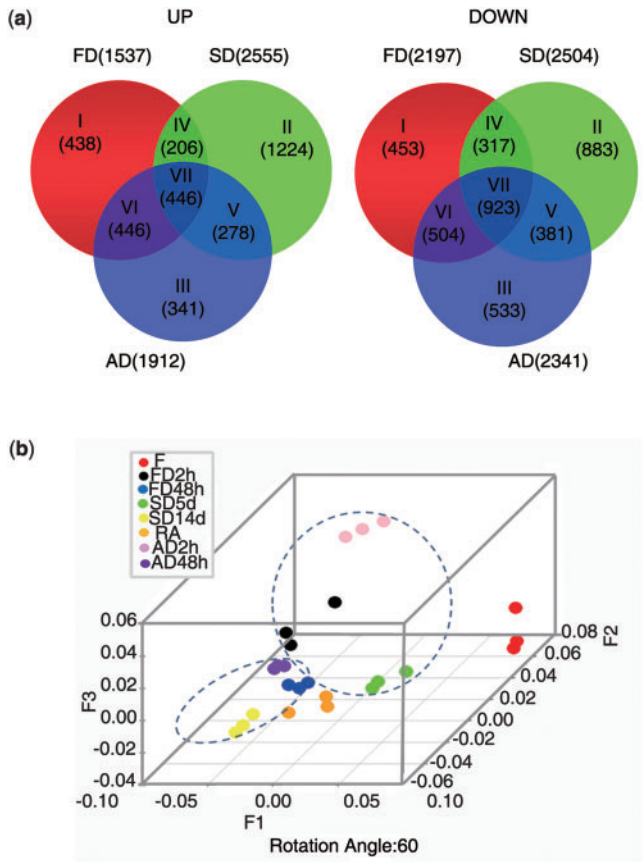

Fig. 2 Venn diagrams showing the distribution of specific and shared responses [induced (>1 fold) and repressed (<−1 fold)] during eight different treatments, and principal component analysis (PCA) of transcript profiles. (a) Venn diagrams. (b) PCA plot. F, hydrated non-acclimated plant; FD, air-drying of non-acclimated plants; SD, soil-drying of non-acclimated plants; RA, acclimated plants; AD, air-drying of acclimated plants. Numbers in parentheses correspond to unigenes identified in a specific cluster.

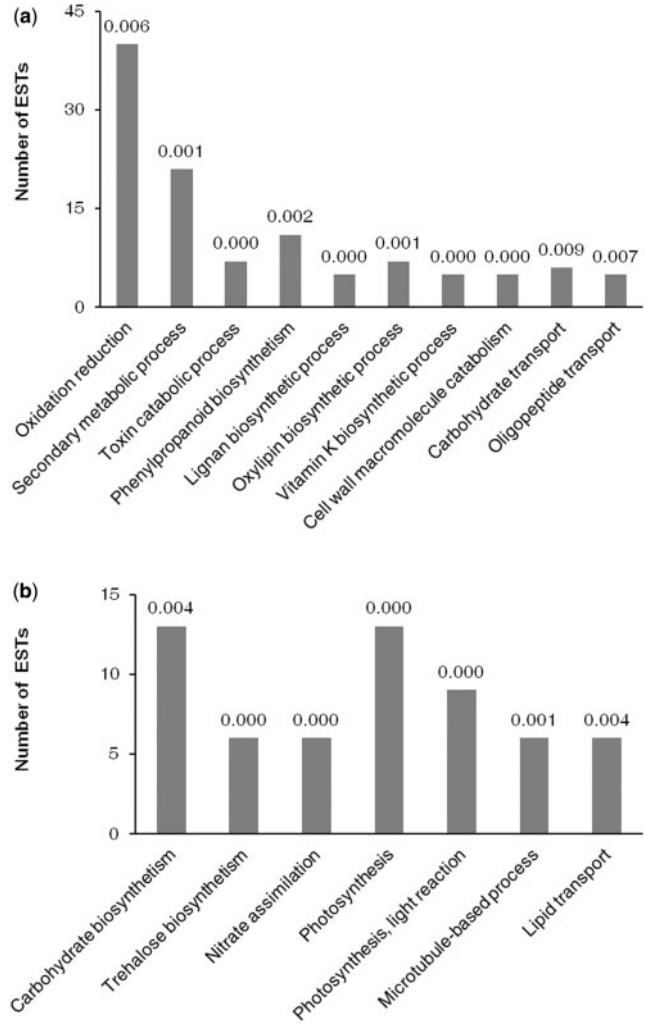

Fig. 3 Gene ontology-enriched categories among acclimation-induced and repressed transcripts. The *P*-value for each category is illustrated above each bar. (a) Processes enriched among acclimation-induced transcripts. (b) Processes enriched among acclimation-repressed transcripts.

GO enrichment analysis revealed that stress and hormone responses, photosynthesis (especially light reaction), nitrate assimilation, biosynthesis of carbohydrate (especially trehalose) and lipid transport were enriched among the down-regulated transcripts, consistent with the retained level of PSII efficiency in rehydrated acclimated plants. Oxidation reduction, secondary metabolism, vitamin biosynthesis, cell wall macromolecule catabolism, carbohydrate and oligopeptide transport categories were enriched among the up-regulated transcripts (**Fig. 3**), indicating that rehydrated acclimated plants had undergone complex transcriptional changes during acclimation.

GO enrichment analysis and KEGG annotation of transcripts responding to air-drying

GO enrichment analysis and KEGG annotation of transcripts were performed to identify processes that accompany air-drying in non-acclimated and acclimated plants. Programmed cell death, protein and amino acid metabolic processes, including biosynthesis of amino acid derivatives, transport of amino acids and protein ubiquitination, were specifically enriched among transcripts that were induced in air-dried non-acclimated plants. They were, however, not enriched among air-drying-induced transcripts in acclimated plants. Similarly, chromatin assembly, gene expression, translation, ribosome biogenesis, carotenoid biosynthesis, lipid transport, generation of precursor metabolites and energy, electron transport chain and photospiration were specifically enriched in air-drying-repressed transcripts in non-acclimated plants, but were not among repressed transcripts in acclimated plants ($P < 0.05$; **Fig. 4a, b**). KEGG pathway enrichment was largely consistent with GO terms, with the addition of endocytosis and ascorbate and aldarate metabolism among up-regulated transcripts (data not shown). These changes indicate that air-drying triggered the activation of ubiquitin-mediated proteolysis and cell death, and the deceleration of energy supply, and carbon and nitrogen assimilation in non-acclimated plants. In addition, processes including biosynthesis of α-tocopherol (vitamin E) and L-ascorbic acid (vitamin C), the ABA metabolic process and

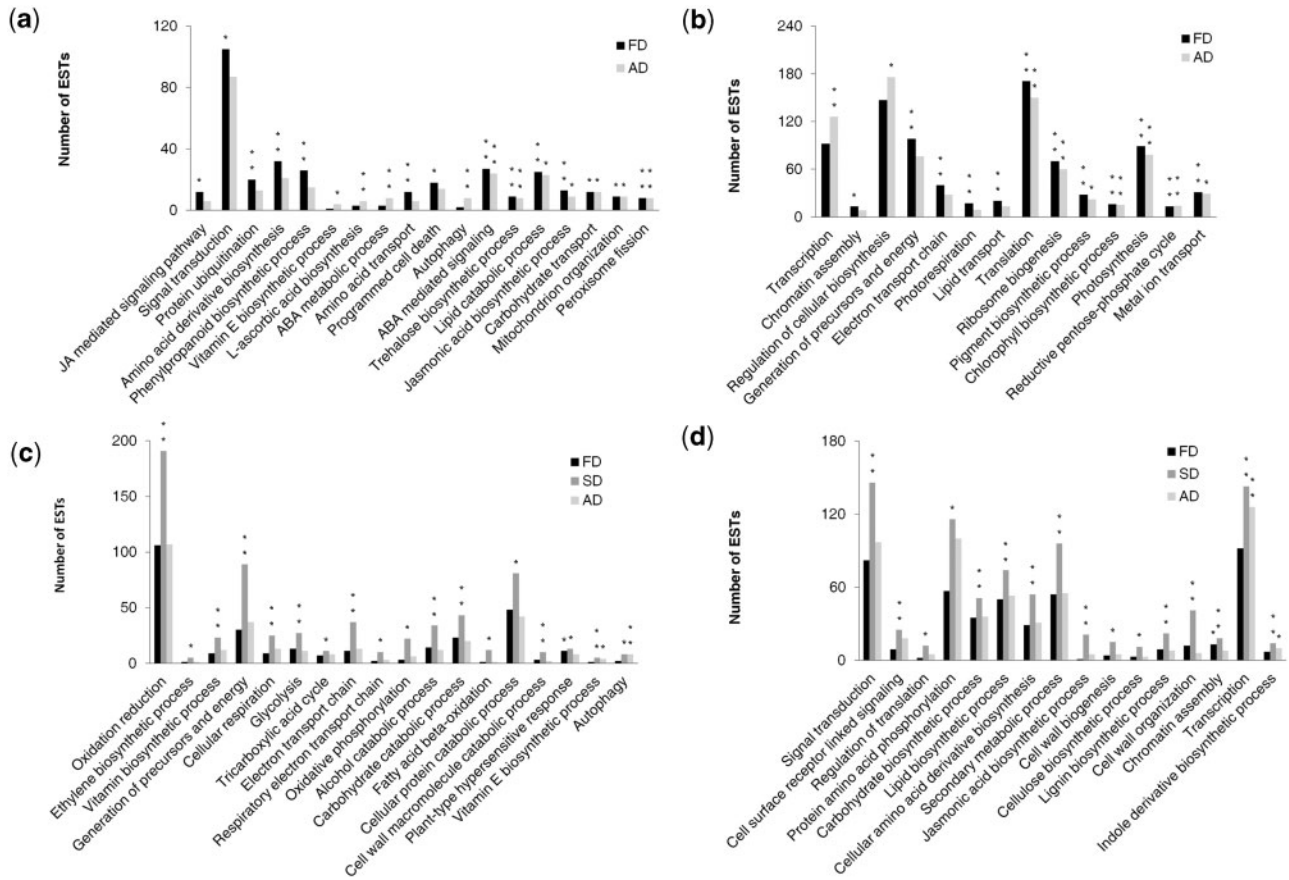

Fig. 4 Gene ontology-enriched categories for biological processes among the transcripts induced or repressed by dehydration. (a) Processes enriched among air-drying-induced transcripts in non-acclimated and acclimated plants. (b) Processes enriched among air-drying-repressed transcripts in non-acclimated and acclimated plants. (c) Processes enriched among soil-drying-induced transcripts in non-acclimated plants. (d) Processes enriched among soil-drying-repressed transcripts in non-acclimated plants. FD, air-drying of non-acclimated plants; SD, soil-drying of non-acclimated plants; AD, air-drying of acclimated plants. $P < 0.05$ or $P < 0.01$ is indicated as * or **, respectively.

autophagy were only enriched among up-regulated transcripts in air-dried acclimated plants; while the response to hormone stimuli (especially to ethylene and salicylic acid), regulation of gene expression and transcription, regulation of cellular biosynthesis and the primary metabolic process were categories enriched among down-regulated transcripts ($P < 0.05$, **Fig. 4a, b**). These findings indicate that air-drying triggers gene expression in non-acclimated vs. acclimated plants differentially.

GO enrichment analysis and KEGG annotation of transcripts responding to soil-drying

Comparison of soil-drying- and air-drying-triggered transcriptome changes in non-acclimated plants led to identification of an overlapping set of GO terms and KEGG pathways. For example, to mitochondrion organization and peroxisome fission were observed among the up-regulated transcripts, and chromatin assembly, Chl biosynthesis and photosynthesis were among the down-regulated transcripts (**Fig. 4c, d**). GO terms and KEGG pathways that were differentially regulated by rapid and gradual dehydration were also identified. For example, in the response to hormone [especially ABA, jasmonic acid (JA) and ethylene], signal transduction, biosynthesis of JA and trehalose were enriched among transcripts activated during air-drying but repressed during soil-drying, while generation of precursors and energy, and electron transport chain activity were enriched among transcripts activated during soil-drying but repressed during air-drying.

Comparison between soil-dried non-acclimated plants and air-dried acclimated plants also revealed specific changes. Overlapping terms were found among differentially regulated transcripts (**Fig. 4c, d**). Autophagy and vitamin (ascorbic acid and α-tocopherol) biosynthesis were not enriched among the induced transcripts in air-dried non-acclimated plants, which suggests that specific reactive oxygen species (ROS)-scavenging agents and autophagy may play important roles in the acquisition of desiccation tolerance (**Fig. 4c**). GO terms related to transcription were enriched among repressed transcripts in both soil-dried non-acclimated plants and air-dried acclimated plants, but not in air-dried non-acclimated plants (**Fig. 4d**). This finding supports the importance of transcriptional regulation of desiccation tolerance. In contrast, GO terms related to chromatin assembly were enriched among repressed transcripts in both soil-dried non-acclimated and air-dried non-acclimated plants, but not in air-dried acclimated plants. Such differences point to different gene regulation mechanisms in acclimated and non-acclimated plants.

Comparison of enriched GO terms also identified processes that were uniquely enriched upon soil-drying, namely vitamin biosynthesis, ethylene biosynthetic process, generation of precursors and energy, and cellular respiration (including glycolysis, the tricarboxylic acid cycle, electron transport chain, oxidative phosphorylation and ATP synthesis, the alcohol catabolic process and the pentose phosphate pathway), oxidation reduction, iron–sulfur cluster assembly, fatty acid β-oxidation, the protein catabolic process and cell wall macromolecule catabolism (**Fig. 4c**). Transcripts related to cell wall loosening and oxidative reduction were also enriched among induced transcripts in rehydrated, acclimated plants.

Gene ontology clustering by expression signatures

A Venn diagram tool was used to compare gene expression in air-dried and soil-dried non-acclimated and acclimated plants and, in turn, to identify genes that were common in two or more conditions. Transcripts were grouped into seven clusters: specifically up- and down- regulated in air-dried non-acclimated plants (cluster I), soil-dried non-acclimated plants (cluster II) and air-dried acclimated plants (cluster III), transcripts that were regulated similarly during air-drying and soil-drying in non-acclimated plants (cluster IV), during soil-drying of non-acclimated plants and air-drying of acclimated plants (cluster V), during air-drying in non-acclimated and acclimated plants (cluster VI) and across all three types of dehydrated plants (cluster VII) (**Fig. 2a**). GO enrichment was also performed to characterize each cluster. Using this approach, protein folding was shown to be enriched in cluster I down-regulated transcripts, and in cluster III and VI up-regulated transcripts, indicating that protein quality control was repressed in air-dried non-acclimated plants and activated in soil-dried non-acclimated plants and air-dried acclimated plants. Translation and ribosome biogenesis GO terms were enriched from cluster VII (down-regulated transcripts), indicating a global decrease in protein biosynthesis in response to drying.

Redox homeostasis and antioxidant accumulation

Oxidation reduction and vitamin antioxidant biosynthesis GO terms were enriched among the induced transcripts in soil-dried non-acclimated plants and air-dried acclimated plants (**Fig. 4**). This supports a role for alterations in redox homeostasis and elevation of antioxidants in desiccation tolerance. Nitro blue tetrazolium (NBT) and diaminobenzidine (DAB) staining confirmed the low levels of the O_2^- radical and H_2O_2 in the acclimated plants that were hydrated and air-dried, in contrast to the intense staining observed in air-dried, non-acclimated plants (**Fig. 5a, b**).

Both ROS-scavenging enzymes and accumulation of antioxidants have been detected in *Craterostigma plantagineum*, *Sporobolus stapfianus* and *Selaginella lepidophylla* (Rodriguez et al. 2010, Oliver et al. 2011, Yobi et al. 2012) and are implicated in decreasing ROS, lipid peroxidation and ion leakage

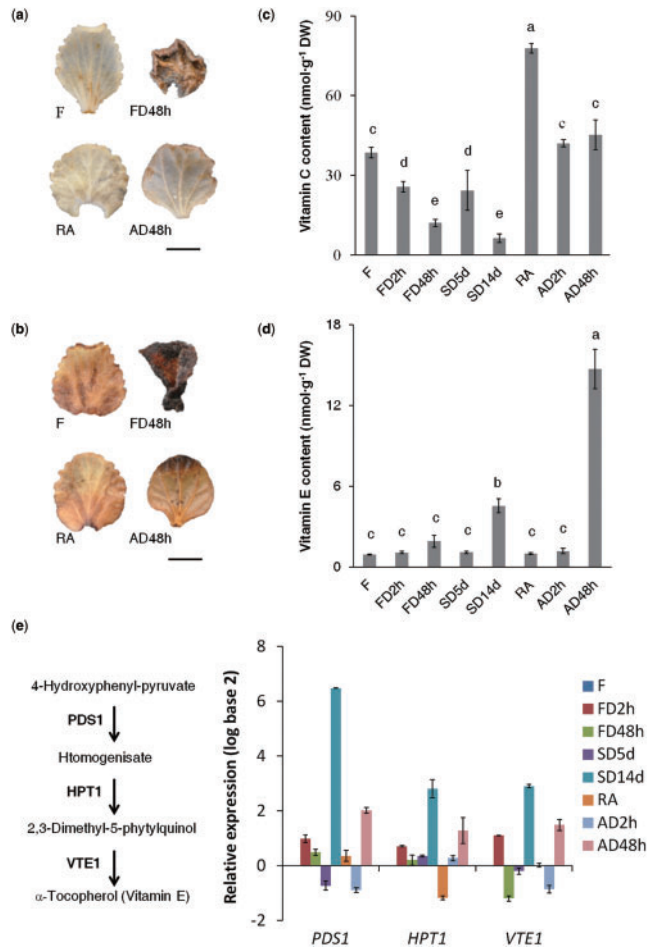

Fig. 5 ROS staining, contents of L-ascorbic acid and α-tocopherol, and expression of key genes in α-tocopherol biosynthesis in *B. hygrometrica* leaves. (a) Detection of H_2O_2 by NBT staining. (b). Detection of O_2^- levels by DAB staining. (c) Content of L-ascorbic acid. (d) Content of α-tocopherol. (e) Schematic plot of the key genes in α-tocopherol biosynthesis and RT-PCR analysis of the expression of the indicated genes. Three-month-old non-acclimated and acclimated plants were subjected to desiccation for up to 48 h. A total of 3–5 plants per treatment in each were measured. F, hydrated non-acclimated plant; FD, air-drying of non-acclimated plants; SD, soil-drying of non-acclimated plants; RA, acclimated plants; AD, air-drying of acclimated plants. *PDS1*, *phytoene desaturation 1*; *HPT1*, *homogentisate phytyltransferase 1*; *VTE1*, *tocopherol O-methyltransferase 1*.

(Abbasi et al. 2007, Jin and Daniell 2014). ROS scavenging enzymes, including glutathione peroxidase, glutathione *S*-transferase and polyphenol oxidase, were found to be increased during dehydration in *B. hygrometrica* (Jiang et al. 2007). In this study, L-ascorbic acid increased in rehydrated, acclimated plants and remained at a high level during subsequent air-drying. In contrast, α-tocopherol accumulation correlated closely with water status, with the highest levels observed in dried tissues (**Fig. 5c, d**). RT-PCR confirmed that key enzymes catalyzing α-tocopherol biosynthesis in plants were highly expressed in soil-dried non-acclimated plants and air-dried acclimated plants, but were unchanged or repressed in air-dried non-acclimated plants (**Fig. 5e**).

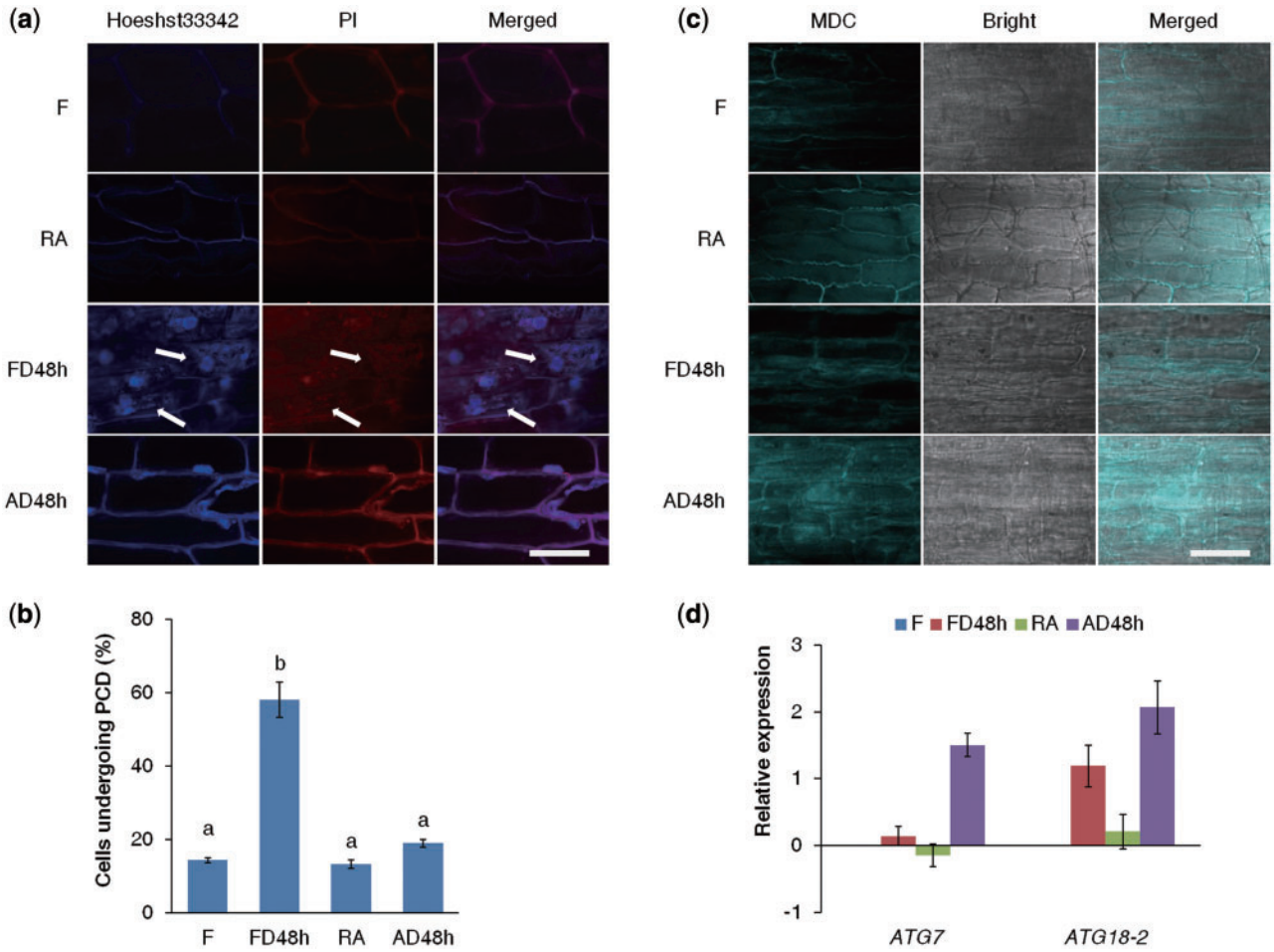

Fig. 6 Detection of cell death and autophagy during air-drying in *B. hygrometrica*. (a) Cell death. (b) Percentages of dying cells. (c) Autophagy. (d) RT-PCR analysis of the expression of representative autophagy-related genes. Roots of 3-month-old plants were co-stained with Hoechst 33342 and PI for cell death and with MDC for autophagy. Fluorescence was observed using a multiphoton laser scanning microscope. The percentage of cells undergoing apoptosis relative to the total cell number was estimated from three different samples, each of which contained at least 30 cells. Statistical significance was determined with Student's *t*-test ($P < 0.05$), and is labeled with lower case letters. F, hydrated non-acclimated plant; FD, air-drying of non-acclimated plants; RA, acclimated plants; AD, air-drying of acclimated plants. Scale bar = 45 μm. Arrows indicate dead cells. *ATG7*, *Autophagy-related protein 7*; *ATG18-2*, *Autophagy-related protein 18-2*.

Cell death and anti-cell death

GO enrichment analysis indicated that cell death was induced in air-dried non-acclimated plants, but not in soil-dried non-acclimated plants or air-dried acclimated plants (Fig. 4). To estimate cell death in the context of rapid desiccation tolerance, cell viability and nuclear morphology in air-dried non-acclimated and acclimated plants were compared by Hoechst 33342 (a fluorescent DNA-binding dye for detecting nuclear morphology) and propidium iodide (PI; a nuclear dye that is membrane impermeable and generally excluded from viable cells) co-staining assays. Faint red fluorescence was observed in nuclei in hydrated non-acclimated plants, rehydrated acclimated plants and air-dried acclimated *B. hygrometrica* plants, whereas more intense staining was observed in nuclei from air-dried non-acclimated plants (Fig. 6a). A total of 58.1% of cells were non-viable in air-dried non-acclimated plants, compared with 18.9% in air-dried acclimated plants (Fig. 6b). Together, these results confirmed that cell death was mitigated in desiccation-tolerant plants but not in sensitive plants upon air-drying.

Autophagy is a major form of programmed cell death and simultaneously plays an important role in anti-cell death (Patel et al. 2006). In *B. hygrometrica*, autophagy was enriched among the induced transcripts in soil-dried non-acclimated plants and air-dried acclimated plants (Fig. 4). Monodansylcadaverine (MDC; an autofluorescent amine that specifically stains autophagosomes) staining was conducted to study autophagy further under various treatments. After air-drying, cells in non-acclimated tissues shrank with decreased fluorescence signals, while cells in acclimated tissues retained morphology with enhanced fluorescence signals (Fig. 6c). Two key genes of autophagy, *ATG7* and *ATG18-2*, were also shown to accumulate in acclimated plants in response to air-drying (Fig. 6d). These data support that autophagy is activated specifically in desiccation-tolerant plants.

Protein homeostasis and quality control

Protein homeostasis is central to normal cellular function, which is often disturbed under abiotic stress conditions

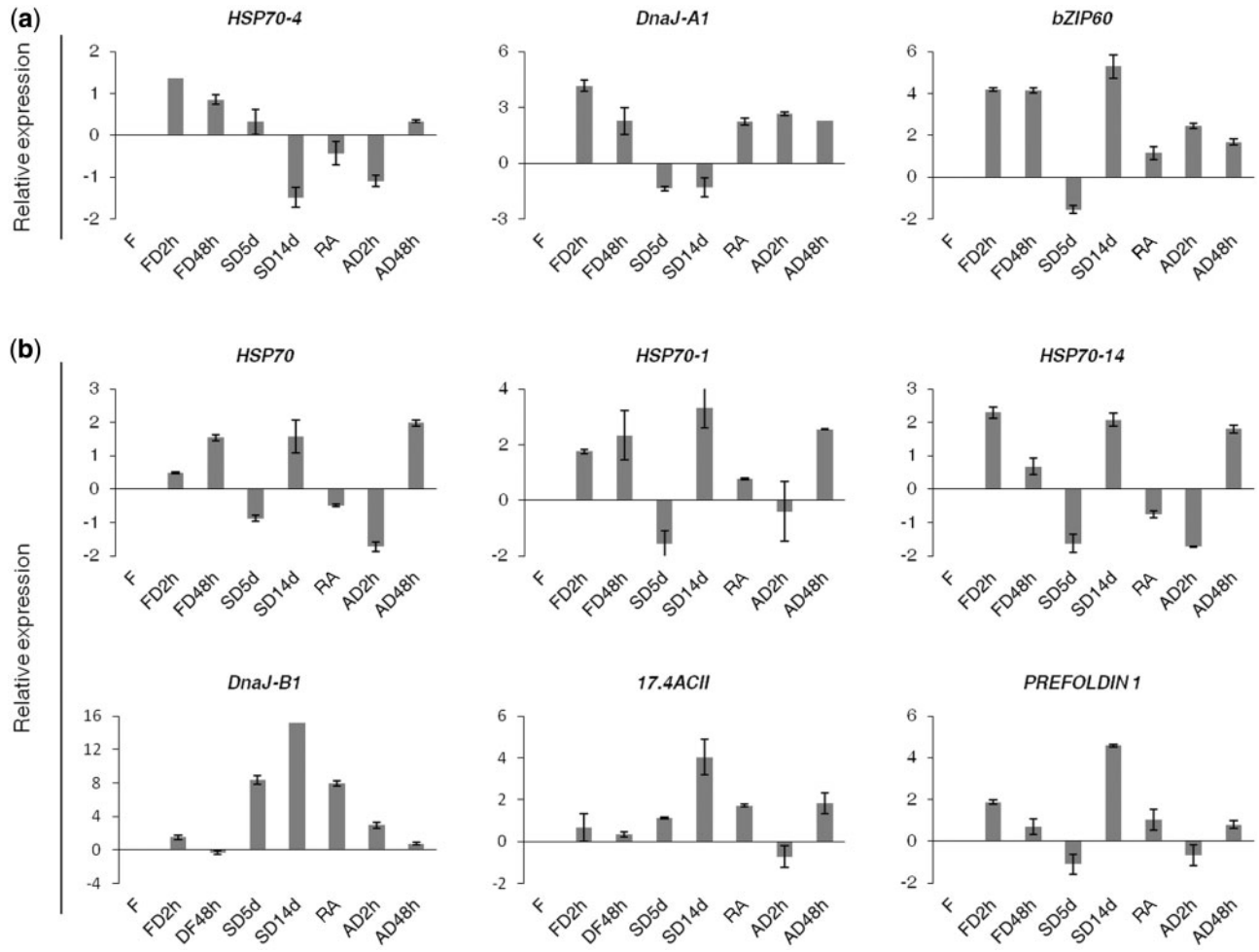

Fig. 7 RT-PCR of the expression patterns of genes related to unfolded protein stress and protein quality control in *B. hygrometrica* during air-drying and soil-drying of non-acclimated plants and air-drying of acclimated plants. (a) Marker genes and a regulatory gene of unfolded protein stress in the ER. (b) Chaperone genes that may be involved in protein quality control. An asterisk denotes $P < 0.05$. Three independent biological samples with three technical replicates were used. At least three plants per treatment were used in each sample.

(Liu and Howell 2010). GO analysis illustrates several processes related to protein synthesis, degradation, folding and modifications that were enriched in different clusters. Translation and ribosome biogenesis were simultaneously enriched among the down-regulated genes in air-dried non-acclimated plants, soil-dried non-acclimated and air-dried acclimated plants (cluster VII, down-regulated), suggesting the arrest of protein biosynthesis upon dehydration in both acclimated and non-acclimated plants, irrespective of air- or soil-drying.

Unfolded protein stress marker genes *BiP2* [*HSP70-4*; a molecular chaperone functioning in unfolded protein response in the endoplasmic reticulum (ER)], *ERDJ3A1* (a co-chaperones of BiP2) and unfolded protein stress signaling component *bZIP60* (a transcription factor) were up-regulated earlier and to higher levels in air-dried non-acclimated plants than in soil-dried non-acclimated plants and in air-dried acclimated plants (**Fig. 7a**). These results indicate the occurrence of unfolded protein stress in air-dried non-acclimated plants.

In contrast, protein folding was found to be enriched among cluster I (unique for air-dried non-acclimated plants) down-regulated genes, and cluster III (unique for air-dried acclimated plants) and VI (common for soil-dried non-acclimated plants and air-dried acclimated plants) up-regulated genes, indicating that the protein quality control system is activated in desiccation-tolerant plants to mitigate drought-induced unfolded protein stress, compared with the sensitive plants. In agreement, RT-PCR revealed that a subset of genes that encode HSP70s, DnaJ, sHSP and prefoldin chaperones that may function in protein folding in the chloroplast, cytosol and nuclei, accumulated to higher levels in fully soil-dried non-acclimated plants and air-dried acclimated plants, compared with air-dried non-acclimated plants (**Fig. 7b**).

Cell wall dynamics and lignification

Cell wall macromolecule catabolic processes were enriched specifically among up-regulated genes, while cell wall organization, cell wall biogenesis, cellulose biosynthesis, lignin biosynthesis and cell wall modification were enriched specifically among down-regulated genes in soil-dried non-acclimated plants, in comparison with air-dried non-acclimated plants (**Fig. 4c, d**). The decrease in cell wall lignification was confirmed in soil-dried

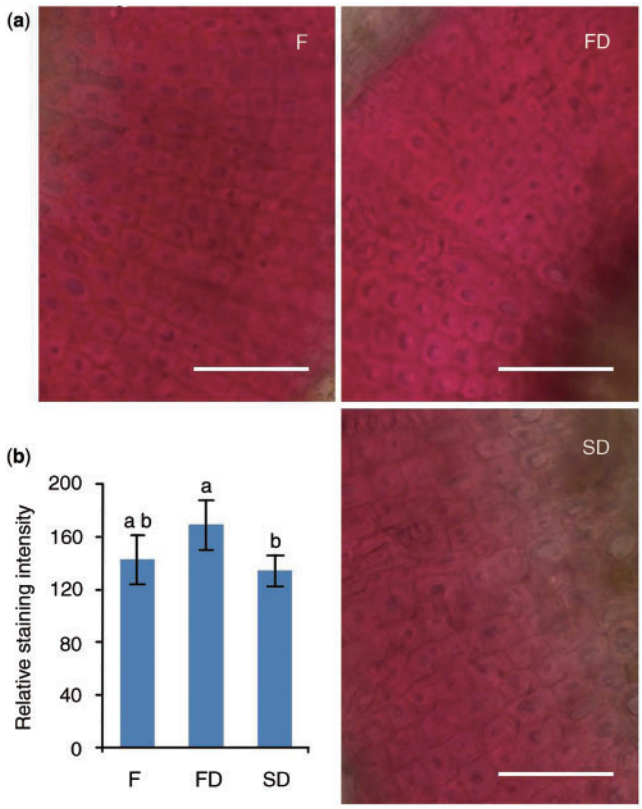

Fig. 8 Histochemical staining of cell wall lignin. (a) Leaves from fresh (F), air-dried (FD) and soil-dried (SD) non-acclimated plants of *B. hygrometrica* were hand-cut into 50–100 μm thick slices for Wiesner staining (Pomar et al. 2002) and observed under a dissecting microscope. At least three different samples for each treatment were stained. Scale bar = 40 μm. (b) Quantification of the intensity of Wiesner staining of plants in (a). Error bars represent 1 SD. Statistical significance was determined with Student's *t*-test ($P < 0.05$), and is labeled with lower case letters.

non-acclimated, plants by phloroglucinol staining (**Fig. 8**). These results showed that modifications occurred to destabilize cell wall architecture during soil-drying acclimation.

Discussion

Tolerance to rapid desiccation is primed by an acclimatory period of gradual water loss

Dehydration tolerance is a quantitative trait involving multiple genes that confer adaptive metabolic and physiological changes in plants (Nakashima et al. 2009). In this study, physiological and cellular processes were identified that correlate with the acquisition of desiccation tolerance in the resurrection plant *B. hygrometrica*. Many GO categories enriched in other resurrection plants during desiccation were also evident in this study, such as responses to abiotic stimuli, stress response pathways, oxidative processes, antioxidant responses, carbohydrate metabolism and cell wall organization, and the induction of protective molecules including LEA (late embryogenesis abundant) and heat shock proteins (Rodriguez et al. 2010, Oliver et al. 2011). Comparison with *H. rhodopensis* also revealed shared responses, such as ubiquitin–proteasome-mediated protein degradation, biosynthesis of antioxidants, energy metabolism, cell wall remodeling and signal transduction (Georgieva et al. 2012, Gechev et al. 2013). It is noted that JA signaling was triggered in the early stage of air-drying in both non-acclimated and acclimated *B. hygrometrica* plants, which is consistent with the quick accumulation of JA in *H. rhodopensis* during air-drying, underlining the initial signaling role of JA in air-drying responses (Djilianov et al. 2013). Some unique responses were observed; for example, dehydration-induced auxin signaling appears specific to *H. rhodopensis*, while dehydration-induced autophagy, trehalose biosynthesis and ascorbic acid accumulation were specific to *B. hygrometrica*. Interestingly, the intensive increase of α-tocopherol was observed at the fully dry and rehydrated state in *H. rhodopensis*, but only at the fully dry state in *B. hygrometrica*. Instead, the ascorbic acid content increased to a high level in rehydrated plants of *B. hygrometrica*, but was not reported in *H. rhodopensis* (Moyankova et al. 2014). These the differences could infer the high value of various antioxidants that contribute to the priming of Gesneriaceae resurrection plants to withstand desiccation.

Immature *B. hygrometrica* plants are uncommon in natural populations during winter, although tolerant accessions have been isolated in summer. Similarly, immature plants seldom survive in natural populations of two European Gesneriaceae resurrection plants, *H. rhodopensis* and *R. myconi* (Xavier Pico and Riba 2002, Bogacheva-Milkoteva et al. 2013). In this study, we demonstrate how pre-exposure to gradual water loss may alter subsequent responses to rapid water loss, implying the existence of so-called acclimation or training. During air-drying, the exposure of roots to air may contribute to some of the observed transcript changes. However, the acclimated plant can tolerate rapid desiccation in both light and dark conditions, when whole or detached (data not shown), indicating that the desiccation response is not significantly affected by exposure of the root under air-drying conditions. Under the conditions tested, 3 d of rehydration was sufficient for plant recovery from soil-drying. In contrast, acclimation-induced tolerance to rapid dehydration is more long-lived (one to several months depending on the sizes of the plants), indicating that acclimation-induced tolerance to rapid dehydration and the differential gene expression regulation is not merely a result of a long-lasting recovery period. This finding provides a foundation to distinguish common responses and/or harmful effects triggered by water loss from protective and adaptive responses for desiccation tolerance by comparing tolerant and sensitive *B. hygrometrica* plants. Thus dehydration-triggered changes of genes that are essential for survival are distinguished from those that lead to loss of plant viability under rapid drying by comparison of the gene expression changes triggered by air-drying with or without preceding soil-drying acclimation. This observation of acclimation-primed gene expression changes is similar to transcriptional stress memory in Arabidopsis, where elevated transcript levels of a subset of the stress response genes (so-called 'trainable

genes') have been described (Kim et al. 2012). To our knowledge, this is the first report specifically considering mechanisms of the benefit of acclimation for revival of air-dried resurrection plants on rehydration.

Ascorbic acid accumulation, cell wall loosening, activated respiration and protein quality control are probably priming mechanisms in support of rapid desiccation tolerance

In this study, we found that the processes of ascorbic acid biosynthesis, cell wall macromolecule catabolism, respiration and protein quality control were differentially regulated by rapid air-drying and slow soil-drying in non-acclimated plants, highlighting the requirement for antioxidants, cell wall flexibility, protein homeostasis and energy supply for maintaining cell viability under desiccation. Furthermore, many transcripts in these processes were kept highly expressed during subsequent rehydration, and L-ascorbic acid accumulated to the highest level in rehydrated plants. These findings indicate that these processes are likely to contribute to the establishment of an optimal physiological and cellular state, and thus facilitate the acquisition of desiccation tolerance.

Excessive ROS levels cause irreversible oxidative damage to proteins, lipids and nucleic acids, and activate signaling pathways leading to unfolded protein stress, mitochondrial dysfunction and ultimately initiate apoptosis (Apel and Hirt 2004, Sanges and Marigo 2006, Y. Zhang et al. 2013). ROS also play an essential role in plant cell wall dynamics and impact the extent of mechanical damage under water deficit. ROS-induced cell death had been observed in water-stressed Arabidopsis root tip cells, accompanied by increased expression of BAX inhibitor-1 (AtBI1, a negative regulator of the ER stress sensor IRE1α) (Duan et al. 2010).

ROS-scavenging enzymes (aldehyde dehydrogenase, glutathione S-transferase and polyphenol oxidase) and low molecular weight antioxidants such as ascorbic acid, α-tocopherol and raffinose oligocarbohydrates accumulate in many plant species, including resurrection plants, in response to water stress (Jiang et al. 2007, Z. Wang et al. 2009, Dinakar and Bartels 2013, Mitra et al. 2013, Moyankova et al. 2014). Inactivation of the photosynthetic apparatus is a common response of plants under drought stress to minimize ROS accumulation in plants, although the antioxidant capacity of the chloroplast stroma is also reduced. In addition, the inactivation of photosynthesis also results in a shortage of energy, which is critical for maintenance of cell viability (De Block et al. 2005).

Water deficit is known to impact protein denaturation (Hampton 2000). Stress also induces the disruption of the ribosome-nascent chain complex (RNC)–translocon seal, resulting in the accumulation of unfolded or misfolded proteins in the ER, and the exposure of nascent polypeptides that enter the ER lumen under a non-stress condition to the cytoplasm (Oyadomari et al. 2006). Protein quality control systems recruit specific genes and pathways to regulate protein repair, which aid in the refolding or degradation of misfolded proteins in the ER, cytosol, and other cell compartments (Ron and Walter 2007). Cytosolic chaperones protect the stressed ER from protein overload and alleviate the level of unfolded protein stress.

Enhanced α-tocopherol biosynthesis and autophagy correlate with the ability to survive rapid desiccation tolerance

Two major processes were activated concurrently with desiccation tolerance: α-tocopherol biosynthesis and autophagy. These processes are differentially regulated during air-drying in non-acclimated and acclimated plants. Different metabolic shifts during dehydration, including the accumulation of protective compounds and antioxidants, have been well documented by comparative analysis of desiccation-tolerant and desiccation-sensitive species from the same genera, notably *Sporobolus* and *Lindernia* (Oliver et al. 2011, Gasulla et al. 2013). Considering the multifunctional roles in detoxification of ROS, responses to unfolded protein stress, generation of ATP and alleviating cell death, α-tocopherol biosynthesis and autophagy appear to play important roles in desiccation tolerance mechanisms. Nevertheless, the increased autophagy and biosynthesis of antioxidants may simply reflect a better metabolic fitness or a metabolic adaptation of the organism during stress. The accumulation of α-tocopherol has also been detected in other desiccated resurrection plants (Rodriguez et al. 2010, Oliver et al. 2011, Yobi et al. 2012), while autophagy was identified in yeast and insects during extreme dehydration, but was first reported to accompany desiccation tolerance in resurrection plants (Ratnakumar et al. 2011, Teets and Denlinger, 2013). Priming of these two processes is probably established during gradual soil drying. The continual activation of ascorbic acid biosynthesis, cell wall loosening and respiration during subsequent rehydration helps maintain the physiological status, which is essential for the reactivation of autophagy and α-tocopherol biosynthesis during subsequent rounds of air-drying. In contrast, air-drying did not lead to transcriptional activation of these processes, which correlated with a loss of cell viability

Taken together, this study provides the first molecular evidence for the transcriptional regulation of critical priming of a co-ordinative mechanism based on enhanced autophagy and antioxidative processes during acclimation, which probably contribute to mitigation of cell death and acquisition of tolerance to cellular desiccation. Giving that stress and ROS-induced cell death have been observed in many economically important plants, for example rice, wheat, tobacco and *Brassica napus* (De Block et al. 2005, Nguyen et al. 2009, Duan et al. 2010, Xu et al. 2011, Lu et al. 2012), further understanding of the priming of the key processes will extend our knowledge of the essentials of acclimation and bring forth new ideas for improving plant drought tolerance to meet agricultural demand.

Materials and Methods

Plant materials and treatments

Boea hygrometrica seeds were collected in natural habitats and germinated directly in $5 \times 5\,cm^2$ pots in well-watered greenhouse conditions (approximately

25°C, 16 h/8 h light period). Seedlings were grown in the same conditions for 3 months to the two-leaf stage for the treatments. For gradual dehydration, irrigation was withdrawn from soil-grown plants for 14 d. For rehydration, soil-dried plants were re-watered for 3 d. For rapid drying, acclimated plants were transferred to Petri dishes and air-dried immediately after 3 d. Untreated plants were used as non-acclimated controls. Acclimated and non-acclimated plants were air-dried in parallel under 50% relative humidity and moderate illumination at 25°C for 48 h in a climate chamber. For re-hydration, the air-dried plants were placed in Petri dishes with wet filter papers for 3 d. At least 4–8 independent plants were prepared for each treatment, and at least three repetitions were collected for each experiment.

Quantification of water content, Chl fluorescence and electrolyte leakage

Leaf RWC and potential PSII photochemical efficiency (F_v/F_m) were monitored as described previously (Liu et al. 2009). RWC (%) = (FW − DW)/(turgid weight − DW) × 100. F_v/F_m was measured for dark-adapted leaves using PAM-101 (Walz), with a saturating light intensity of approximately 800 mmol m^{-2} s^{-1} and duration of 4.5 s. Electrolyte leakage was measured as described (Jiang et al. 2007) using a Hanna EC 215 conductivity meter (Hanna Instruments). Leakage values (μS g dry $mass^{-1}$ min^{-1}) were obtained from the leakage rate corrected by leaf dry mass. Three replicates of individual leaves were used for each treatment.

RNA isolation and real-time PCR

Total RNA was isolated using the RNAiso Plus kit (TAKARA). First-strand cDNA was synthesized from DNase I-treated total RNA using M-MLV reverse transcriptase (Promega). RT-PCR was performed as described (Z. Zhang et al. 2013). Primers used are listed in **Supplementary Table S1**. The relative expression levels were calculated using the relative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) in three independent biological samples with three technical replicates. The raw data (cycle threshold values) were normalized using *B. hygrometrica* 18S rRNA as the internal reference gene for *B. hygrometrica* gene expression. The relative expression of each gene was calculated as the mean fold change compared with the untreated fresh sample in each experiment. Statistical significance was determined by Student's *t*-test ($P < 0.05$).

Library preparation and pyrosequencing

A 3 μg aliquot of each cDNA sample was nebulized to produce fragments of a mean size of between 40 and 800 bp. cDNA fragment libraries were prepared and emulsion PCR conducted according to the manufacturer's instructions (Roche GS FLX). Pyrosequencing was performed on a Roche Genome Sequencer FLX instrument (Roche Diagnostics, http://www.roche.com/).

Cleaning, assembly and annotation of the pyrosequenced reads

The quality of reads was assessed with the SEQCLEAN EST trimming and validation tool (http://sourceforge.net/projects/seqclean/files/). The adaptor and primer sequences were identified and the trim positions were changed in.sff files using SeqClean (http://sourceforge.net/projects/seqclean/) and Newbler v2.5.3. Sequences shorter than 50 nucleotides or containing homopolymers were trimmed by LUCY (1.20 P) (Li and Chou 2004). The clean data were assembled into isotigs using the Roche GS De Novo Assembler (i.e. Newbler assembler) using optimized parameters (minimum read length, 45; use duplicate reads, checked; extend low depth overlaps, checked; reads limited to one contig, checked), and all of the non-redundant isotigs were exported to a file in FASTA format. The assembled isotigs and singlets were combined and clustered using cd-hit (version 4.0) to generate unigenes that represented clusters of genes with >95% sequence identity. In total, 36,365 unigenes were obtained and queried against the NCBI non-redundant protein (nr database, downloaded April 20, 2012) and The Arabidopsis Information Resource (TAIR) database (downloaded June 15, 2012) databases using BLASTX (Camacho et al. 2009). Homologs were chosen based on a cut-off score of 1e-4. GO annotations for TAIR identifiers were retrieved from the Gene Ontology database (Ashburner et al. 2000), and the resulting biological process (BP), molecular functions (MF) and cellular component (CC) at GO level 2 were also derived. KEGG (Kanehisa et al. 2010) annotations for TAIR identifiers were retrieved from the DAVID database (david.abcc.ncifcrf.gov/). Sequence data from this article can be found in the Short Read Archive database at NCBI under accession No. SRR1040631 (experiment accession No. SRX385264).

Microarray design and analysis

Unigenes were submitted to Agilent's eArray web tool (http://earray.chem.agilent.com/earray/) to generate oligonucleotide (60 mer) microarray probe sequences. The study was performed using Agilent 8 × 15K probes custom-made arrays and probe design used the eArray 3′-bias option. The entire EST collection was utilized for cross-reference within eArray to minimize chances of non-specific hybridization. One probe per target was designed. Each array contained 536 controls. Genes that were previously characterized to be dehydration inducible in *B. hygrometrica* were included as positive controls, including *BhGolS1/J-57*, *BhHsf1/OA-41*, *BhLEA1/10-47* and *BhLEA2/OB-44* (Liu et al. 2009, Z. Wang et al. 2009, Zhu et al. 2009).

Total RNA was labeled according to the Low RNA Input Linear Amplification Kit PLUS, one-color (Agilent Technologies). Cyanine 3-labeled cRNA samples from the control and exposed groups ($n = 3$ each) were hybridized to Agilent Custom Gene Expression Microarrays (Agilent Technologies) in accordance with the manufacturer's protocol. Microarray images were analyzed using Agilent's Feature Extraction 10.7 software (Agilent Feature Extraction Reference Guide 2007). The extracted data were analyzed with Bioconductor software (Gentleman et al. 2004). Agilent standard quantile normalizations for Agilent FE (one-color) data were applied to all data sets. The data were filtered to keep features within the dynamic range of the scanner and to retain features of good quality.

For each set of replicated probes, unique probe values were obtained by computing the median of the intensities. A total of 11,476 probes remained for statistical analysis using the packages Limma version 2.2.0 (Smyth 2005). Genes were considered statistically significant at the level of $P < 0.05$ after FDR (false discovery rate) correction, with absolute log2 fold change cut-off set at 1.0. The PCA plot was made using bioconductor's mad 4 package. GOstats version 2.2.0 (Gentleman 2005) from Bioconductor (http://www.bioconductor.org) was used under R version 2.2.0 (R Development Core Team 2005) to compute the hypergeometric test. Functional annotation of the microarray probe sequences was performed by BLASTN searches against EST contigs from the TAIR database (http://www.arabidopsis.org/). The complete array data have been deposited in the NCBI GEO (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession No. GSE53058.

Monodansylcadaverine staining for autophagy

Roots were stained with a 0.05 mM final concentration of MDC (Sigma) in phosphate-buffered saline (PBS) for 10 min (Biederbick et al. 1995) and washed with PBS twice to remove excess MDC. Fluorescence was measured using an Olympus (Japan) FV1000MPE multiphoton laser scanning microscope, with an excitation wavelength of 335 nm and an emission wavelength of 508 nm. The image was processed with FV10-ASW 3.0 Viewer and Adobe Photoshop elements.

Hoechst 33342 and propidium iodide co-staining for apoptosis

Roots were soaked in 1 ml of assay buffer containing Hoechst 33342 and PI for 30 min at 4°C using the Apoptosis and Necrosis Assay Kit (Beyotime Institute of Biotechnology). After washing twice with 10 ml of ddH_2O, samples were observed with a two-photon microscope (Olympus FV1000MPE, Olympus) with an excitation filter of 353–377 nm. The image was processed with FV10-ASW 3.0 Viewer and Adobe Photoshop elements. The percentage of cells undergoing apoptosis compared with the total cell number was estimated from three different samples, each containing at least 30 cells. One-way analysis of variance (ANOVA) was performed with SPSS13.0.

L-Ascorbic acid and α-tocopherol determination

Reduced ascorbic acid was measured using the Plant Vitamin C ELISA kit (Hongyue). The kit uses an antibody–antigen–enzyme–antibody complex to

determine the plant vitamin C level at a wavelength of 450 nm using a Multiskan MK3 Spectrum (Thermo Scientific). The content of α-tocopherol was measured using a Plant Vitamin E (VE) ELISA Kit [Rapidbio (RB)] according to the manufacturer's protocol.

H_2O_2 and O_2^- radical detection

H_2O_2 and O_2^- levels were measured by DAB and NBT staining, respectively (Orozco-Cardenas and Ryan 1999, Kawai-Yamada et al. 2004). Seedlings were immersed in freshly prepared 0.1% (w/v) DAB-HCl (pH 3.8) and 0.1% (w/v) NBT in 10 mM potassium phosphate buffer (pH 7.8) containing 10 mM NaN_3, respectively. After vacuum infiltration, samples were incubated in darkness at 22°C for 10 h for DAB staining and 1 h for NBT staining. For H_2O_2 determination, seedlings were placed in acetic acid : glycerol : ethanol (1 : 1 : 3, by vol.) solution at 95°C for 10 min and then stored in 95% ethanol until photographed. For O_2^- detection, stained plants were bleached in acetic acid : glycerol : ethanol (1 : 1 : 3, by vol.), boiled for 5 min, and stored in 95% ethanol until photographed.

Histochemical staining of cell wall lignin

Boea hygrometrica leaves were hand-cut into 50–100 μm sections prior to Wiesner staining (Pomar et al. 2002). Sections were incubated in 1% phloroglucinol (w/v) in 6 mol l^{-1} HCl for 5 min, and observed under a dissecting microscope. The intensity of Wiesner staining was quantified using Image J software. One-way ANOVA was performed with SPSS13.0.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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References

Abbasi, A.R., Hajirezaei, M., Hofius, D., Sonnewald, U. and Voll, L.M. (2007) Specific roles of alpha- and gamma-tocopherol in abiotic stress responses of transgenic tobacco. *Plant Physiol.* 143: 1720–1738.

Apel, K. and Hirt, H. (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* 55: 373–399.

Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M. et al. (2000) Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat. Genet.* 25: 25–29.

Bartels, D. and Salamini, F. (2001) Desiccation tolerance in the resurrection plant *Craterostigma* plantagineum. A contribution to the study of drought tolerance at the molecular level. *Plant Physiol.* 127: 1346–1353.

Biederbick, A., Kern, H.F. and Elsässer, H.P. (1995) Monodansylcadaverine (MDC) is a specific in vivo marker for autophagic vacuoles. *Eur. J. Cell Biol.* 66: 3–14.

Bogacheva-Milkoteva, K., Kozuharova, E. and Claßen-Bockhoff, R. (2013) Breeding systems of Haberlea rhodopensis (Gesneriaceae), a Tertiary relict endemic to the Balkan Peninsula. *Phytol. Balcanica* 19: 201–208.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K. et al. (2009) BLAST+: architecture and applications. *BMC Bioinformatics* 10: 421.

Chiara, M., Horner, D.S. and Spada, A. (2013) De novo assembly of the transcriptome of the non-model plant *Streptocarpus rexii* employing a novel heuristic to recover locus-specific transcript clusters. *PLoS One* 8: e80961.

De Block, M., Verduyn, C., De Brouwer, D. and Cornelissen, M. (2005) Poly(ADP-ribose) polymerase in plants affects energy homeostasis, cell death and stress tolerance. *Plant J.* 41: 95–106.

Dinakar, C. and Bartels, D. (2013) Desiccation tolerance in resurrection plants: new insights from transcriptome, proteome and metabolome analysis. *Front. Plant Sci.* 4: 482.

Djilianov, D., Dobrev, P., Moyankova, D., Vankova, R., Georgieva, D., Gajdošová, S. et al. (2013) Dynamics of endogenous phytohormones during desiccation and recovery of the resurrection plant species *Haberlea rhodopensis*. *J. Plant Growth Regul.* 32: 564–574.

Duan, Y., Zhang, W., Li, B., Wang, Y., Li, K., Sodmergen. et al. (2010) An endoplasmic reticulum response pathway mediates programmed cell death of root tip induced by water stress in Arabidopsis. *New Phytol.* 186: 681–695.

Gaff, D.F. and Oliver, M. (2013) The evolution of desiccation tolerance in angiosperm plants: a rare yet common phenomenon. *Funct. Plant Biol.* 40: 315–328.

Gasulla, F., Dorp, K., Dombrink, I., Zähringer, U., Gisch, N., Dörmann, P. et al. (2013) The role of lipid metabolism in the acquisition of desiccation tolerance in Craterostigma plantagineum: a comparative approach. *Plant J.* 75: 726–741.

Gechev, T.S., Benina, M., Obata, T., Tohge, T., Sujeeth, N., Minkov, I. et al. (2013) Molecular mechanisms of desiccation tolerance in the resurrection glacial relic *Haberlea rhodopensis*. *Cell Mol. Life Sci.* 70: 689–709.

Gentleman, R. (2005) GOstats: Tools for Manipulating GO and Microarrays, R Package Version 1.1.3. URL http://www.bioconductor.org.

Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S. et al. (2004) Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* 5: R80.

Georgieva, T., Christov, N. and Djilianov, D. (2012) Identification of desiccation-regulated genes by cDNA-AFLP in *Haberlea rhodopensis*—a resurrection plant. *Acta Physiol. Plant.* 34: 1055–1066.

Hampton, R.Y. (2000) ER stress response: getting the UPR hand on misfolded proteins. *Curr. Biol.* 10: R518–R521.

Heilmeier, H. and Hartung, W. (2011) *Chamaegigas intrepidus* DINTER: an aquatic poikilohydric angiosperm that is perfectly adapted to its complex and extreme environmental conditions. *In* Plant Desiccation Tolerance. Edited by Lüttge, U., Beck, E. and Bartels, D. pp. 233–251. Springer, Berlin.

Jiang, G., Wang, Z., Shang, H., Yang, W., Hu, Z., Phillips, J. et al. (2007) Proteome analysis of leaves from the resurrection plant *Boea*

hygrometrica in response to dehydration and rehydration. *Planta* 225: 1405–1420.

Jin, S. and Daniell, H. (2014) Expression of γ-tocopherol methyltransferase in chloroplasts results in massive proliferation of the inner envelope membrane and decreases susceptibility to salt and metal-induced oxidative stresses by reducing reactive oxygen species. *Plant Biotechnol. J.* 12: 1274–1285.

Kanehisa, M., Goto, S., Furumichi, M., Tanabe, M. and Hirakawa, M. (2010) KEGG for representation and analysis of molecular networks involving diseases and drugs. *Nucleic. Acids Res.* 38: D355–D360.

Kawai-Yamada, M., Ohori, Y. and Uchimiya, H. (2004) Dissection of Arabidopsis Bax inhibitor-1 suppressing Bax-, hydrogen peroxide-, and salicylic acid-induced cell death. *Plant Cell* 16: 21–32.

Kim, J.M., To, T.K., Ishida, J., Matsui, A., Kimura, H. and Seki, M. (2012) Transition of chromatin status during the process of recovery from drought stress in *Arabidopsis thaliana*. *Plant Cell Physiol.* 53: 847–856.

Li, S. and Chou, H.H. (2004) LUCY2: an interactive DNA sequence quality trimming and vector removal tool. *Bioinformatics* 20: 2865–2856.

Liu, J.X. and Howell, S.H. (2010) Endoplasmic reticulum protein quality control and its relationship to environmental stress responses in plants. *Plant Cell* 22: 2930–2942.

Liu, X., Wang, Z., Wang, L., Wu, R., Phillips, J. and Deng, X. (2009) LEA 4 group genes from the resurrection plant *Boea hygrometrica* confer dehydration tolerance in transgenic tobacco. *Plant Sci.* 176: 90–98.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). *Methods* 25: 402–408.

Lu, H., Wan, Q., Wang, H., Na, X., Wang, X. and Bi, Y. (2012) Oxidative stress and mitochondrial dysfunctions are early events in narciclasine-induced programmed cell death in tobacco Bright Yellow-2 cells. *Physiol. Plant.* 144: 48–58.

Mitra, J., Xu, G., Wang, B., Li, M. and Deng, X. (2013) Understanding desiccation tolerance using the resurrection plant *Boea hygrometrica* as a model system. *Front. Plant Sci.* 4: 446.

Moyankova, D., Mladenov, P., Berkov, S., Peshev, D., Georgieva, D. and Djilianov, D. (2014) Metabolic profiling of the resurrection plant *Haberlea rhodopensis* during desiccation and recovery. *Physiol. Plant.* 152: 675–687.

Nakashima, K., Ito, Y. and Yamaguchi-Shinozaki, K. (2009) Transcriptional regulatory networks in response to abiotic stresses in Arabidopsis and grasses. *Plant Physiol.* 149: 88–95.

Nguyen, G.N., Hailstones, D.L., Wilkes, M. and Sutton, B.G. (2009) Drought-induced oxidative conditions in rice anthers leading to a programmed cell death and pollen abortion. *J. Agron. Crop Sci.* 195: 157–164.

Oliver, M.J. and Bewley, J.D. (1997) Desiccation-tolerance of plant tissues: a mechanistic overview. *Hortic. Rev.* 18: 171–213.

Oliver, M.J., Guo, L., Alexander, D.C., Ryals, J.A., Wone, B.W. and Cushman, J.C. (2011) A sister group contrast using untargeted global metabolomic analysis delineates the biochemical regulation underlying desiccation tolerance in *Sporobolus stapfianus*. *Plant Cell* 23: 1231–1248.

Orozco-Cardenas, M. and Ryan, C.A. (1999) Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway. *Proc. Natl Acad. Sci. USA* 96: 6553–6557.

Oyadomari, S., Yun, C., Fisher, E.A., Kreglinger, N., Kreibich, G., Oyadomari, M. et al. (2006) Cotranslocational degradation protects the stressed endoplasmic reticulum from protein overload. *Cell* 126: 727–739.

Patel, S., Caplan, J. and Dinesh-Kumar, S.P. (2006) Autophagy in the control of programmed cell death. *Curr. Opin. Plant Biol.* 9: 391–396.

Pomar, F., Merino, F. and Barcel Ros, A. (2002) O-4-Linked coniferyl and sinapyl aldehydes in lignifying cell walls are the main targets of the Wiesner (phloroglucinol–HCl) reaction. *Protoplasma* 220: 17–28.

Proctor, M. and Pence, V. (2002) Vegetative tissues: bryophytes, vascular resurrection plants and vegetative propagules. *In* Desiccation and Survival in Plants: Drying Without Dying. Edited by Black, M. and Pritchard, H. pp. 207–237. CABI Publishing, New York.

R Development Core Team. (2005) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, www.R-project.org.

Ratnakumar, S., Hesketh, A., Gkargkas, K., Wilson, M., Rash, B.M., Hayes, A. et al. (2011) Phenomic and transcriptomic analyses reveal that autophagy plays a major role in desiccation tolerance in *Saccharomyces cerevisiae*. *Mol. Biosyst.* 7: 139–149.

Rodriguez, M.C., Edsgärd, D., Hussain, S.S., Alquezar, D., Rasmussen, M., Gilbert, T. et al. (2010) Transcriptomes of the desiccation-tolerant resurrection plant *Craterostigma plantagineum*. *Plant J.* 63: 212–228.

Ron, D. and Walter, P. (2007) Signal integration in the endoplasmic reticulum unfolded protein response. *Nat. Rev. Mol. Cell Biol.* 8: 519–529.

Sanges, D. and Marigo, V. (2006) Cross-talk between two apoptotic pathways activated by endoplasmic reticulum stress: differential contribution of caspase-12 and AIF. *Apoptosis* 11: 1629–1641.

Smyth, G.K. (2005) Limma: linear models for microarray data. *In* Bioinformatics and Computational Biology Solutions using R and Bioconductor. Edited by Gentleman, R., Carey, V., Huber, W., Irizarry, R. and Dudoit, S. pp. 397–420. Springer, New York.

Sun, W. and Leopold, A.C. (1997) Cytoplasmic vitrification and survival of anhydrobiotic organisms. *Comp. Biochem. Physiol.* 117: 327–333.

Teets, N.M. and Denlinger, D.L. (2013) Autophagy in Antarctica: combating dehydration stress in the world's southernmost insect. *Autophagy* 9: 629–631.

Wang, L., Shang, H., Liu, Y., Zheng, M., Wu, R., Phillips, J. et al. (2009) A role for a cell wall localized glycine-rich protein in dehydration and rehydration of the resurrection plant *Boea hygrometrica*. *Plant Biol.* 11: 837–848.

Wang, Z., Zhu, Y., Wang, L., Liu, X., Liu, Y., Phillips, J. et al. (2009) A WRKY transcription factor participates in dehydration tolerance in *Boea hygrometrica* by binding to the W-box elements of the galactinol synthase (BhGolS1) promoter. *Planta* 230: 1155–1166.

Xavier Pico, F. and Riba, M. (2002) Regional-scale demography of *Ramonda myconi*: remnant population dynamics in a preglacial relict species. *Plant Ecol.* 161: 1–13.

Xu, F.J., Jin, C.W., Liu, W.J., Zhang, Y.S. and Lin, X.Y. (2011) Pretreatment with H_2O_2 alleviates aluminum-induced oxidative stress in wheat seedlings. *J. Integr. Plant Biol.* 53: 44–53.

Yobi, A., Wone, B.W., Xu, W., Alexander, D.C., Guo, L., Ryals, J.A. et al. (2012) Comparative metabolic profiling between desiccation-sensitive and desiccation-tolerant species of Selaginella reveals insights into the resurrection trait. *Plant J.* 72: 983–999.

Zhang, Y., Zhao, L., Li, X., Wang, Y., Yao, J., Wang, H. et al. (2013) V8, a newly synthetic flavonoid, induces apoptosis through ROS-mediated ER stress pathway in hepatocellular carcinoma. *Arch. Toxicol.* 88: 97–107.

Zhang, Z., Wang, B., Sun, D. and Deng, X. (2013) Molecular cloning and differential expression of sHSP gene family members from the resurrection plant *Boea hygrometrica* in response to abiotic stresses. *Biologia* 68: 651–661.

Zhao, Y., Xu, T., Shen, C.Y., Xu, G.H., Chen, S.X., Song, L.Z. et al. (2014) Identification of a retroelement from the resurrection plant *Boea hygrometrica* that confers osmotic and alkaline tolerance in *Arabidopsis thaliana*. *PLoS One.* 9: e98098.

Zhu, Y., Wang, Z., Jing, Y.J., Wang, L.L., Liu, X., Liu, Y. et al. (2009) Ectopic over-expression of BhHsf1, a heat shock factor from the resurrection plant *Boea hygrometrica*, leads to increased thermotolerance and retarded growth in transgenic Arabidopsis and tobacco. *Plant Mol. Biol.* 71: 451–467.