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ORIGINAL ARTICLE

Transcriptional and metabolic changes in the desiccation tolerant plant *Craterostigma plantagineum* during recurrent exposures to dehydration

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Abstract

Main conclusion **Multiple dehydration/rehydration treatments improve the adaptation of *Craterostigma plantagineum* to desiccation by accumulating stress-inducible transcripts, proteins and metabolites. These molecules serve as stress imprints or memory and can lead to increased stress tolerance.**

It has been reported that repeated exposure to dehydration may generate stronger reactions during a subsequent dehydration treatment in plants. This stimulated us to address the question whether the desiccation tolerant resurrection plant *Craterostigma plantagineum* has a stress memory. The expression of four representative stress-related genes gradually increased during four repeated dehydration/rehydration treatments in *C. plantagineum*. These genes reflect a transcriptional memory and are trainable genes. In contrast, abundance of chlorophyll synthesis/degradation-related transcripts did not change during dehydration and remained at a similar level as in the untreated tissues during the recovery phase. During the four dehydration/rehydration treatments the level of ROS pathway-related transcripts, superoxide dismutase (SOD) activity, proline, and sucrose increased, whereas H_2O_2 content and electrolyte leakage decreased. Malondialdehyde (MDA) content did not change during the dehydration, which indicates a gain of stress tolerance. At the protein level, increased expression of four representative stress-related proteins showed that the activated stress memory can persist over several days. The phenomenon described here could be a general feature of dehydration stress memory responses in resurrection plants.

Keywords Dehydration stress · Resurrection plants · Stress memory · Stress-related genes

Abbreviations

ALAD	5-Aminolevulinic acid dehydratase
APX	Ascorbate peroxidase
BHT	Butylated hydroxytoluene
CAT	Catalase
CHLG	Chlorophyll synthase
CHLM	Mg-protoporphyrin IX methyltransferase
CPO	Coprogen oxidase
Cu/Zn-SOD	Copper/zinc superoxide dismutase
EDR1	Early dehydration responsive 1
EF1a	Elongation factor 1-alpha
GC/MS	Gas chromatography/mass spectrometry
GSA	Glutamate-1-semialdehyde aminotransferase
LEA	Late embryogenesis abundant
MDA	Malondialdehyde
Mn-SOD	Manganese superoxide dismutase
NBT	4-Nitro-blue tetrazolium chloride
NYE1	Non-yellowing 1
OD	Optical density
PaO	Pheophorbide a oxygenase
PBGD	Porphobilinogen deaminase
PPH	Pheophytinase

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RCCR	Red chlorophyll catabolite reductase
ROS	Reactive oxygen species
RWC	Relative water content
SOD	Superoxide dismutase
TBA	Thiobarbituric acid

Introduction

Due to global warming, air temperature, precipitation capacity and rainfall distribution change more frequently, which possibly leads to more irregular and multiple abiotic stresses, such as drought, heat and cold stress (Wang et al. 2014; Niinemets et al. 2017). Drought is one of the major factors, which can affect growth, survival and productivity of plants throughout their life cycle repeatedly (Avramova 2015; Todaka et al. 2017). It has been observed that tolerance to biotic and/or abiotic stress can be increased when plants are initially exposed to a mild stress (Ramírez et al. 2015). This phenomenon is termed "priming" and it is used in the context of application of chemical agents and biotic stress, and "acclimation" or "hardening" in the context of abiotic stress (Sinclair and Roberts 2005; Hilker et al. 2016; Savvides et al. 2016). Information may be stored from the initial first stress until the subsequent stress; this stored information influences the response to the subsequent stress so that plants can respond more quickly and efficiently and thus tolerance can be enhanced (Bruce et al. 2007; Ding et al. 2012; Li et al. 2014; Ramírez et al. 2015; Liu et al. 2016). A modulated response to a subsequent second stress represents the concept of a stress memory or imprint, which is responsible for providing mechanisms for acclimation and adaptation (Avramova 2015). Since 'plant memory' has been described as 'an ability to access past experience so that new responses incorporate relevant information from the past' (Pintó-Marijuan et al. 2017), we therefore use 'memory' in this work. A plant stress memory is associated with changes in physiology and biochemical parameters. Walter et al. (2011) reported that the relative leaf water content does not change significantly between two recurrent drought stresses, but the maximum quantum efficiency (Fv/Fm) and maximum fluorescence (Fm) are reduced. Studies demonstrated that a pre-exposition to cold stress can activate the sub-cellular antioxidant systems by increasing activities of SOD, APX and CAT which depress the oxidative burst in the photosynthetic apparatus, and thus the tolerance to a subsequent cold stress is enhanced (Li et al. 2014). It was observed that the photosynthetic rate and the sugar content increase, the stomata aperture decreases, the ROS homeostasis sustains and the grain yield increases when plants were exposed to a second abiotic stress (Wang et al. 2014, 2015; Li et al. 2015; Virlouvet and Fromm 2015; Hu et al. 2016). Plant stress memory is mediated by proteins, transcription factors and modifications on the epigenetic level such as DNA methylation and demethylation, histone modifications and small RNAs (Chinnusamy and Zhu 2009). Besides increasing stress tolerance priming for a stress memory has also sometimes negative impacts as it may compromise growth of plants and biomass production (Chen and Arora 2012). The transcriptional changes induced by the pre-treatment may contribute to cope with the subsequent stress treatments by involving stress memory effects (Hu et al. 2016). Previous experiments demonstrated that different genes (*RD29A*, *RD29B*, *COR15A*, *RAB18*, *WRKY70*, jasmonic acid-associated genes and 'revised-response' memory genes) behave differently during repeated exposures to dehydration and memory and non-memory genes can be distinguished (Liu et al. 2016). Memory genes (or trainable genes) alter transcriptional responses to a subsequent stress, while 'non-memory' genes (or non-trainable genes) respond in the same way to each stress (Alvarez-Venegas et al. 2007; Ding et al. 2012; Liu et al. 2014, 2016; Avramova 2015). However, the process is so far not well studied and a more detailed analysis is required of the physiology and the molecular basis in plants.

Understanding the response of plants to stresses which occur repeatedly within short intervals may lead to beneficial treatments also in crop plants. Memory responses have been identified in several angiosperm plants (such as potato, *Arabidopsis* or perennial ryegrass) (Ramírez et al. 2015; Hu et al. 2016; Liu et al. 2016).

Craterostigma plantagineum is a desiccation-tolerant plant which looses nearly all cellular water and goes to a quiescent state during desiccation. Upon water availability, the plant rehydrates and restores full metabolic activity (Bartels et al. 1990). This is a good experimental system to study the dehydration stress-induced memory responses in resurrection plants. We were interested to know how the desiccation-tolerant resurrection plant *C. plantagineum* responds to repeated dehydration treatments with short time intervals and whether this plant has the capacity to recover from repeated stress treatments. Therefore, we analyzed whether memory responses exist in *C. plantagineum* under repeated dehydration treatments on the physiological and molecular levels and how long the memory persists after the stress relief. Representative genes from different pathways were selected and it was investigated whether they are trainable genes and could be involved in dehydration stress memory.

Materials and methods

Plant growth and stress treatments

Craterostigma plantagineum was propagated vegetatively and grown in artificial clay pebbles (Lanstedt Ton, Gebr.

Lenz GmbH, Bergneustadt, Germany) in individual pots with nutrient solutions according to Bartels et al. (1990). The artificial clay pebbles are porous small granules (8–12 mm). Dehydration stress was applied to 12-week-old plants by removing the plants from the artificial clay pebbles. The roots were gently washed to remove any remaining pebbles. Then plants were air-dried for 6 h in light conditions. This exposure was recorded as stress 1 (S1) and resulted in the decrease of the fresh tissue weight to 65% of the untreated state (F). It was established in preliminary experiments that if the fresh tissue weight decreased to 65% of the F state, plants could recover fully after rehydration, indicating that this level of water loss is reversible and non-lethal. After dehydration, plants were placed in a controlled growth chamber (120–150 µmol/m^2/s light, 22 °C and 30% humidity) with their roots in water for 18 h (including a dark period of 6 h) to recover (R1). The same treatments (dehydration and rehydration) were repeated 4 times until stress 4 (S4). After S4 plants were re-watered for 2, 4 or 6 days and these samples are termed R2d, R4d and R6d. Samples were harvested at each time point for further analysis. The treatments are depicted in the scheme in Fig. 1a. In each experiment, at least 30 plants were used, of which 6 plants were used as control. The control plants were kept under the same light and temperature conditions as the treated plants. At least three independent biological replications were done for each experiment and three to five technical repetitions in each biological experiment.

Relative water content measurement

To check the water status of the plants at the dehydration and rehydration stages, the relative water content was measured, according to the formula: RWC (%) = [(FW − DW)/(TW − DW)] × 100% where FW is the fresh weight of leaves, TW is turgid weight of the

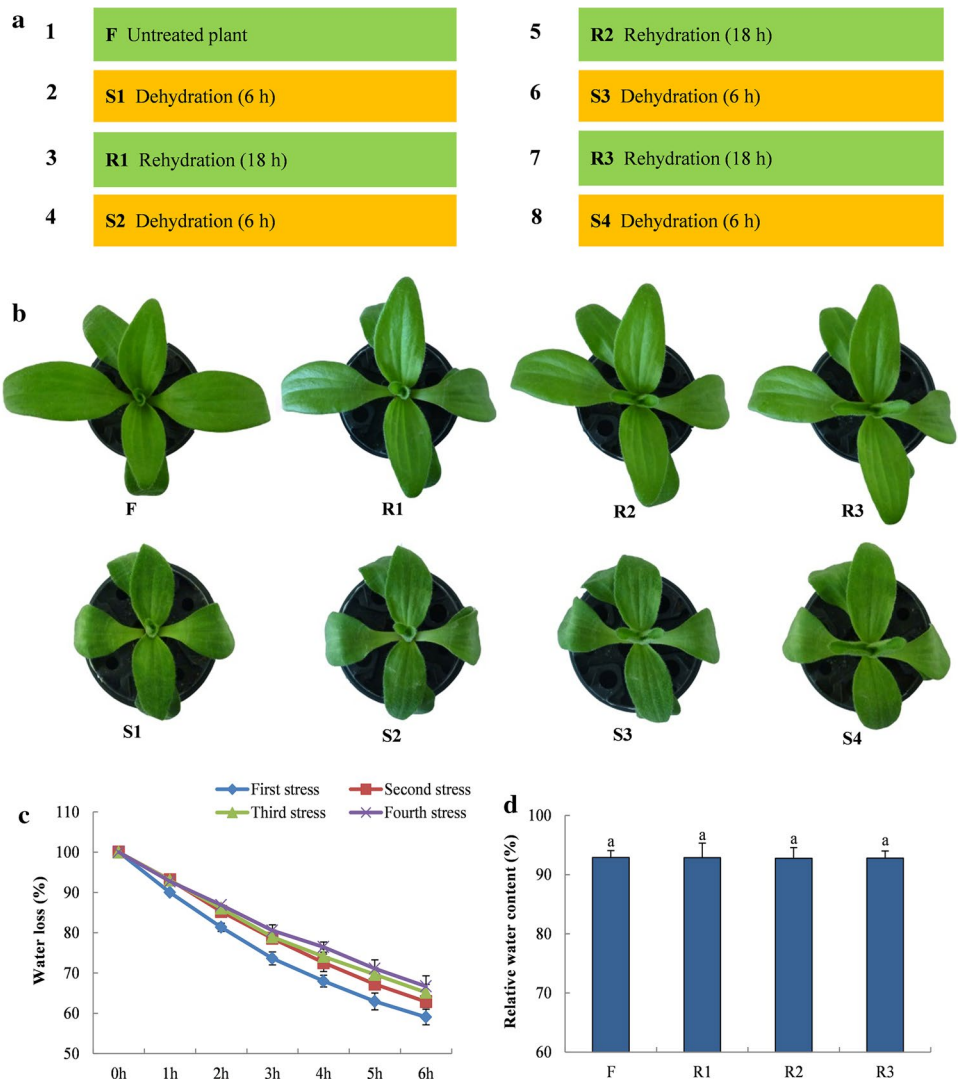


Fig. 1 The assessment of dehydration tolerance and water status during four cycles of dehydration. **a** The experimental design of dehydration/rehydration applications (see details in Materials and methods). **b** Appearance of *C. plantagineum* plants during four consecutive cycles of stress (S1–S4) and subsequent recovery (R1–R3) treatments. **c** Fresh weight of plants after experiencing the first dehydration (blue line), second (red line), third (light green line) and fourth (purple line) stress cycles. **d** Relative water content of plant leaves in untreated plants before the start of the dehydration (F) state and after recovery (R1–R3) phases. Values in (**c**, **d**) were calculated from three independent biological repetitions and five technical repetitions within each biological experiment (mean ± SE). The vertical bars with different lower-case letters are significantly different from each other at $P < 0.05$ (one-way ANOVA). Other details are given in Materials and methods

same leaves after submerged in water for 24 h and DW is the dry weight of the same leaves after 24 h incubation at 80 °C (Ding et al. 2012; Quan et al. 2016a).

Assay of electrolyte leakage

For electrolyte leakage assays, at least 4 leaves were used in one repetition, and 5 technical repetitions and 3 biological repetitions were done. Leaves were cut into squares of about 1 cm side length and shaken at 160 rpm (Innova 4000 incubator shaker, New Brunswick Scientific, USA) in Milli-Q water for 4 h at room temperature before measuring the initial conductivity using a conductivity meter (Qcond 2400, VWR International, USA). Then samples were boiled for 30 min. The conductivity of the samples was measured after they had been cooled down to room temperature. Electrolyte leakage was calculated as the ratio of initial conductivity and the conductivity of the boiled samples (Quan et al. 2016b).

Determination of chlorophyll content

Plant leaves (dry weight: 20–60 mg) were ground under liquid nitrogen and homogenized in 2 ml of 80% (v/v) acetone. The mixtures were incubated for 30 min in the dark with shaking and centrifuged for 5 min at 12,000*g* at room temperature. The chlorophyll content was measured at 663 nm (OD 663) and 645 nm (OD 645) and calculated using the following formula:

$$C(\text{mg/g DW}) = 0.002 \times (20.2 \times \text{OD } 645 + 8.02 \times \text{OD } 663)/\text{g DW},$$

where *C* corresponds to the total chlorophyll content (Missihoun 2011).

Quantification of proline content

Proline levels were determined from leaves of treated plants according to Liu and Chan (2015). Proline was extracted from 0.5 g leaf tissue by adding 5 ml of 3% (*w*/*v*) sulfosalicylic acid and subsequently boiled for 10 min. Two ml supernatant were transferred to a mixture of 2 ml of 100% (v/v) glacial acetic acid and 2 ml of ninhydrin acid (1.25 g ninhydrin dissolved in 30 ml of 100% (v/v) glacial acetic acid and 20 ml of 6 mol/L phosphoric acid). Then the compounds were boiled for 30 min. After cooling to room temperature, 4 ml of toluene was added. The upper phase was transferred to a new tube after vortexing for 30 s. After centrifugation for 5 min at 3000*g*, the upper organic phase was collected and the OD was measured at 520 nm. In parallel, L-proline was used for preparing a standard curve (Liu and Chan 2015). The proline content was calculated based on the standard curve as follows:

$$\text{Proline}(\mu\text{g/g DW}) = (\text{Concentration derived from a standard curve} \times \text{volume of the extract})/\text{g DW}.$$

Assay of H_2O_2 content

The H_2O_2 content was measured according to Velikova et al. (2000). Leaf material (20–60 mg) was ground with liquid nitrogen and homogenized in 2 ml of 0.1% (*w*/*v*) trichloroacetic acid. The mixture was incubated on ice for 5 min and centrifuged for 10 min at 13,000*g* at 4 °C. After centrifugation, 0.5 ml of the supernatant was transferred into a mixture of 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M potassium iodide to start the reaction. After keeping the compounds in the dark for 20 min at room temperature, the absorbance was read at 390 nm. In parallel H_2O_2 standards (5, 10, 25, 50 µM) were prepared for a standard curve. The H_2O_2 content was calculated based on the standard curve as follows:

$$C(\mu\text{mol/g DW}) = (\text{Concentration derived from a standard curve} \times \text{volume of the extract})/\text{g DW},$$

where *C* corresponds to the H_2O_2 content.

Lipid peroxidation assay

The MDA was measured as the end product of the lipid peroxidation process according to Hodges et al. (1999) and Kotchoni et al. (2006). Briefly, 1 ml pre-chilled 0.1% (*w*/*v*) TCA solution was used to homogenize the leaf tissue (20–60 mg) which was ground with liquid nitrogen. The homogenate was centrifuged at 13,000 rpm at 4 °C for 5 min. One volume of 20% (*w*/*v*) TCA, 0.01% (*w*/*v*) butylated hydroxytoluene (BHT) and 0.65% (*w*/*v*) thiobarbituric acid (TBA) mixture was added to 500–600 µl supernatant for the last step. The compound was incubated at 95 °C in a water bath for 25 min. Absorbances were read at 440 nm (OD 440), 532 nm (OD 532) and 600 nm (OD 600), respectively. The MDA content was determined by the following formula:

$$\text{MDA equivalents (nmol/g DW)} = [(A-B)/157000] \times 1000000 \times \text{total volume of the extracts ((ml))/g DW},$$

where $A = [(\text{OD } 532 - \text{OD } 600)]$ and $B = [(\text{OD } 440 - \text{OD } 600) \times 0.0571]$.

Determination of superoxide dismutase activity

Superoxide dismutase activity was measured by the 4-nitro-blue tetrazolium chloride (NBT) illumination method as described by Liu and Chan (2015).

Sugar extraction and analysis

Sugars were extracted from leaf tissues of *C. plantagineum* as described by Zhang et al. (2016). Leaf material (1 g) was

ground to a fine powder in liquid nitrogen and extracted twice with 80% (v/v) methanol at 4 °C. After centrifugation for 5 min at 5000*g* at 4 °C, the supernatant was transferred to a new tube. A dry sediment was obtained after evaporation at 25 °C under reduced pressure for about 3 h. The sediment was dissolved in water and extracted three times with chloroform. The aqueous phase was centrifuged at 10 000*g*, 4 °C for 30 min. The exchange resin (Dowex 50 WX8 and Dowex IX8) was added to the aqueous phase (5 g/100 ml) to remove charged molecules. Then the aqueous phase containing the sugar fraction was stirred for 1 h before the gas chromatography/mass spectrometry (GC/MS) analysis.

The extracted sugars were first analyzed by thin layer chromatography according to Zhang et al. (2016)

For the sucrose and octulose content measurement, 10 µg xylitol was used as internal standard. The following steps were done according to Zhang et al. (2016). Data analysis was performed with GC ChemStation [Rev.B.03.02 (341), Agilent Technologies, Böblingen, Germany].

RNA isolation and reverse transcription-PCR

Total RNA was extracted according to Valenzuela-Avendaño et al. (2005). Reverse transcription (RT)-PCR was performed as described by Zhao et al. (2017). Gene-specific primers are listed in Table S1. The transcript of the *EF1α* gene was used as the internal reference for *C. plantagineum* (Giarola et al. 2015). The relative gene expression level was determined by Image-J (https://imagej.nih.gov/ij/). Fold changes of each gene at different time points were analyzed by setting F phase as 1.

Protein analysis

Crude protein extracts were prepared according to Laemmli (1970) using Laemmli-sample buffer [62.5 mM Tris–HCl, pH 6.8, 10% (v/v) glycerol, 2% (*w*/*v*) SDS, 0.1 M DTT, 0.005% (*w*/*v*) bromophenol blue]. Pre-chilled transfer buffer was used to transfer proteins separated on a 10% polyacrylamide SDS gel onto a nitrocellulose Protran BA-85 membrane (Towbin et al. 1979). The transferred proteins were visualized by staining the membrane with 0.2% (*w*/*v*) Ponceau S-red solution for 30 min. After destaining, the membrane was incubated in blocking solution (1 × TBS, 4% (*w*/*v*) skimmed milk powder and 0.1% (v/v) Tween-20) at room temperature for 60 min. Polyclonal antibodies were used for protein detection. The presence of the target protein was detected on the membrane by chemiluminescence under a CCD camera (Intelligent Dark Box II, Fujifilm Corp.). The relative protein expression level was determined by Image-J (https://imagej.nih.gov/ij/). Fold changes of each protein at different time points were analyzed by setting F phase as 1.

Cluster and correlation analysis

Hierarchical cluster analyses were performed using the Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) by the uncentered correlation and average linkage method and the resulting tree figures were displayed using Java Treeview (http://jtreeview.sourceforge.net/) (Shi et al. 2014).

Correlation analysis was done in Excel (Microsoft 2010). Electrolyte leakage is given on the *x*-axis and the measurements for the other parameters are given on the *y*-axis (Shi et al. 2012).

Statistical analysis

SPSS 20.0 software (IBM, USA) was used in this study for statistical analyses. The differences were performed by one-way ANOVA, and at least three independent biological replicates and three to five technical replicates within each biological experiment were done for each parameter. The results shown are mean ± SE. The vertical bars with different lower-case letters are significantly different from each other at $P < 0.05$.

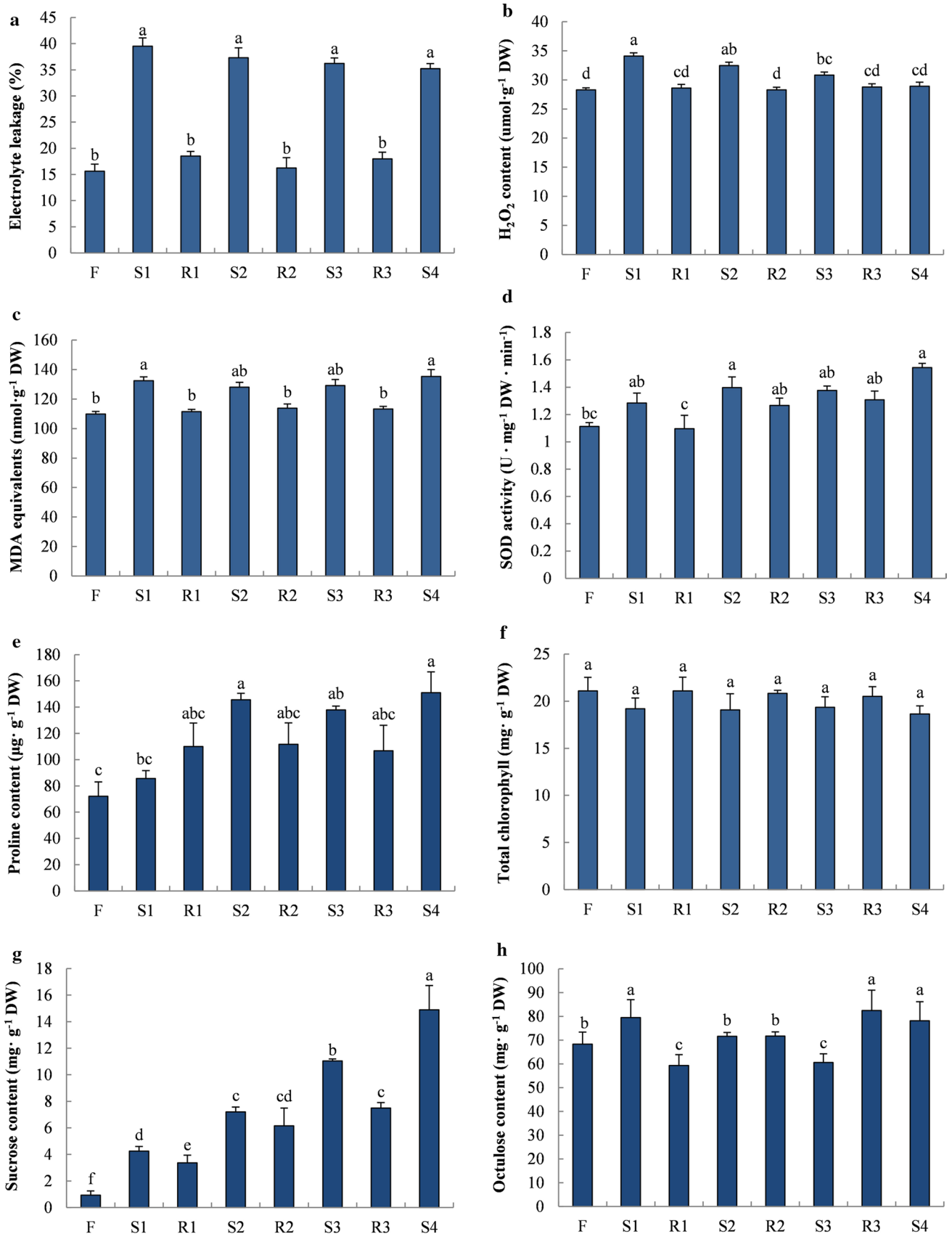
Results

Dehydration stress-trained *C. plantagineum* plants

The phenotype of *C. plantagineum* plants subjected to four cycles of 6 h air-drying and 18 h rehydration is shown in Fig. 1b. During the four consecutive cycles of stress (S1–S4) and recovery (R1–R3), the plants lost water faster and wilted faster in the S1 phase than plants in the S2, S3, and S4 phases (Fig. 1c). The RWC in the R1, R2 and R3 phases were always similar to the RWC of untreated leaves (F) (Fig. 1d). This shows that the plants had fully recovered after each rehydration (R1, R2 and R3), although they had lost water during each air-drying treatment.

Changes in physiological parameters during repeated dehydration and rehydration in leaves of *C. plantagineum*

After repetitive dehydration-stress/recovery, electrolyte leakage and H_2O_2 content decreased from the S1 phase to the S4 phase. The H_2O_2 content at the S4 phase did not differ from the untreated state. Electrolyte leakage and H_2O_2 content stayed at the same level as in the untreated state during all phases of recovery (Fig. 2a, b). MDA levels, SOD activity and proline content gradually increased during the four dehydration treatments and remained at a relatively low level in F and R1–R3 phases (Fig. 2c–e). The increase of the


a
Electrolyte leakage (%)
b
Hydrogen peroxide content (umol·g-1 DW)
c
MDA equivalents (nmol·g-1 DW)
d
SOD activity (U · mg-1 DW · min-1)
e
Proline content (μg· g-1 DW)
f
Total chlorophyll (mg· g-1 DW)
g
Sucrose content (mg· g-1 DW)
h
Octulose content (mg· g-1 DW)
F
S1
R1
S2
R2
S3
R3
S4

◂**Fig. 2** Quantitative comparison of physiological parameters in *C. plantagineum* during multiple exposures to dehydration. Changes in electrolyte leakage and H_2O_2 content during four consecutive cycles of stress (S1–S4) and recovery (R1–R3) treatments (**a**, **b**). Changes in MDA content and SOD activity during four dehydration and recovery treatments (**c**, **d**). Changes in proline and total chlorophyll content during four stress/recovery treatments (**e**, **f**). Changes of sucrose and octulose content were analyzed during four dehydration and recovery treatments (**g**, **h**). Values in **a**–**f** were calculated from three independent biological repetitions and five technical repetitions within each biological experiment (mean ± SE); and values in (**g**, **h**) were calculated from three independent biological repetitions and three technical repetitions within each biological experiment (mean ± SE). The vertical bars with different lower-case letters are significantly different from each other at $P < 0.05$ (one-way ANOVA). Other details are given in Materials and methods

MDA and SOD activity was similar during the four dehydration phases, respectively (Fig. 2c, d). The total chlorophyll content did not change significantly throughout the treatments (Fig. 2f).

Changes in sucrose and octulose are major metabolic alterations in *C. plantagineum* during dehydration/rehydration (Bianchi et al.1991). Therefore, sugars were analyzed during the recurrent stress experiment according to Zhang et al. (2016). The sucrose content gradually increased during the dehydration stages, even though the content declined at each recovery stage, while the octulose content stayed at a high level during the whole process (Fig. 2g, h, Fig. S1, Fig. S2).

Correlation of electrolyte leakage with other physiological parameters under the repetitive dehydration and rehydration conditions

The correlation was analyzed between electrolyte leakage and other physiological parameters during four cycles of dehydration. The leaf RWC and chlorophyll content showed a negative linear correlation with the electrolyte leakage during the dehydration process ($R^2 = 0.99$, 0.89, respectively) (Fig. 3a, b) which means an association with dehydration-induced cell injury in *C. plantagineum* during the S1–S4 phases. However, there was no linear correlation between electrolyte leakage and the accumulation of SOD activity and osmolytes (proline, sucrose and octulose) ($R^2 = 0.47$, 0.26, 0.27, and 0.02, respectively) (Fig. 3c–f). A positive correlation was observed for the MDA and H_2O_2 content with electrolyte leakage ($R^2 = 0.92$, 0.66, respectively) (Fig. 3g, h).

Transcript accumulation during recurrent stress treatments

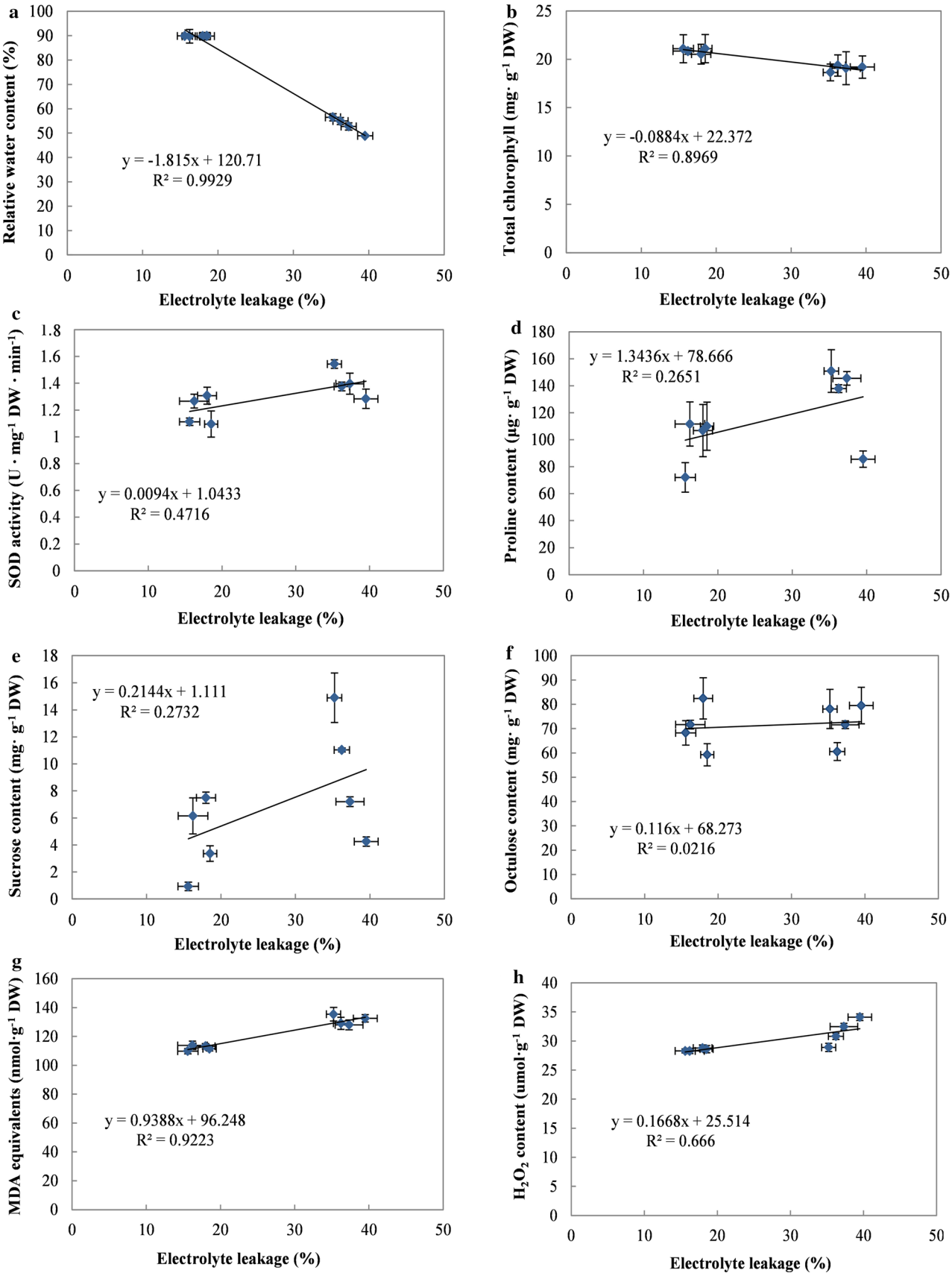
Transcript levels of four selected stress-induced genes were determined during the repeated dehydration/rehydration treatments (Fig. 4). Figure 4a shows the transcript abundance of late embryogenesis abundant (LEA) genes (*LEA-like 11-24*, *LEA2 6-19* and *LEA 13-62*), and early dehydration responsive 1 (*EDR1*). The results show that after S1 the later dehydration treatments induced higher expression levels of the four selected genes than during the first dehydration. The *LEA-like 11-24*, *LEA2 6-19* and *EDR1* gene reached the highest expression level at the S2 stage, whereas the *LEA 13-62* gene had a transcription peak at the S3 stage during the four cycles of dehydration. The four stress-induced transcripts accumulated during the repeated dehydration treatments in the S1, S2, S3 and S4 phases (Fig. 4b–e).

A decline of photosynthesis during dehydration and its recovery during rehydration is characteristic for the homiochlorophyllous resurrection plant *C. plantagineum*. Therefore, chlorophyll synthesis and degradation-related genes were analyzed during the dehydration/rehydration cycles. Transcript abundance of chlorophyll synthesis-related genes is shown in Fig. 5a. During dehydration the transcript levels of Mg protoporphyrin IX methyltransferase (*CHLM*), porphobilinogen deaminase (*PBGD*), glutamate-1-semialdehyde aminotransferase (*GSA*), 5-aminolevulinic acid dehydratase (*ALAD*), coprogen oxidase (*CPO*) and chlorophyll synthase (*CHLG*) decreased and during rehydration the transcripts of all six genes remained at a similar level as in the untreated state, except for *PBGD*, *GSA* and *CPO* which were reduced in R1, R3, R1 and R2, respectively (Fig. 5b–g).

The changes in transcript abundance of the chlorophyll degradation-related genes red chlorophyll catabolite reductase (*RCCR*), pheophorbide a oxygenase (*PaO*), pheophytinase (*PPH*) and non-yellowing 1 (*NYE1*) showed an opposite trend to the synthesis-related genes during four cycles of dehydration. During the dehydration treatments, the transcript levels of the four genes (*RCCR*, *PaO*, *PPH* and *NYE1*) increased at each time point, except for *PaO* in the S4 phase which was not significantly different from the untreated state (Fig. 6).

As markers for stress defense, four ROS pathway-related genes, i.e., manganese superoxide dismutase (*Mn-SOD*), copper/zinc superoxide dismutase (*Cu/Zn-SOD*), catalase (*CAT*) and ascorbate peroxidase (*APX*) were analyzed. The transcript patterns of the four genes changed at each time point (Fig. 7a). At the S4 stage, the transcript levels of *Mn-SOD*, *Cu/Zn SOD* and *CAT* were reduced compared to the untreated state, whereas the *APX* transcript did not change between the S4 and F phases (Fig. 7b–e).

To compare the transcripts of the different pathways, a hierarchical cluster analysis was done. The graph shows that the transcript levels of the four stress-related genes (*LEA-like 11-24*, *LEA2 6-19*, *EDR1* and *LEA 13-62*) accumulated throughout dehydration treatments. However, the genes (*CHLM*, *PBGD*, *GSA*, *ALAD*, *CPO*, *CHLG*, *RCCR*, *PaO*, *PPH*, *NYE1*, *Mn SOD*, *Cu/Zn SOD*, *CAT* and *APX*) stayed at


a
Relative water content (%)
y = -1.815x + 120.71
R² = 0.9929
Electrolyte leakage (%)
b
Total chlorophyll (mg· g-1 DW)
y = -0.0884x + 22.372
R² = 0.8969
Electrolyte leakage (%)
c
SOD activity (U · mg-1 DW · min-1)
y = 0.0094x + 1.0433
R² = 0.4716
Electrolyte leakage (%)
d
Proline content (μg· g-1 DW)
y = 1.3436x + 78.666
R² = 0.2651
Electrolyte leakage (%)
e
Sucrose content (mg· g-1 DW)
y = 0.2144x + 1.111
R² = 0.2732
Electrolyte leakage (%)
f
Octulose content (mg· g-1 DW)
y = 0.116x + 68.273
R² = 0.0216
Electrolyte leakage (%)
g
MDA equivalents (nmol·g-1 DW)
y = 0.9388x + 96.248
R² = 0.9223
Electrolyte leakage (%)
h
H2O2 content (umol·g-1 DW)
y = 0.1668x + 25.514
R² = 0.666
Electrolyte leakage (%)

◂**Fig. 3** Correlation between electrolyte leakage and other physiological parameters during multiple exposures to dehydration stress. Correlation of electrolyte leakage with RWC (**a**), total chlorophyll (**b**), SOD activity (**c**), proline content (**d**), sucrose content (**e**), octulose (**f**), MDA content (**g**), and H_2O_2 content (**h**) during four consecutive cycles of dehydration and rehydration treatments. Values in (**a**–**h**) were calculated from three independent biological repetitions and three to five technical repetitions within each biological experiment (mean ± SE)

similar levels or were even reduced by the end of the treatment compared with the untreated state (Fig. 8a).

Expression patterns of stress-induced proteins

The abundance of four stress-induced proteins (LEA-like 11-24, LEA2 6-19, EDR1 and LEA 13-62) was analyzed

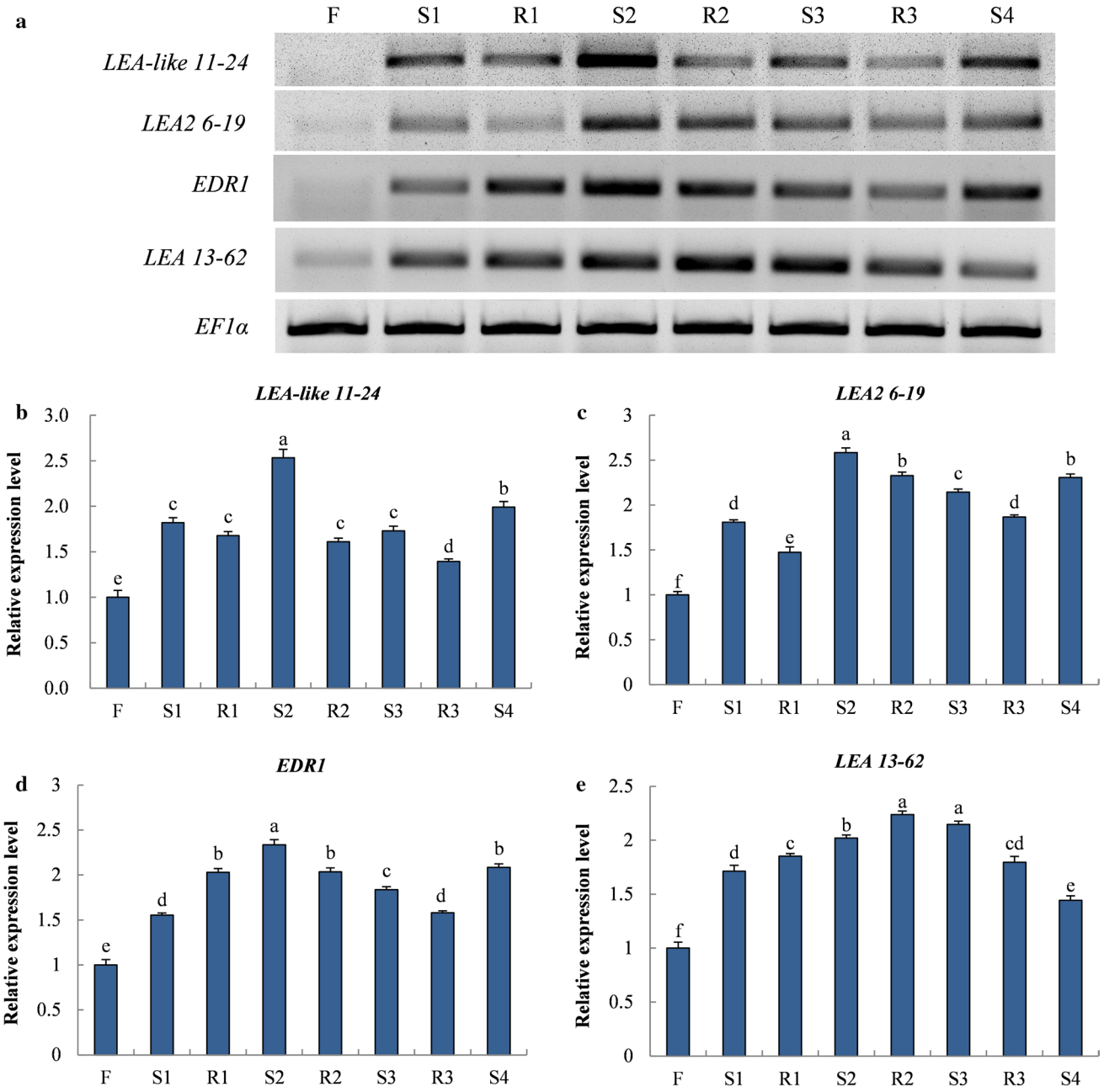
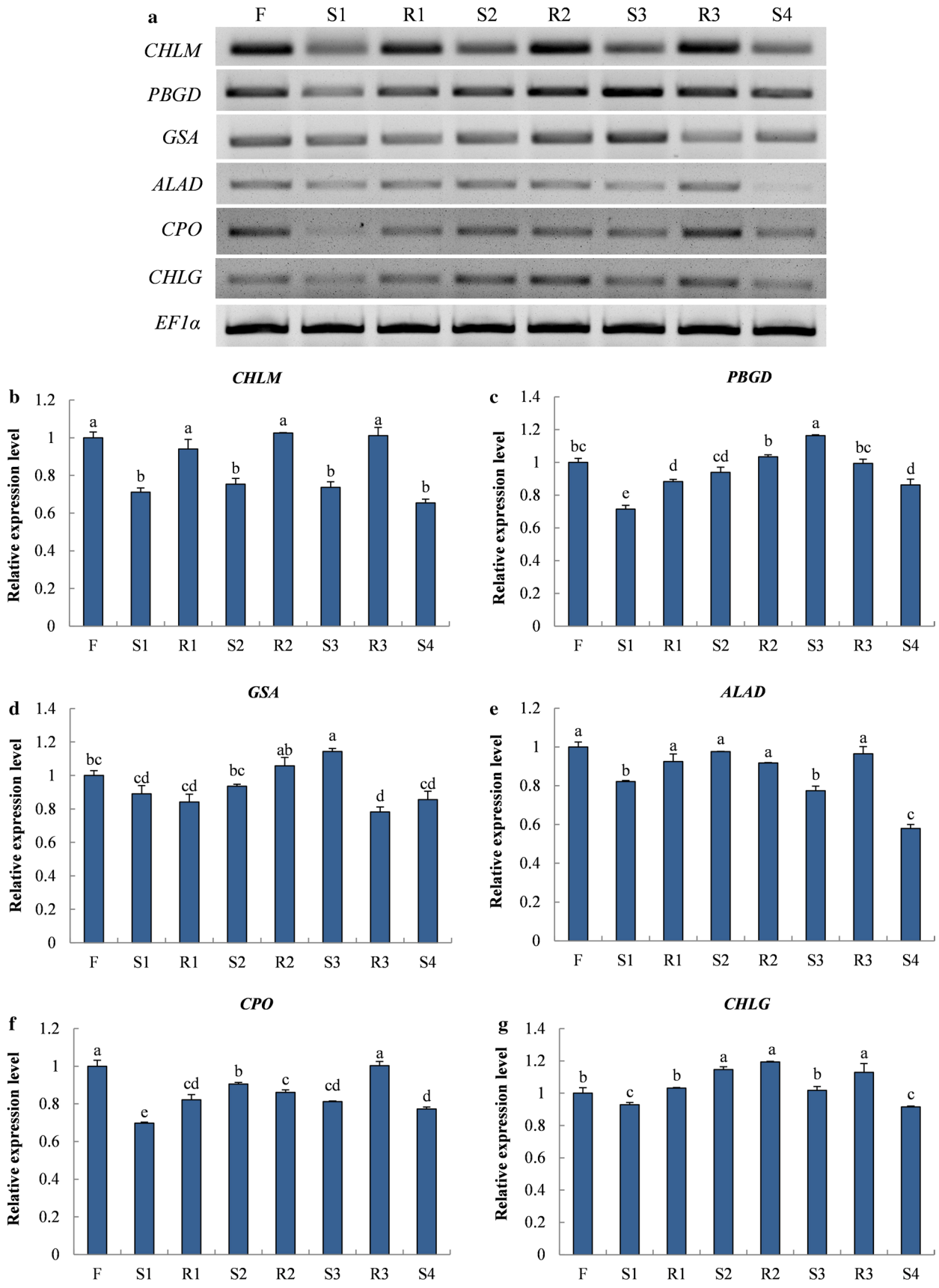


Fig. 4 **a** Transcript levels of stress-induced genes in *C. plantagineum* during multiple exposures to dehydration stress. Transcript abundance of *LEA-like 11–24* (**b**), *LEA2 6–19* (**c**), *EDR1* (**d**) and *LEA 13–62* (**e**) genes measured by semi-quantitative PCR. Fold changes of each gene at different time points were analyzed by setting the level at F phase as 1 using the Image-J software (**b**–**e**). *EF1α* was used as an internal control. Values in (**b**–**e**) were calculated from three independent biological repetitions (mean ± SE). The vertical bars with different lower-case letters are significantly different from each other at $P < 0.05$ (one-way ANOVA)


a
F S1 R1 S2 R2 S3 R3 S4
CHLM
PBGD
GSA
ALAD
CPO
CHLG
EF1α
b
CHLM
Relative expression level
c
PBGD
d
GSA
e
ALAD
f
CPO
g
CHLG

◂**Fig. 5** **a** Transcript levels of chlorophyll synthesis-related genes in *C. plantagineum* during multiple exposures to dehydration stress. Transcript abundance of *CHLM* (**b**), *PBGD* (**c**), *GSA* (**d**), *ALAD* (**e**), *CPO* (**f**) and *CHLG* (**g**) genes was measured by semi-quantitative PCR. Fold changes of each gene at different time points were analyzed by setting the level at F phase as 1 using Image-J software (**b**–**g**). *EF1α* was used as an internal control. Values in (**b**–**g**) were calculated from three independent biological repetitions (mean ± SE). The vertical bars with different lower-case letters are significantly different from each other at $P < 0.05$ (one-way ANOVA)

by immunoblots (Fig. 9). Equal loading of proteins was monitored by staining the membrane with Ponceau S (red bands) (Fig. 9a). The relative fold change of each protein was measured by setting the untreated state as 1. The results showed that the abundance of the four stress-induced proteins gradually increased during four cycles of dehydration. The abundance of LEA-like 11–24, LEA2 6–19 and EDR1 proteins increased and reached the highest level at S4. The

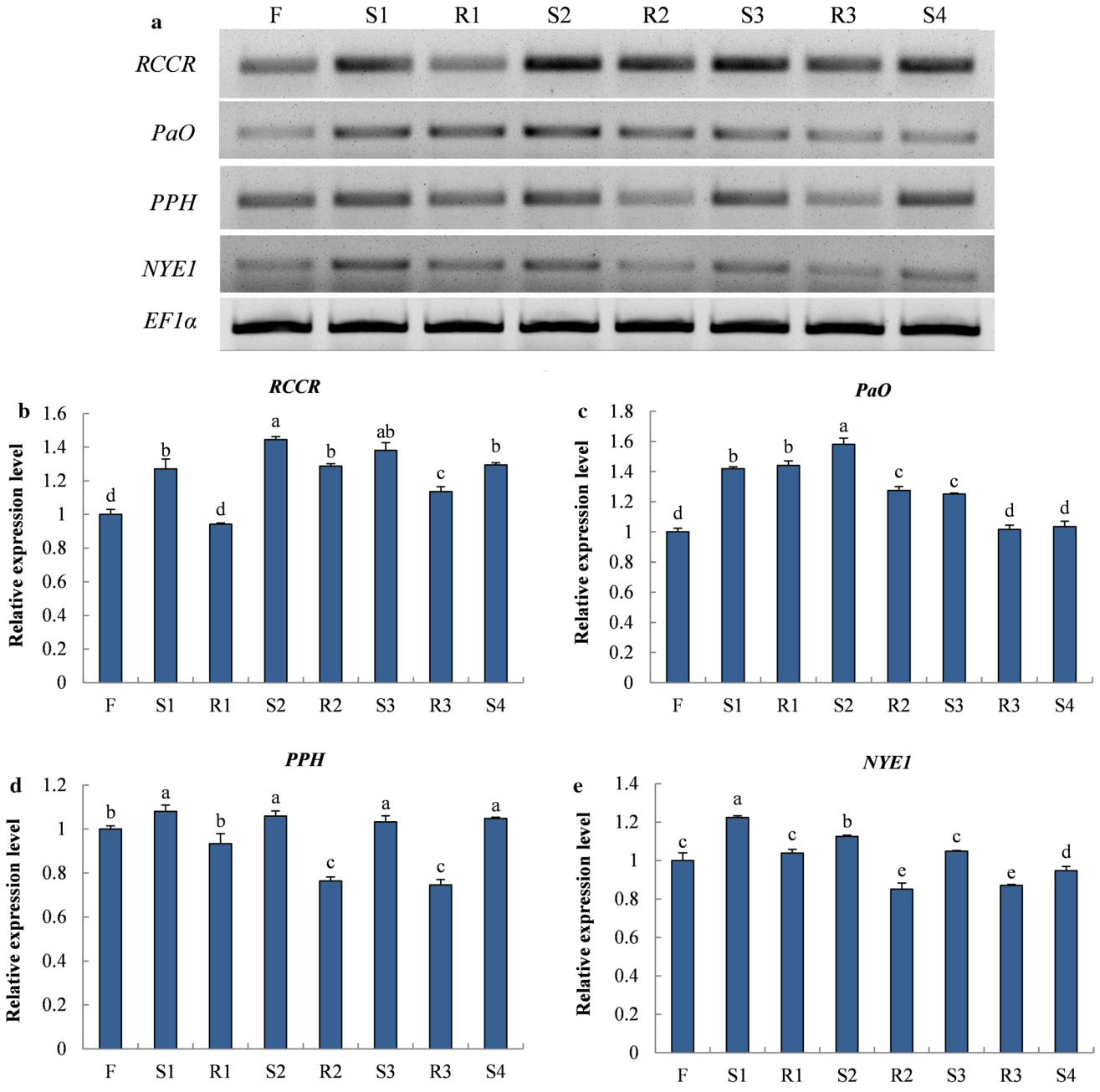


Fig. 6 **a** Transcript levels of chlorophyll degradation-related genes in *C. plantagineum* during multiple exposures to dehydration stress. Transcript abundance of *RCCR* (**b**), *PaO* (**c**), *PPH* (**d**) and *NYE1* (**e**) genes was measured by semi-quantitative PCR. Fold changes of each gene at different time points were analyzed by setting the level at F phase as 1 (**b**–**e**). *EF1α* was used as an internal control. Values in (**b**–**e**) were calculated from three independent biological repetitions (mean ± SE). The vertical bars with different lower-case letters are significantly different from each other at $P < 0.05$ (one-way ANOVA)

expression level of LEA 13–62 had a peak at the S2 phase, and then gradually decreased in the following treatments, but the expression level in S4 was still higher than that at the untreated state (Fig. 9b–e). A hierarchical cluster analysis of the four stress-induced proteins showed that the abundance of LEA-like 11–24, LEA2 6–19 and EDR1 proteins increased during the repeated dehydration treatments except for LEA13–62, which slightly decreased after the R2 phase, but was still higher than that at the untreated state (Fig. 8b). These results demonstrate that the stress-related proteins increased during the treatment and the protein changes correlate directly with the transcript levels.

Dehydration stress memory

The question was raised as how long the stress memory can persist in the absence of stress stimulation. The protein abundance of four stress-induced genes is shown in Fig. 10a. The LEA-like 11-24 and LEA 13-62 proteins were retained for 2 and 4 days, and the relative protein levels at R2d and R4d were both significantly higher than at the untreated state, but at R6d the expression level of the two proteins did not differ from the untreated state (Fig. 10b, e). The EDR1 protein persisted for 3 days, and the protein disappeared after 4 days of re-watering (Fig. 10d). The LEA2 6–19 protein gradually decreased from R2d to R6d, but was significantly higher during re-watering conditions than in the untreated state (Fig. 10c).

Discussion

In nature, plants are usually exposed to repeated cycles of dehydration that differ in duration and intensity. Responses of plants to reiterated dehydration are different from those to a single exposure to dehydration (Fleta-Soriano and Munné-Bosch 2016). Previous stress encounters are remembered through the accumulation of transcripts and some metabolites (involved in carbon and energy metabolism as well as amino acid or fatty acid biosynthesis and others) after pre-exposure to stress conditions (Bruce et al. 2007; Hu et al. 2016; Sun et al. 2018).

Metabolites during dehydration/rehydration

Minimizing water loss is a key factor for survival of plants experiencing dehydration, and plants can acclimate to dehydration by reducing the water loss rate (Yoo et al. 2010; Hu et al. 2012; Shi et al. 2012). During the four repetitive dehydration/rehydration treatments in this study, the water loss rate in *C. plantagineum* leaves decreased from S1 to S4, and the RWC reached the same level as in the untreated state after each recovery (R1, R2 and R3) (Fig. 1c, d). A similar trend was also observed in desiccation-sensitive *A. thaliana* plants during mild dehydration and rehydration conditions (Ding et al. 2012).

During dehydration the electrolyte leakage is increased due to disturbance of cell membranes (Zhao et al. 2011). Oxidative stress is caused by excessive production of ROS which can lead to damage of nucleic acids and proteins as well as lipid peroxidation. The SOD activity increases to scavenge the over-production of ROS and protects plants from oxidative damage, functioning as the first stage of antioxidant defense (Mittler 2002; Apel and Hirt 2004; Mittler et al. 2004; Liu and Chan 2015). In the study presented here, the SOD activity gradually increased during S1–S4, the levels of electrolyte leakage and H_2O_2 content decreased and no significant changes of MDA content were observed during the four cycles of dehydration treatments (Fig. 2a–d). Repeated dehydration did not lead to increased accumulation of stress metabolites, but the opposite behavior was observed which implies that repeated stress treatments make the *C. plantagineum* plants more robust to dehydration.

Osmotic adjustment and cellular compatible solute accumulation are widely recognized as plant adaptation to dehydration (Blum 2017). According to previous studies, high proline content provides an advantage for plants under dehydration stress (Lu et al. 2009; Zhao et al. 2011). An increase of proline and sucrose was observed in *C. plantagineum* leaves during the four cycles of dehydration and rehydration (Fig. 2e, g). Electrolyte leakage as an indicator of cell membrane stability has been used to evaluate the extent of cell injury (Hu et al. 2010; Shi et al. 2012). The analysis of the H_2O_2 and MDA values as well as RWC and total chlorophyll content showed the highest positive and negative correlation with electrolyte leakage, respectively (Fig. 3).

Gene expression patterns

Multiple consecutive stress/recovery treatments can alter transcriptional responses to a subsequent stress (Goh et al. 2003; Ding et al. 2012; Liu et al. 2014, 2016; Hu et al. 2016). Genes involved in ABA/abiotic stress responses are signatures for memory genes in which trainable LEA genes represent a major component (Ding et al. 2013). The genes *LEA 2 6-19* and *LEA-like 11-24* are involved in ABA signaling pathways (Michel et al. 1994; Van den Dries et al. 2011). The increasing accumulation of transcripts and proteins of stress-related genes and an altered accumulation of dehydration-responsive metabolites during repetitive exposures to dehydration suggest that an earlier stress exposure is memorized by *C. plantagineum* (Figs. 2, 4 and 9). The hierarchical cluster analysis of the accumulation of stress-related genes/proteins during repetitive dehydration and rehydration indicates the formation of a stress memory during the accumulation process (Fig. 8). It has been suggested that the stress

memory improves tolerance to subsequent stress exposures. This includes tolerance to dehydration-related stresses but it may also be beneficial for the desiccation-tolerant plants to cope with other stresses than dehydration.

Photosynthesis-related processes

Photosynthesis is very sensitive to dehydration stress in both desiccation-tolerant and -sensitive species (Challabathula et al. 2018). The photosynthetic apparatus is susceptible to injury during dehydration but can quickly be repaired upon rehydration and photosynthetic activity is

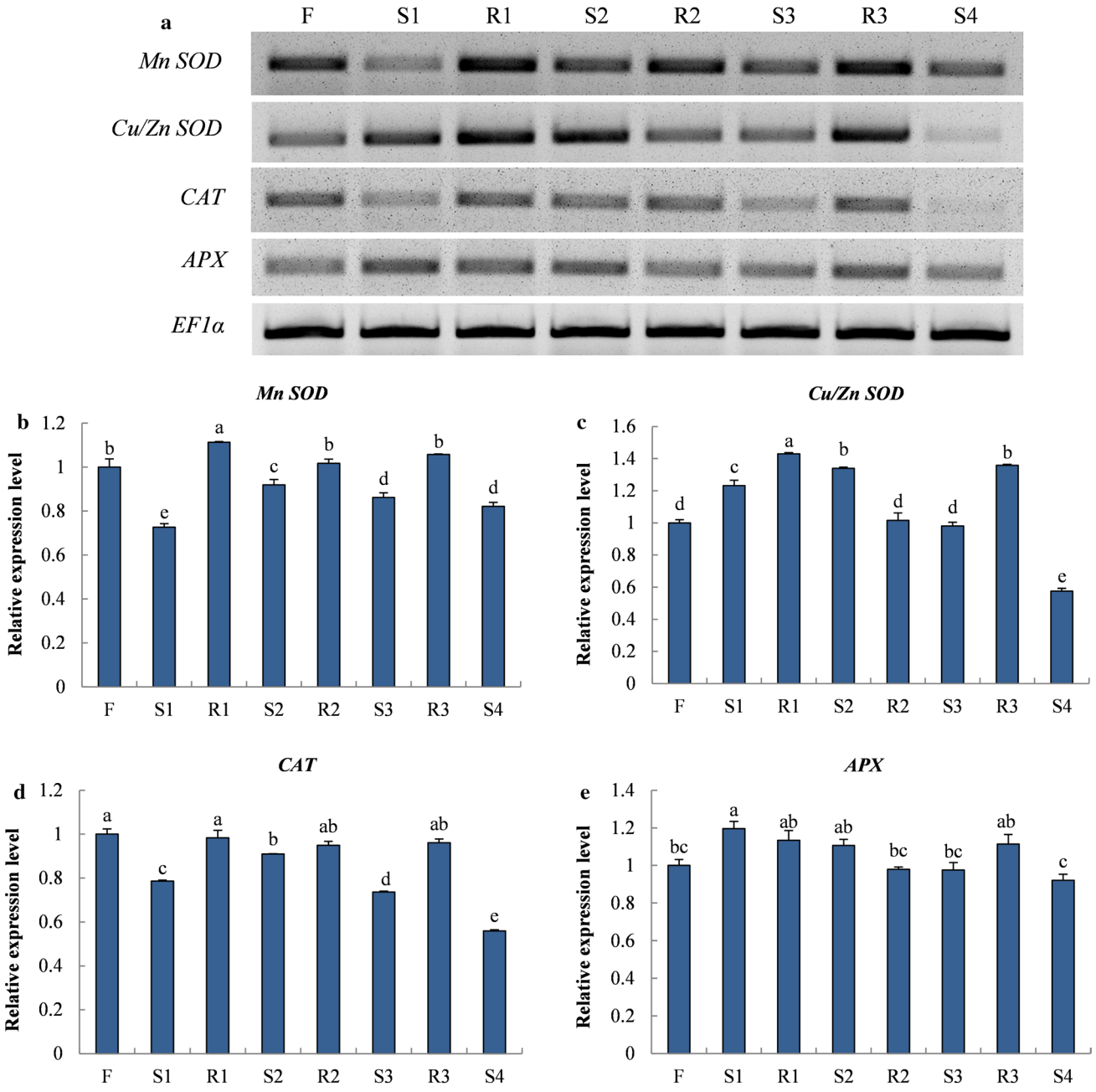


Fig. 7 **a** Transcript levels of ROS pathway-related genes in *C. plantagineum* during multiple exposures to dehydration stress. Transcript abundance of *Mn-SOD* (**b**), *Cu/Zn-SOD* (**c**), *CAT* (**d**) and *APX* (**e**) genes was measured by semi-quantitative PCR. Fold changes of each gene at different time points were analyzed by setting the level at F phase as 1 (**b**–**e**). *EF1α* was used as an internal control. Values in (**b**–**e**) were calculated from three independent biological repetitions (mean ± SE). The vertical bars with different lower-case letters are significantly different from each other at $P < 0.05$ (one-way ANOVA)

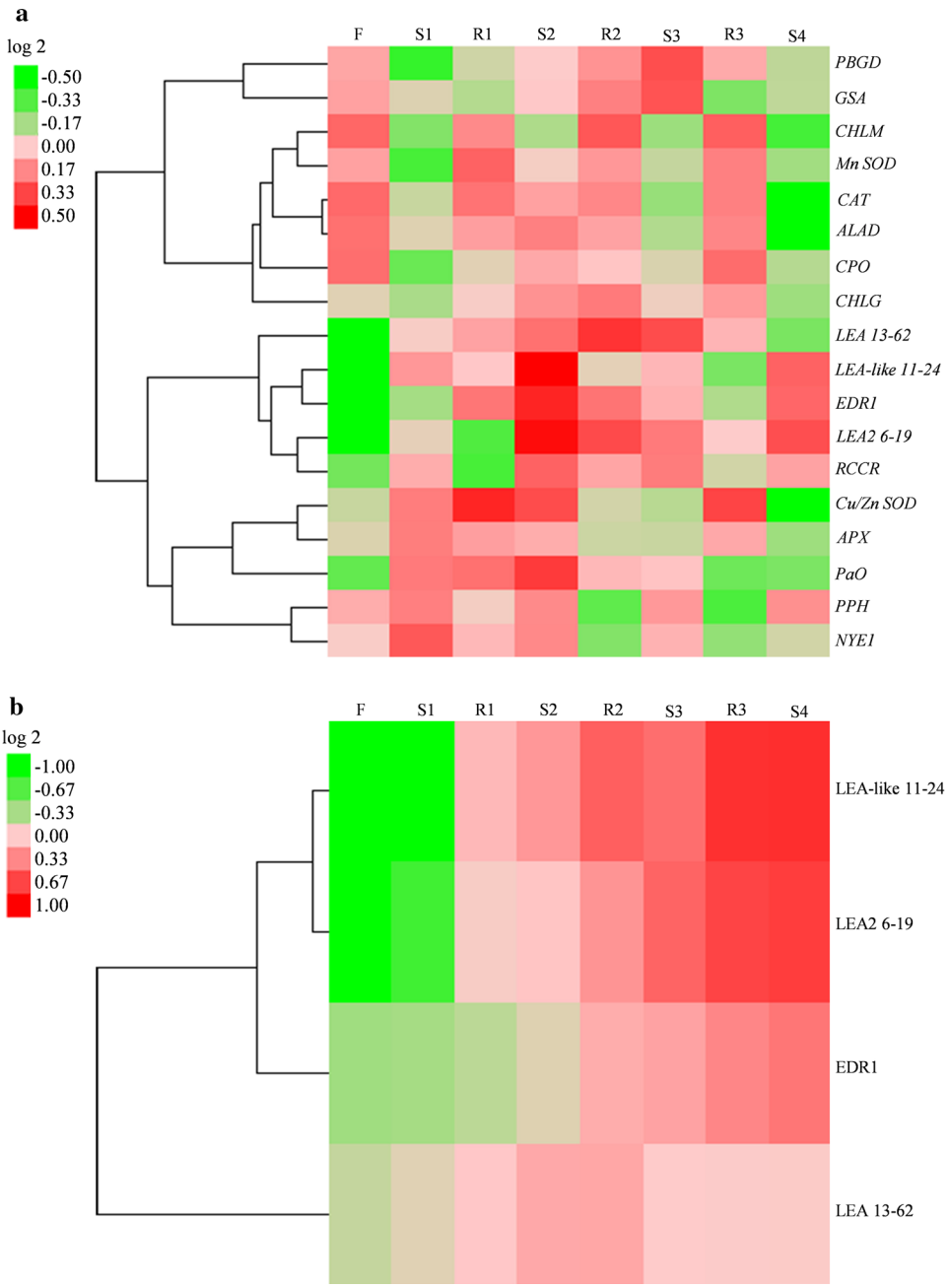


Fig. 8 **a** Hierarchical cluster analyses of different kinds of genes/proteins responding to four cycles of dehydration stress. **b** The relative data of transcript levels of all tested genes were chosen for cluster analysis. The relative expression abundance of four stress-induced proteins was chosen for cluster analysis. All these data were quantified as fold change relative to the untreated state (F) which was set as 1. The tree figure was obtained using Cluster 3.0 software and Java Treeview. The cluster color bar was shown as $\log_2$ fold change

restored in *C. plantagineum* in contrast to most angiosperm plants (Challabathula et al. 2012). Changes in chlorophyll content can be considered as an indicator for plant dehydration tolerance (Li et al. 2006). In our consecutive dehydration/rehydration treatment system, the total chlorophyll content in *C. plantagineum* leaves was at a similar level as the untreated state during the recovery (R1, R2 and R3), indicating that the plants had fully recovered after each treatment which is consistent with phenotypic observations and relative water content measurements (Figs. 1b, d, 2f). Consistently, the transcript levels of chlorophyll synthesis-related genes decreased during dehydration and increased during recovery, whereas degradation-related transcripts and proteins increased at each dehydration stage and declined during recovery (Figs. 5, 6).

Stress memory

The results presented here are in agreement with a dehydration stress memory as already observed in *Arabidopsis* or as observed for drought hardening in horticultural plants (Kozlowski and Pallardy 2002; Ding et al. 2012). Stress memory will fade off, if the signals of stress stimulation disappear. The duration of the transcriptional memory

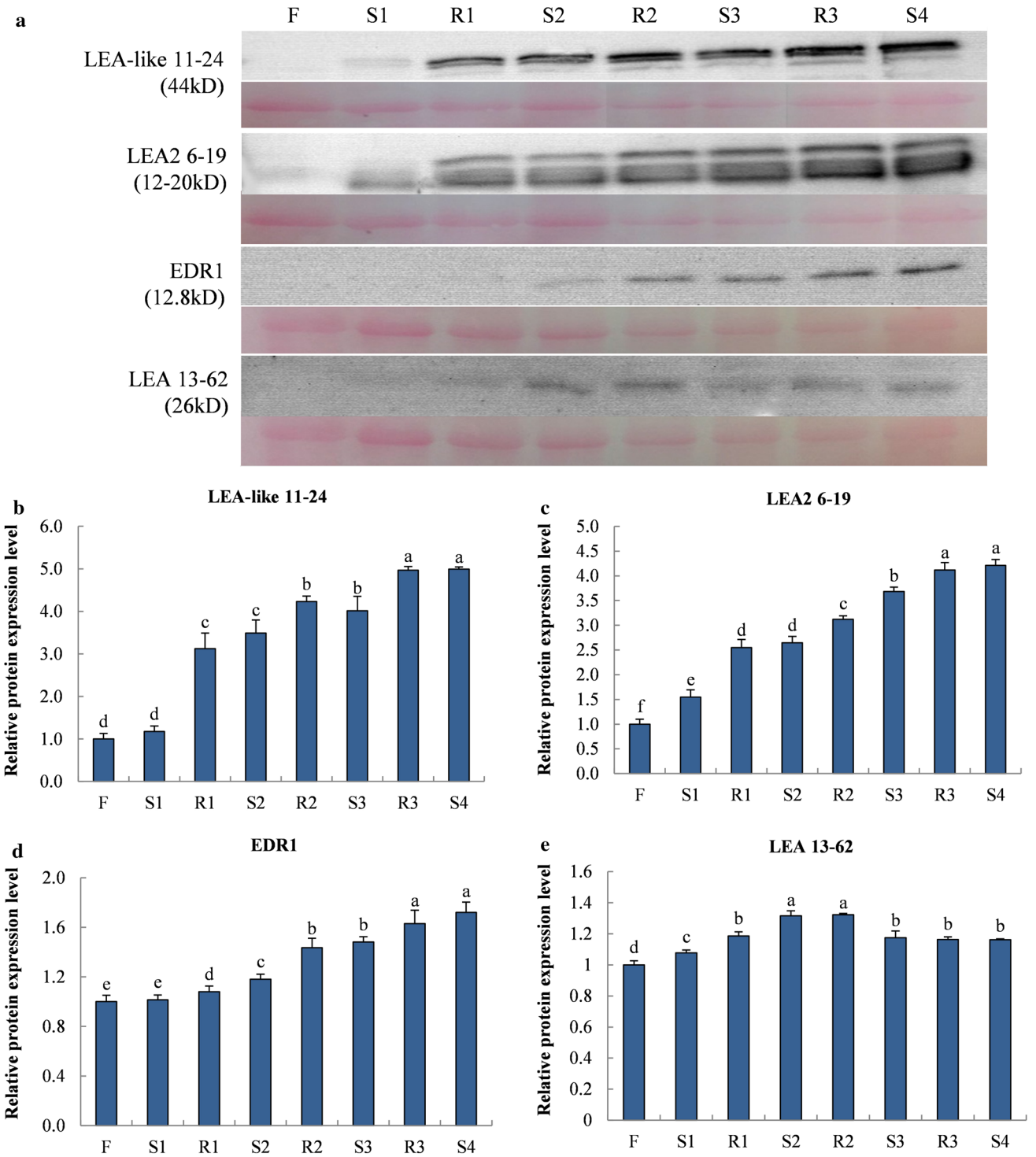


Fig. 9 **a** Expression abundance of stress-induced proteins in *C. plantagineum* during multiple exposures to dehydration stress. **b** Expression levels of LEA-like 11–24, **c** LEA2 6–19, **d** EDR1 and **e** LEA 13–62 proteins were measured by Western blot. Equal loading of proteins was monitored by staining the membrane with Ponceau S (red bands). Fold changes of each protein at different time points were analyzed by setting the level at the F phase as 1 (**b**–**e**). Values in (**b**–**e**) were calculated from three independent biological repetitions (mean ± SE). The vertical bars with different lower-case letters are significantly different from each other at $P < 0.05$ (one-way ANOVA)

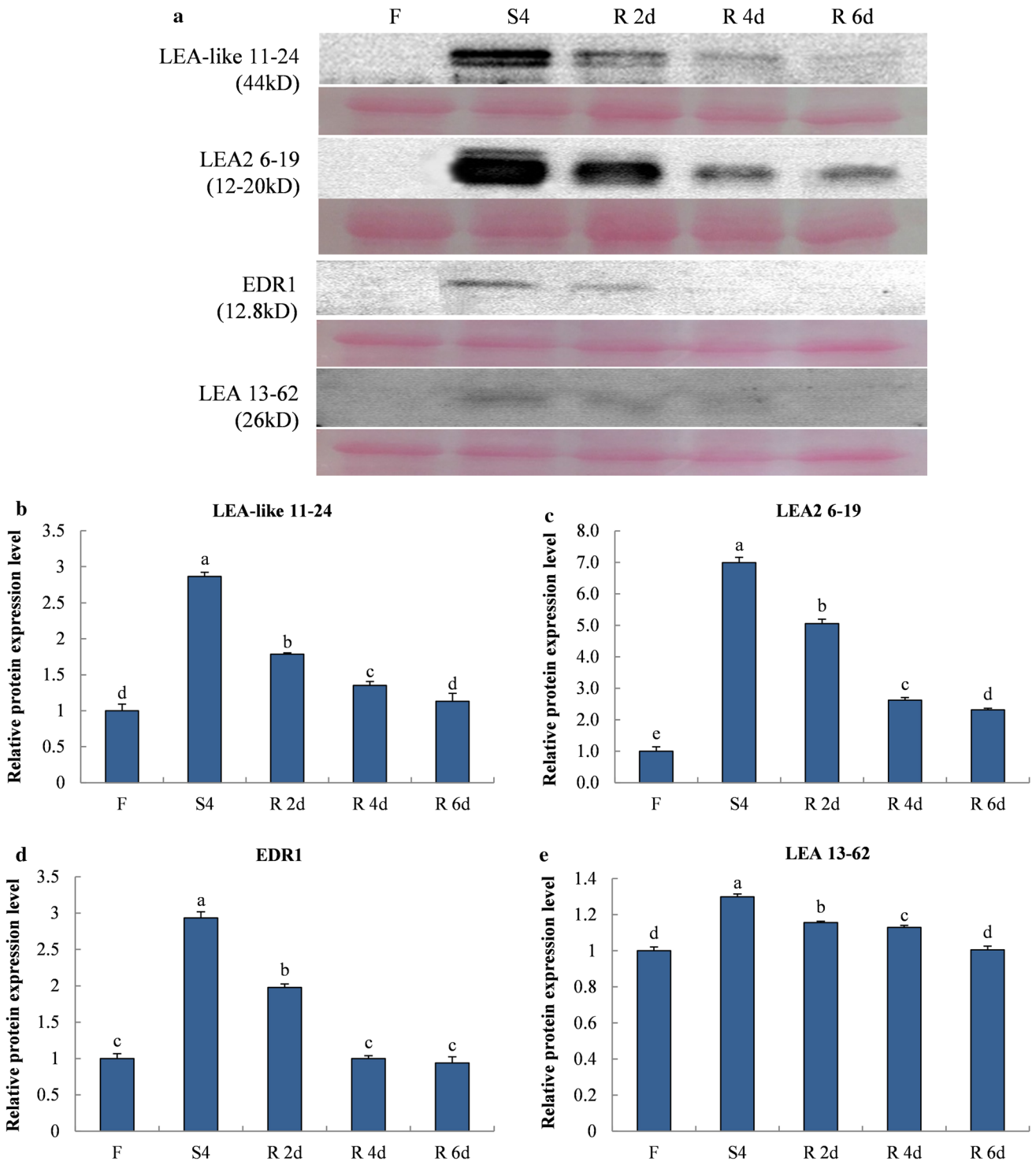


Fig. 10 Dehydration stress memory. **a** Expression levels of LEA-like 11–24 (**b**), LEA2 6–19 (**c**), EDR1 (**d**) and LEA 13–62 (**e**) proteins were analyzed by Western blot after 2d, 4d or 6d recovery. Equal loading of proteins was monitored by staining the membrane with Ponceau S (red bands). Fold changes of each protein at different time points were analyzed by setting the level at the F phase as 1 (**b**–**e**). Values in (**b**–**e**) were calculated from three independent biological repetitions (mean ± SE). The vertical bars with different lower-case letters are significantly different from each other at $P < 0.05$ (one-way ANOVA)

for stress-related genes *RD29B*, *RAB18*, *ACO1*, *JAZ9* and *TRAF1* in desiccation sensitive *Arabidopsis* plants persisted for 5 days under dehydration conditions (Ding et al. 2012; Liu et al. 2016). In another study, the transcriptional memory in trained perennial ryegrass plants sustained for up to 4 days after cycles of salt stress (Hu et al. 2016). In *C. plantagineum* dehydration stress-induced memory responses could persist up to 6 days after the stress stimulation disappeared under the conditions applied here (Fig. 10).

The present study supports the notion that pre-exposure to multiple dehydration treatments could improve the stress response to the subsequent encounter at physiological and molecular levels. Upon the first dehydration signal, plants respond by expressing stress-related transcripts/proteins (Fig. 11). Upon subsequent exposures to stress dehydration responsive transcripts/proteins accumulate further which suggests that plants memorize the first stress and are able to prepare for the subsequent stress. This shows that memory responses exist in *C. plantagineum* for stress-responsive transcripts/proteins and that it takes at least 6 days for the memory to fade off, if the stress is not re-imposed. This phenomenon is most likely a general feature of the dehydration stress memory response in resurrection plants and may induce cross-protection to other environmental cues besides dehydration.

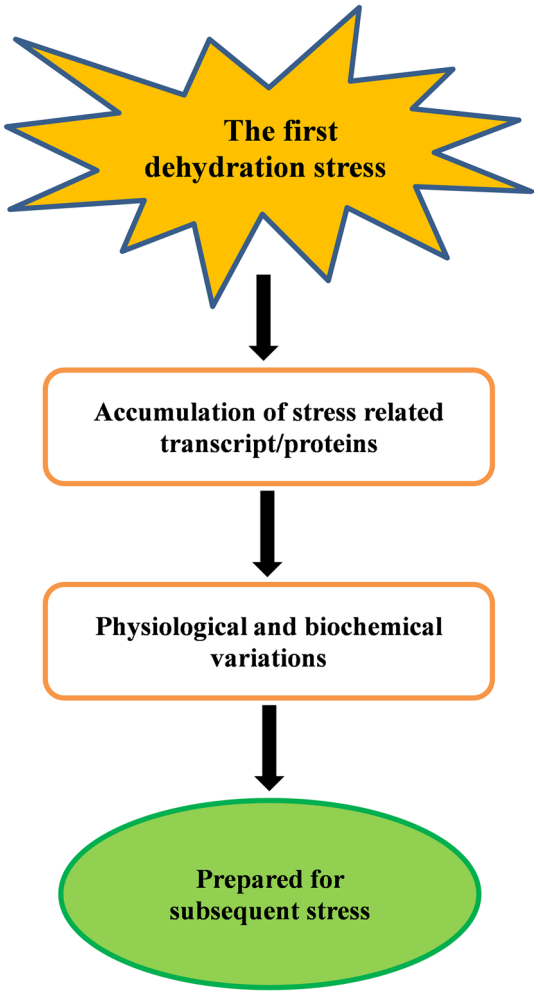


Fig. 11 A model for dehydration stress memory in *C. plantagineum*

Author contribution statement DB, DC and XL conceived and designed the experiments. XL performed the experiments and wrote the article. XL and WQ analyzed the data. DB, DC and WQ revised the article. DB supervised and corrected the writing.

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