Genome-wide analysis of BURP genes and identification of a BURP-V gene RcBURP4 in Rosachienesis

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Abstract

BURP proteins are unique to plants and may contribute greatly to growth, development, and stress responses of plants. Despite the vital role of BURP proteins, little is known about these proteins in rose (*Rosa* spp.). In the present study, nine genes belonging to the BURP family in *R. chienesis* were identified by using multiple bioinformatic approaches against the rose genome database. The nine *RcBURP*s, with diverse structures, were located on all chromosomes of the rose genome, except for Chr2 and Chr3. Phylogenic analysis revealed that these *RcBURP*s can be classified into eight subfamilies, including BNM2-like, PG1β-like, USP-like, RD22-like, BURP-V, BURP-VI, BURP-VII, and BURP-VIII. Conserved motif and exon-intron analyses indicated a conserved pattern within the same subfamily. The presumed *cis*-regulatory elements (CREs) within the promoter region of each *RcBURP* were analyzed and the results showed that all *RcBURP*s contained different types of CREs, including abiotic stress-, light response-, phytohormones response-, and plant growth and development-related CREs. Transcriptomic analysis revealed that a BURP-V member, *RcBURP4*, was induced in rose leaves and roots under mild and severe drought treatments. We then overexpressed *RcBURP4* in *Arabidopsis* and examined its role under abscisic acid (ABA), NaCl, polyethylene glycol (PEG), and drought treatments. Furthermore, *RcBURP4*-silenced rose plants exhibited decreased tolerance to dehydration. The results obtained from this study provide the first comprehensive overview of *RcBURP*s and highlight the importance of *RcBURP4* in rose plant.

Introduction

The environments where plants live are ever-changing and adverse conditions can influence plant growth and development. The unfavorable environmental conditions include internal and external stimuli, such as heat, drought, cold, nutrient deficiency, and excess salt or toxic metals in the soil (Zhu, 2016). Of these, drought stress serves as a main environmental factor, affecting plant natural distribution, limiting plant productivity, and threatening food security (Rampino, 2006). In response, plants have evolved a complicated network involving stress-related pathways that consist of regulatory proteins such as protein phosphatases, protein kinases, and transcription factors, as well as functional proteins including chaperones and signaling components (Zhu, 2002; Broun, 2004; Bohnert et al., 2006; Hirayama & Shinozaki, 2010). Of the two categories of stress-related proteins, functional proteins play a vital role.

The BURP proteins are plant-specific and known as functional proteins, which play an important role in growth, development, and stress responses of plants (Phillips et al., 2017). The BURP domain, comprised of conserved amino acid sequences, is initially named after BNM2, USP, RD22, and PG1β (Hattori et al., 1998). The BURP proteins contain three domains, including a hydrophobic domain at the N-terminus, a C-terminal BURP domain, and a variable internal region specific to individual members. The motifs of C-terminal amino acids in BURP proteins are highly conserved, including certain conserved amino acids and four repeat cysteine-histidine (CH) motifs, namely, CHX10, CHX23-37, CHX23-26, and CHX8W (X = any amino acid residue) (Sun et al., 2019). The *BURP* genes have been investigated among numerous plants, including alfalfa (Li et al., 2016), cotton (Sun et al., 2019), rice (Ding et al., 2009), poplar (Shao et al., 2011), sorghum (Gan et al., 2010), soybean (Xu et al., 2010), and maize (Gan et al., 2010). Phylogenetic

analysis further divided BURP proteins into various subfamilies including BNM2-like, USP-like, RD22-like, PG1β-like, and others.

Many BURP proteins have been identified and classified according to their sequences; however, the knowledge of their functions remains scarce. Previous studies have revealed that *BURP*s may play a vital role in growth, development, and response to abiotic or biotic stress of plants. A fairly large number of *BURP*s were proven to participate in plant response to environmental stimuli. *AtRD22*, a drought-responsive *BURP* gene in *Arabidopsis*, is upregulated by drought and involved in abscisic acid (ABA) signaling and biosynthesis pathways. The *AtRD22* expression is proven to be regulated via the interaction of *AtMYB2* and *AtRD22-BP1* (Abe et al., 2003). The biosynthesis of proteins participating in ABA-mediated gene expression is vital in *AtRD22* response to drought stress (Yamaguchi-Shinozaki et al., 1993). *AtUSP1* is also suggested to be a suppressor in ABA-mediated response to moisture stress (Van Son et al., 2009). In *A. thaliana*, drought stress response is suppressed by proteins encoded by *AtRD22* and *AtUSPL1* belonging to the *BURP* gene family (Harshavardhan et al. 2014). *SBIP-355* of tobacco, a homologous gene to *AtRD22*, may participate in plant defense via the salicylic acid (SA) pathway (Almazroue et al., 2014). *BnBDC1* is specifically expressed in the shoot of *Brassica napus*; it is induced by ABA, mannitol, and NaCl, while suppressed by SA and UV irradiation (Yu et al. 2004). *BgBDC1-4* of *Bruguiera gymnorrhiza*, with sequences similar to *AtRD22*, are upregulated by drought, ABA, and high salt treatments (Banzai et al. 2002). The above findings suggest that *BURP*s possess critical functions in response to abiotic stimuli and may participate in phytohormone signaling pathways, such as ABA and SA.

Rose (*Rosa* spp.), as one of the most important horticultural and economic crops, is characterized by high commercial values in pharmaceutical and perfume industries (Raymond et al. 2018). Rose plants are usually influenced by adverse conditions (Jiang et al. 2009), among which drought is the main factor affecting the growth, development, and survival of rose plants. Drought stress also restricts the fully utilizations of rose plants, especially in arid or semi-arid areas. The rose genome has been sequenced; however, a comprehensive investigation of *BURP*s in the rose genome has not been performed. In this study, we carried out a genome-wide identification of *RcBURP*s and investigated their structures, chromosomal localizations, *cis*-regulatory elements (CREs), and phylogeny. In addition, we overexpressed *RcBURP4* in *Arabidopsis* and examined its role under ABA, NaCl, polyethylene glycol (PEG), and drought stress. We also silenced *RcBURP4* in rose using the virus-induced gene silencing (VIGS) method. The results obtained from this study reveal the critical role of *RcBURP*s in rose response to drought stress and unravel the molecular mechanism of *RcBURP4*, providing a theoretical foundation for future studies on the role of *RcBURP*s.

Materials And Methods

Identification and sequence analysis of RcBURPs

The hidden Markov model (HMM) profile of the BURP domain (PF03181) was downloaded from the Pfam database (Finn et al., 2016). Putative RcBURP proteins were searched using HMMER 3.0 with a threshold of $1e^{-10}$ against the *R. chinensis* (Raymond et al., 2018) and *R. multiflora* genomes (Nakamura et al., 2017). The online program Expasy (https://web.expasy.org/compute_pi/) was used to determine the molecular weight (Mw) and isoelectric point (pI) of identified RcBURP proteins. The subcellular localizations and potential signal peptides of RcBURPs were predicted by using CELLO v2.5 server (http://cello.life.nctu.edu.tw/) (Yu et al., 2006) and SignalP 4.0 server (Wilkins et al., 1999), respectively.

Analyses of gene structure and conserved motifs

The structure (intron/exon) of the *RcBURP* genes was predicted by TBtools (Chen et al., 2020) based on the comparison between the coding sequence (CDS) and the genomic sequence. The online MEME program (Bailey et al., 2009) was used to analyze the conserved motifs of RcBURP proteins; the parameter settings were: the occurrence rate of a single motif was no greater than one per sequence; the motif width was between 10 and 300 amino acids; the maximum number of identified motifs was 10; all other parameters were default.

Multi-sequence alignment, phylogenetic, and CRE analyses

Approximately 166 presumed BURP proteins from 11 plant species, including *R. chinensis*, *R. multiflora*, *Gossypium raimondii*, *G. arboreum*, *G. hirsutum*, *A. thaliana*, *Glycine max*, *Oryza sativa*, *Populus trichocarpa*, *Zea mays*, and *Sorghum bicolor*, were aligned by ClustalX v2.0 with default parameters (Larkin et al., 2007). MEGA X (Kumar et al., 2018) was utilized to build a phylogenetic tree using the neighbor-joining (NJ) method, the p-distance model, and 2000 bootstrap replications. The resulting phylogenic tree was visualized in IToL v5.7 (http://itol.embl.de) (Letunic et al., 2019). CREs were predicated from approximately 2000 bp upstream of the transcription start site (TSS) of each *RcBURP* with the PlantCARE database (Lescot et al., 2002). The three-dimensional (3D) structure of RcBURPs was predicted by Phyre2 (Kelley et al., 2015) homology modeling.

Chromosomal distribution, gene duplication, and syntenic analyses

Chromosomal distributions of *RcBURP*s were determined by searching against the rose genome database with Circos (Krzywinski et al., 2009). Gene duplication events were examined with MCScanX (Wang et al., 2012) with default parameters. The syntenic relationships among the orthologous *BURP*s obtained from *R. chinensis*, *Fragaria vesca*, and *Malus domestica* were generated using Dual Systeny Plotter (Chen et al., 2020). KaKs_Calculator v2.0 (Chen et al., 2020) was adopted to calculate the non-synonymous (Ka) and synonymous substitution rate (Ks) of each duplicated *BURP* gene, and the results were visualized in TBtools.

Transcriptomic analysis

The expression profiles of *RcBURP*s have been generated from our previous study (Li et al., 2020). Gene expression was measured by the reads per kilobase per million mapped reads (RPKM) value for each

RcBURP. The expression level was normalized by log2 and visualized in TBtools.

Vector construction and Arabidopsis **plant transformation**

The full-length CDS of *RcBURP4* was cloned and recombinated into the pCAMBIA1300 vector controlled by the cauliflower mosaic virus (CaMV) 35S promoter. The binary vector was introduced into the *Agrobacterium tumefaciens* strain GV3101, which was then transformed into *A. thaliana* (Col-0) using the floral dip method (Clough and Bent 1998). Seeds of transgenic *A. thaliana* were collected and sown on Murashige and Skoog (MS) medium containing 50 mg L^{-1} hygromycin. Surviving plants were obtained, and the *RcBURP4* expression was confirmed with qRT-PCR using specific primers (Supplementary Table 1). Three independent transgenic lines (OE# 1, 2, and 3) were used for further analyses.

Seed germination and root phenotype assays

For seed germination assays, 20 seeds from each of the three *RcBURP4*-overexpressing lines (OE# 1, 2, and 3) and vector control (VC) were incubated on MS media containing 0, 0.5, or 1.0 μM ABA; 0, 100, 150, or 200 mM NaCl; or 0, 4%, 8%, or 12% (w/v) PEG6000; respectively. The seed germination was defined as cotyledon turns green and recorded after 7 d.

For root growth phenotyping, the sterilized seeds from VC and *RcBURP4*-OEs were placed on MS solid media. Seeds were vernalized at 4℃ for 3 d, and then transferred to normal growth conditions (light intensity: 100 mol m^{-2} s^{-1}; relative humidity: 40–65 %; 23 ± 2 ℃; 16/8 h light/dark) for 7 d. One-week-old seedlings were transferred to MS plates supplemented with NaCl (0, 100, or 200 mM) or ABA (0, 30, or 50 μM). After incubation for 10 d, the primary root length and lateral root number were measured by ImageJ (http://rsbweb.nih.gov/ij/). Six plant individuals of each treatment was used, and the experiments included at least three replicates.

In situ histochemical localization of hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-)

The H2O2 and $O2^-$ levels were determined in one-week-old seedlings of *RcBURP4*-OEs and VC by diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining (Shi et al., 2010). *RcBURP4*-OE and VC seedlings were immersed into 200 mM NaCl or 50% (w/v) PEG6000 for 1 h, with H_2O as control. For H_2O_2 detection, the samples were vacuum-infiltrated with fresh DAB (1 mg mL^{-1}, pH 3.8) in darkness until brown sediment were observed. For O_2^- detection, seedlings were vacuum-infiltrated using fresh NBT in phosphate buffer (1 mg mL^{-1}, pH 7.8) under natural light until blue sediment appeared. Seedlings were then destained with 90% ethanol before photographs were taken by a Stemi DV4 light microscope (Carl Zeiss, Gottingen, Germany). H_2O_2 and O_2^- were quantitatively analyzed as per Wang et al. (2017). Each tested condition included ten technical and three biological replicates.

Determination of chlorophyll, malondialdehyde (MDA), and superoxide dismutase (SOD) contents

One-week-old seedlings of *RcBURP4*-OEs and VC were transferred into a tube containing 50% (w/v) PEG6000 or 200 mM NaCl, with H_2O used as control. The tubes were then vacuum-infiltrated. Chlorophyll content was assayed as described previously by Faragó et al. (2018). MDA content was determined as per Jouve et al. (2007). SOD activity was measured as described by Meng et al. (2014). At least six seedlings from each independent line were included in each experiment.

Drought tolerance assessment

Three-week-old seedlings of *RcBURP4*-OEs and VC were grown in trapezoidal pots containing a mixture of vermiculite and humus (1:1, v/v) and maintained under long day conditions (16/8 h light/dark). *RcBURP4*-OEs and VC were grown without watering for 15 d, watered again, and recovered for 7 d; the survival rate was then calculated. Each comparison included more than 20 plants. For water loss assays, leaves of three-week-old seedlings of *RcBURP4*-OEs and VC were assayed at designated time intervals (light intensity: 100 mol m^{-2} s^{-1}; relative humidity: 30–40%; 23–25℃). Water loss rate was calculated as (W0-Wn)/W0, where W0 represents the initial weight of detached leaves and Wn represents the fresh weight of leaves measured at each time point. Three independent biological replicates were conducted for the experiment, with six seedlings from each independent line.

Silencing of RcBURP4 by VIGS

VIGS of *RcBURP4* was conducted as per the procedures previously described (Jiang et al. 2014). A specific 324-bp fragment of *RcBURP4* was inserted into the pTRV2 vector (Liu et al., 2002) to generate the TRV-*RcBURP4* construct. Discs with a diameter of 1 cm were collected from the center of immature leaves of rose with a hole punch. These leaf discs were immersed into a bacterial suspension containing the tobacco rattle virus (TRV) control (pTRV1 and pTRV2) or TRV-*RcBURP4* (pTRV1-*RcBURP4* and pTRV2-*RcBURP4*), and were then vacuum-infiltrated at 0.5 MPa for 30 s. After release of the vacuum, leaf discs were washed and kept in deionized water at 4°C for 3 d, then at 23°C for 1 d. Discs were dehydrated for 12 h and then rehydrated in deionized water for 24 h. The fresh weight and disc area were measured at 12 h dehydration, and 4 h, 8 h, 12 h, and 24 h rehydration. Discs were sampled after 12-h rehydration to obtain the VIGS efficiency by qRT-PCR. Cell counting of samples after 12-h rehydration was performed as per Jiang et al. (2014). The experiments included five replicates, with at least 16 discs in each replicate.

Statistical analyses

All statistical analyses were performed in SPSS v25.0 (SPSS Inc., USA). Tukey's honestly significant difference (HSD) test was conducted to compare the data. The differences at $P < 0.05$ were considered significant.

Results

Identification of BURPs in R. Chinensis

To identify *RcBURP*s, the HMM model and BLASTp (e-value < = $1e^{-5}$) searches were performed against the rose genome (https://www.rosaceae.org/) using *O. sativa* (Ding et al., 2009) 18 BURP amino acid sequences as queries. A total of nine *RcBURP*s were identified in the rose genome, which were then confirmed to contain the BURP domain by the Pfam and SMART databases. These nine *RcBURP*s were designated as *RcBURP1* to *RcBURP9* according to their locations on chromosomes and previously reported homologous groups in other plant species. The characteristics of the nine identified RcBURP proteins, including the signal peptide, pI, Mw, locus ID, physical position, subcellular localization, and some additional features, are shown in Supplementary Table 2. The lengths of RcBURPs ranged from 243 amino acids (aa) (RcBURP7) to 636 aa (RcBURP8). Five RcBURPs (RcBURP1, RcBURP3, RcBURP4, RcBURP7, and RcBURP8) were localized in the extracellular space, whereas two RcBURPs (RcBURP2 and RcBURP9) were localized in the mitochondrial, moreover two RcBURPs (RcBURP5 and RcBURP6) were localized in the nucleus. Different subcellular localizations of RcBURPs suggested that these proteins may function diversely in rose growth and development.

Phylogeny and classification of RcBURPs

To investigate the phylogenetic relationship of BURPs, we constructed an unrooted phylogenetic tree with other 166 predicted BURP proteins from three monocot plants: *O. sativa* (14), *S. biocolor* (11), and *Z. mays* (10); and eight dicot plants: *A. thaliana* (5), *G. max* (21), *G. arboretum* (17), *G. hirsutum* (30), *G. raimondii* (18), *P. trichocarpa* (18), *R. chinensis* (9), and *R. multiflora* (13), using MEGA-X with the NJ method (Fig. 1 and Supplementary Table 3). Based on the tree topology, these BURPs can be classified into eight distinct subfamilies: BNM2-like, PG1β-like, RD22-like, USP-like, BURP-V, BURP-VI, BURP-VII, and BURP-VIII (Fig. 1). The nine RcBURPs were present in the BNM2-like (RcBURP5, RcBURP6, and RcBURP7), PG1β-like (RcBURP3 and RcBURP8), RD22-like (RcBURP9), and BURP-V (RcBURP1, RcBURP2, and RcBURP4) subfamilies. Subfamilies BURP-VI, VII, and VIII were only comprised of members from monocot plants (e.g., SbBURP11, OsBURP 13, and ZmBURP9) and members of USP-like subfamilies only has *G. max*, while no RcBURP was found in these four subfamilies. The members of RD22-like and PG1β-like subfamilies contained monocots and dicots plants indicated BURP proteins were conserved differentiation between monocot and dicot plants. These results demonstrated conserved evolution of BURPs between monocot and dicot plants.

Gene structure and conserved motifs of RcBURPs

We next examined the gene structure and conserved motifs of the nine RcBURPs and 14 RmBURPs from *R. multiflora*, which is closely related to *R. chinenesis* (Fig. 2a). Results of gene structure analyses revealed that the numbers of exon(s) and/or intron(s) were different between members belonging to different subfamilies. As shown in Fig. 2a, *RcBURP*s contained one to three exons, with most *RcBURP*s comprising of one (3/9) or two exons (4/9), while two *RcBURP*s (RcBURP4 and RmBURP9) had three exons. We further investigated conserved motifs of RcBURPs and RmBURPs using the MEME program. Ten conserved motifs were randomly distributed among these BURPs, and most RcBURPs belonging to the same subfamily had similar motifs. Motifs 1–5 were present in all BURPs, except for RmBURP1,

RmBURP3, and RmBURP5 (Fig. 2a). Motif 6 and motif 10 were observed in RD22-like, PG1β-like, and BURP-IV subfamilies. In addition, motif 7 was found in both RD22-like and PG1β-like subfamilies. It is worth noting that motif 8 and motif 9 were only present in the PG1β-like subfamily, and members in the PG1β-like subfamily contained all motifs (1–10). Overall, the gene structure and conserved motif composition were similar in members from the same subfamily, which confirmed the reliability of the subfamily classification based on phylogenetic analysis.

Three-dimensional modeling was also conducted on the nine RcBURPs (Fig. 2b). All RcBURPs contained multiple α-helix and coil structures (Fig. 2b). In RcBURPs, the most predicted secondary structure was β-strand (9–36% in RcBURPs), whereas the proportion of α-helix was only 8–25%. Results from 3D modeling revealed tertiary structure similarity in these RcBURPs, indicating that they may evolve from the same ancestral sequence and/or be under purifying selection for stabilization during long-term acclimation after the initial divergence.

CRE prediction for RcBURP**s**

To further investigate the possible role of CREs in the promoter regions of *RcBURP*s, we used PlantCARE (Rombauts et al., 1998) to identify CREs in the 2000 bp upstream of the TSS in the nine *RcBURP*s. Based on their functions, the identified CREs were classified into four groups: abiotic stress-, light response-, phytohormone response-, and plant growth and development-related (Fig. 3a). Phytohormone-responsive CREs included nine types: ABA response elements (ABRE), auxin response elements (AuxRR-core and TGA-element), methyl jasmonate (MeJA) response elements (CGTCA-motif and TGACG-motif), gibberellin response elements (P-box, TATC-box, and GARE-motif), and SA response elements (TCA-element). CREs responding to abiotic stresses contained five groups, including TC-rich repeats (defense and stress responsiveness), MBS (drought stress), LTR (cold stress), ARE (anaerobic induction), and CCAAT-box (MYB binding site) (Fig. 3a). Additionally, six CREs were identified in terms of light response (i.e., GT1-motif, G-box, 3-AF3, Sp1, MRE, and ACE). Only a few CREs were identified to participate in plant growth and development; no CRE of this kind was found in *RcBURP6*. Besides, as shown in Fig. 3a, the numbers of G-box (30), ABRE (25), and ARE (24) were greater than those of other CREs. Specifically, all *RcBURP*s contained ARE, with the highest number of 5 in *RcBURP3* and *RcBURP6*. Therefore, *RcBURP*s may participate in rose response to abiotic stresses, light, and phytohormones.

Chromosomal distribution and evolution of RcBURPs

To study the relationship between the expansion and duplication of *RcBURP*s, all *RcBURP*s were mapped onto the five chromosomes of the rose genome (Fig. 3b). Our results showed that the chromosomal distributions of *RcBURP*s were significantly heterogeneous, with most *RcBURP*s distributed in the distal regions of the chromosomes. Moreover, Chr6 contained four *RcBURP*s (*RcBURP5*, *RcBURP6*, *RcBURP7*, and *RcBURP9*), whereas Chr1 contained two (*RcBURP1* and *RcBURP2*), and each of Chr4, Chr5, and Chr7 contained only one *RcBURP*. To explore the relationship among *RcBURP*s, gene duplication analysis (e.g., segmental duplication and tandem duplication) was performed by including *BURP*s from *R. chinensis*, *M.*

domestica, and *F. vesca* (Fig. 3c). In *R. chinensis*, only one segmental duplication event between *RcBURP3* and *RcBURP8* was found. Six and seven homologous gene pairs were identified among *R. chinensis*, *M. domestica*, and *F. vesca* (Supplementary Table 4), respectively. To further exam the selection pressure in the divergence of *BURP*s, we calculated the Ka/Ks ratio for the orthologous gene pairs (Supplementary Table 4). The average Ka/Ks ratio was 0.28 for the 15 identified orthologous gene pairs, which suggested that *BURP*s across the species have undergone purifying or stabilizing selection during the evolutionary process.

Expression of RcBURP**s and sequence analysis of** RcBURP4

To gain insight into the expression pattern of *RcBURP*s under drought stress, we searched the gene expression data from our previous transcriptomic study (Li et al., 2020). Six treatments included normal conditions (ND) in leaves (NL) and roots (NR), mild drought stress (MD) in leaves (ML) and roots (MR), and severe drought stress (SD) in leaves (SL) and roots (SR). Different expression patterns were observed for the nine *RcBURP*s during drought stress. All *RcBURP*s exhibited induced expression levels in rose leaves and roots under the MD condition, while three *RcBURP*s (*RcBURP1*, *RcBURP3*, and *RcBURP8*) showed repressed expression in leaves under the SD condition. Compared with ND, *RcBURP3*, *RcBURP5*, and *RcBURP7* showed lower expression levels in rose roots under the SD condition. Interestingly, *RcBURP4* showed higher transcript abundance under SR vs. NR, ML vs. NL, MR vs. NR, ML vs. MR, and SL vs. SR, suggesting that this gene may contribute greatly in rose response to drought stimulus. We then selected *RcBURP4* for further analysis. Seven BURP-domain containing proteins of six kinds of plants sequence alignments were also performed (Fig. 4b). The results showed that highly conserved amino acids were evenly distributed, including five prolines, six phenylalanines, an arginine, three threonines, two cystines, three glycines, two glutamic acids, two serines, an aspartic acid, a histidine, two tryptophans, two valines, two lysines, an alanine, a tyrosine, and four CH motifs. The conserved C-terminal sequence of RcBURP4 can be described as $CHX_3YX_3VX_2CHX_{12}LX_4GX_6AXCHX_2TX_2WX_3HX_2FX_2LX_3PX_3PXCH$.

RcBURP4 **-OE** Arabidopsis **was susceptible to ABA during germination and growth**

RcBURP4 contained six ABREs and three abiotic stress response CREs (Fig. 3a), indicating that it may contribute greatly in response to drought stimulus through the ABA-dependent pathway. Therefore, we first overexpressed *RcBURP4* in *A. thaliana*, and three independent *RcBURP4*-overexpressing lines (OE#1, 2, and 3) with high expression of *RcBURP4* were selected for further study. Firstly, we compared the seed germination rates of *RcBURP4*-OEs and VC on MS media containing different concentrations of ABA (0, 0.5, and 1.0 μM) (Fig. 5a). As shown in Fig. 5b, no significant differences were observed in seed germination rates between *RcBURP4*-OEs and VC without ABA treatment. However, when supplemented with 1.0 μM ABA, the germination rates of OE#1, 2, and 3 were 72.5%, 50.5%, and 39.5%, respectively, significantly lower than that of VC (94.5%). Next, to further analyze the root morphology of *RcBURP4*-OEs under ABA treatment, 7-day-old seedings were placed in MS media containing 0, 30, or 50 μM ABA, and incubated for 10 d (Fig. 5c). The root length increments of OE#1, 2, and 3 were 14, 10, and 9.5 mm, respectively, under 50 μM ABA treatment, which were significantly lower than that of VC (3.5 mm; Fig. 5d).

Moreover, no significant difference was observed between *RcBURP4*-OEs and VC in terms of lateral root number (Fig. 5e). In summary, these results suggested that overexpression of *RcBURP4* conferred increased ABA susceptibility in germination and post germination stages in transgenic *Arabidopsis*.

Overexpression of RcBURP4 **improved tolerance to salinity in** Arabidopsis

To further characterize *RcBURP4* function in response to abiotic stress, we investigated *RcBURP4*-transgenic *Arabidopsis* performance under salt stress. Seeds of *RcBURP4*-OEs were sown on MS media containing different concentrations of NaCl (0, 100, 150, and 200 mM) (Fig. 6a). The germination rate of *RcBURP4*-OEs (18.8–41.7%) was significantly inhibited after 150 and 200 mM NaCl treatments compared with that of VC (85–95%) (Fig. 6b). We also measured the root length increment and lateral root number of *RcBURP4*-OEs and VC seedlings under NaCl treatments (Fig. 6c). VC plants maintained higher root length increment under NaCl treatment than did the *RcBURP4*-OEs lines (Fig. 6d), and no significant difference was found between VC and *RcBURP4*-OEs in terms of lateral root number (Fig. 6e). Moreover, we also tested the accumulated reactive oxygen species (ROS) level of *RcBURP4*-OEs and VC. Both *RcBURP4*-OEs and VC plants showed light brown (DAB) and blue (NBT) colors under normal growth conditions. However, when supplemented with 200 mM NaCl, *RcBURP4*-OEs exhibited darker brown (DAB) and blue (NBT) colors than VC did (Supplementary Fig. 1). Besides, the H_2O_2 content (Fig. 6f) and O_2^- production rate (Fig. 6g) of *RcBURP4*-OEs were 0.16–0.23 μmol g^{-1} and 0.48–0.57 nmol g^{-1} min^{-1}, respectively, significantly higher than those of VC. The above results suggest that overexpressing *RcBURP4* leaded to decreased ability of plants to scavenge ROS under salt stress.

Performance of RcBURP4**-OE lines under osmotic stress**

We also investigated seed germination of *RcBURP4*-OE lines and VC plants under PEG treatment. As shown in Fig. 7a, no significant differences were found when plants were grown without PEG6000. However, when supplemented with 12% PEG6000, the germination rates of OE#1, 2, and 3 were 67%, 63%, and 74%, significantly higher than that of VC (41%) (Fig. 7b). Additionally, after 50% PEG6000 treatment, brown and blue (Fig. 7c) colors were lighter in *RcBURP4*-OE lines than in VC plants. Moreover, the H_2O_2 content and O_2^- production rate of *RcBURP4*-OEs were 0.11–0.13 μmol g^{-1} (Fig. 7d) and 0.33–0.36 nmol g^{-1} min^{-1}, respectively (Fig. 7e), significantly lower than those of VC plants, indicating that *RcBURP4*-OE lines accumulated less ROS and reduced cell membrane damage under osmotic stress. Besides, *RcBURP4*-OEs showed higher levels of chlorophyll content (Fig. 7f) and SOD activity (Fig. 7g), but lower levels of MDA content (Fig. 7h) compared to VC plants. The above results demonstrated that *RcBURP4* conferred enhanced resistance to osmotic stress in transgenic *Arabidopsis*.

Overexpression of RcBURP4 **increased resistance to drought stress in** Arabidopsis

We further examined the performance of *RcBURP4*-OEs and VC under drought stress. As shown in Fig. 8a, after 15-d drought stress, VC exhibited obvious symptoms of water loss (e.g., withered leaves), while a small amount of green color was observed in *RcBURP4*-OEs leaves. After 7-d rewatering, a fair number of

RcBURP4-OE lines recovered (Fig. 8a), and the survival rates of OE#1, 2, and 3 were 75%, 62.5%, and 87.5%, respectively, significantly higher than that of VC (Fig. 8b). Additionally, changes in physiological indices including MDA content were also evaluated. Fifteen days after drought treatment, *RcBURP4*-OEs exhibited a significantly lower MDA content (40.1–52.6 nmol g^{-1}) than VC (87.7 nmol g^{-1}) (Fig. 8c). The water loss rate was lower in *RcBURP4*-OEs than in VC at the same time points (Fig. 8d). Therefore, these results suggest that the tolerance to drought stress was stronger in *RcBURP4*-OEs than in VC.

Silencing of RcBURP4 decreased tolerance to dehydration in rose

To further reveal the potential role of *RcBURP4*, we silenced the *RcBURP4* expression in rose leaves using the VIGS approach. *A. tumefaciens* carrying TRV-*RcBURP4* and TRV1 (Liu et al., 2002) were co-infiltrated into rose leaf discs to generate *RcBURP4*-silenced samples. The infiltrated rose leaves experienced 12-h dehydration and 24-h rehydration (Fig. 9a). Firstly, we confirmed the efficiency of VIGS with qRT-PCR. A ~ 65% decrease in the *RcBURP4*expression was observed in *RcBURP4*-silenced samples compared to the control (Fig. 9b). Comparing to the control (TRV), rose plants inoculated with TRV-*RcBURP4* showed more serious withering (Fig. 9a). Furthermore, silencing of *RcBURP4* resulted in significantly smaller disc areas during the 12-h dehydration, a reduction of 10.5%, compared to the TRV control (Fig. 9c). In addition, a more significant decline was observed in the fresh weight of *RcBURP4*-silenced discs after the 12-h rehydration compared with the TRV control (Fig. 9d). However, silencing of *RcBURP4* led to a greater cell number per microscope visual area of 28% in abaxial, compared to the TRV control (Fig. 9e), indicating that silencing of *RcBURP4* reduced tolerance to dehydration in rose.

Discussion

*BURP*s have been extensively studied in diverse plants (Van Son et al., 2009; Gan et al., 2011; Dinh et al., 2017; Sun et al., 2019), including *A. thaliana, Z. mays, Coffea arabica, G. hirsutum*, and *S. vulgare*. Previous studies have revealed that *BURP*s could be classified into eight subfamilies, including RD22-like, PG1β-like, USP-like, BNM2-like, BURP-V, BURP-VI, BURP-VII, and BURP-VIII (Li et al., 2016). The phylogenic tree constructed in this study supported the classification of *BURP*s, with five distinctive subfamilies being emphasized in our assays (Fig. 1). Our results showed that subfamilies BURP-VI, VII, and VII had close relationships with the RD22-like subfamily, and subfamily USP-like was close to subfamily BURP-V. This is different from results obtained from previous studies (Li et al., 2016), which may be ascribed to the different methods utilized in phylogenetic analyses. Among these subfamilies, the BURP-V subfamily was only present in monocotyledons, whereas the BNM2-like subfamily was only found in dicotyledons, indicating that these *BURP*s might have originated before the divergence of monocot and dicot plants. Interestingly, the BURP-V subfamily was only found exclusively in woody plants, such as *R. chinenesis, R. multiflora*, and *G. hirsutum*. Taken together, these results suggest that *BURP*s may share a common ancestor prior to the divergence of higher and lower land plants.

Chromosomal distribution analysis revealed that the nine *RcBURP*s were not evenly distributed on rose chromosomes; 44.4% of *RcBURPs* were located on Chr6 and 22.2% were distributed on Chr1 and Chr7,

respectively; no *RcBURP* genes were identified on two chromosomes (Chr2 and Chr3). Meanwhile, the *BURP*s in *F. vesca* and *M. domestica* were also unevenly distributed. These results clearly demonstrate a conserved evolution pattern across the Rosaceae plants. Moreover, gene structure and motif analysis of *R. multiflora* and *R. chienesis* showed that the BURP proteins contained a highly conserved BURP domain in the C-terminus, especially the four notable CH motifs (Supplementary Fig. 2). The component and arrangement of motifs identified by MEME implied conserved patterns within subfamilies and divergence among subfamilies. Ten motifs were identified in subfamily PG1β-like, and more than five motifs were detected in other subfamilies (Fig. 2a). Meanwhile, members of the BNM2-like subfamily likely missed some motifs in their N-termini. Our results obtained from motif analysis are consistent with those obtained from the gene structure analysis of *BURP*s.

*BURP*s have been identified in various plants. However, the function of these genes remains largely unknown. Extensive studies have indicated that *BURP*s may play diverse roles in growth, development, and response to environmental stresses of plants. Members of subfamily USP-like are often related to plant development. In *Arabidopsis*, *AtUSPL1* is expressed in cellular compartments that are crucial for seed and storage protein synthesis in parenchyma cells (Van Son et al., 2009). Members of the RD22-like subfamily are likely to participate in plant response to abiotic or biotic stress. For example, *B. napus BnBDC1* is up-regulated by both cold and salt stresses; overexpression of *BnBDC1* in *Arabidopsis* promotes the expression of its downstream genes, conferring enhanced resistance to drought and freezing (Yu et al. 2004). Overexpression of *G. max GmRD22* improves tolerance to salt stress in both *Arabidopsis* and rice. Moreover, *GmRD22* is likely to regulate cell wall peroxidases, thereby strengthening cell wall integrity during salt stress (Wang et al. 2012). *AtRD22* and *AtUSPL1* are induced as part of the ABA-mediated moisture stress response, and their products act to suppress the drought stress response (Harshavardhan et al. 2014). The *CaBDP1* expression is highly regulated in *C. arabica* under cold, drought, ABA, or salt stress. The overexpression of *CaBDP1* leads to delayed germination of transgenic *Arabidopsis* under abiotic stress with the presence of ABA (Dinh et al., 2017), implying *CaBDP1* may participate in ABA signaling pathway. The research on the function of members in the BURP-V subfamily is scarce. In our study, overexpression of *RcBURP4*, a member belonging to subfamily BURP-V conferred higher sensitivity in transgenic *Arabidopsis* to ABA, salinity, and PEG in germination and post-germination stages. In addition, *RcBURP4*-silenced rose plants displayed decreased tolerance to dehydration, while *Arabidopsis* overexpressing *RcBURP4* showed improved tolerance to drought at the seedling stage. These results clearly demonstrate that *RcBURP4* plays a positive role in plant response to drought stress.

Conclusions

In the present study, *BURP*s in the rose genome were systematically analyzed. *BURP*s could be classified into eight subfamilies according to results from analyses of phylogeny, gene structure, and conserved motifs. Gene duplication results indicated that segmental duplication may contribute to the expansion of *RcBURP*s. *Arabidopsis* overexpressing *RcBURP4*, a member of the BURP-V subfamily, showed improved sensitivity under high salinity, ABA, and PEG treatments in the germination stage. Moreover, *RcBURP4* conferred enhanced tolerance to drought stress in transgenic *Arabidopsis*. *RcBURP4*-silenced rose plants

displayed decreased tolerance to dehydration. The results obtained from our study expand the understanding of the *BURP* gene family and provide a candidate gene *RcBURP4* for improving rose tolerance to stresses.

Declarations

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Contributions

JXQ and LQH conceived and designed the experiments; FLF, ZZJ, WH, ZXJ, SL and LYZ carried out the experiments; JXQ, FLF, ZZJ, GLF, LYZ, and TBQ conducted the data analysis. FLF and ZZJ wrote the manuscript; JXQ revised the manuscript and contributed the plant material. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no competing interests.

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Figures

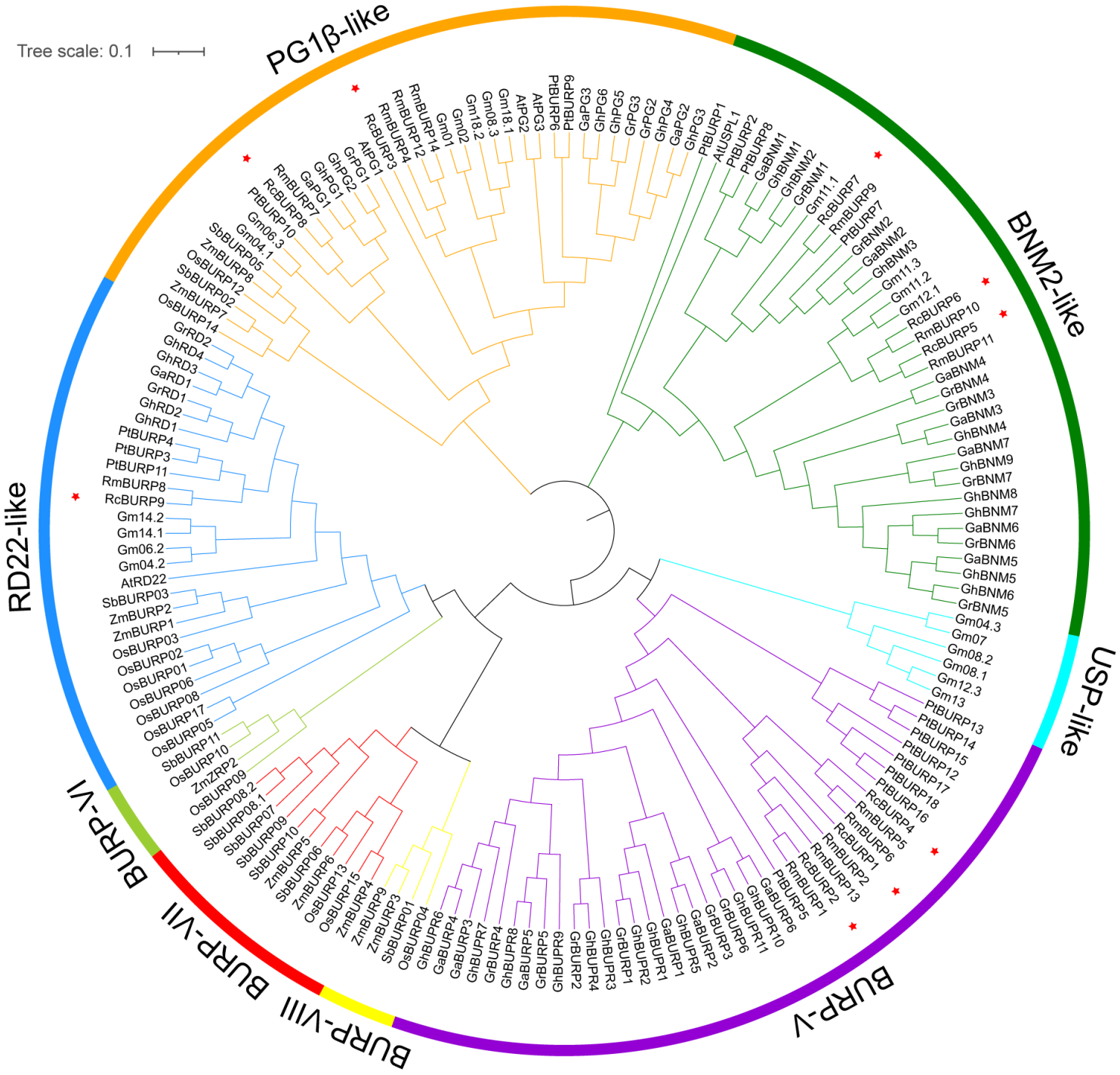

Figure 1

Phylogenetic analysis of BURPs. Five AtBURPs from Arabidopsis thaliana, 30 GrBURPs from Gossypium raimondii, 17 GaBURPs from G. arboretum, 21 GmBURPs from Glycine max, 14 OsBURPs from Oryza sativa, 18 PtBURPs from Populus trichocarpa, nine RcBURPs from Rosa chinensis, 11 SbBURPs from Sorghum bicolor, and ten ZmBURPs from Zea mays were used for constructing an unrooted tree using MEGA X and visualized by iTOL. Distinctive subfamilies are indicated with different colors. RcBURPs are indicated with red stars.

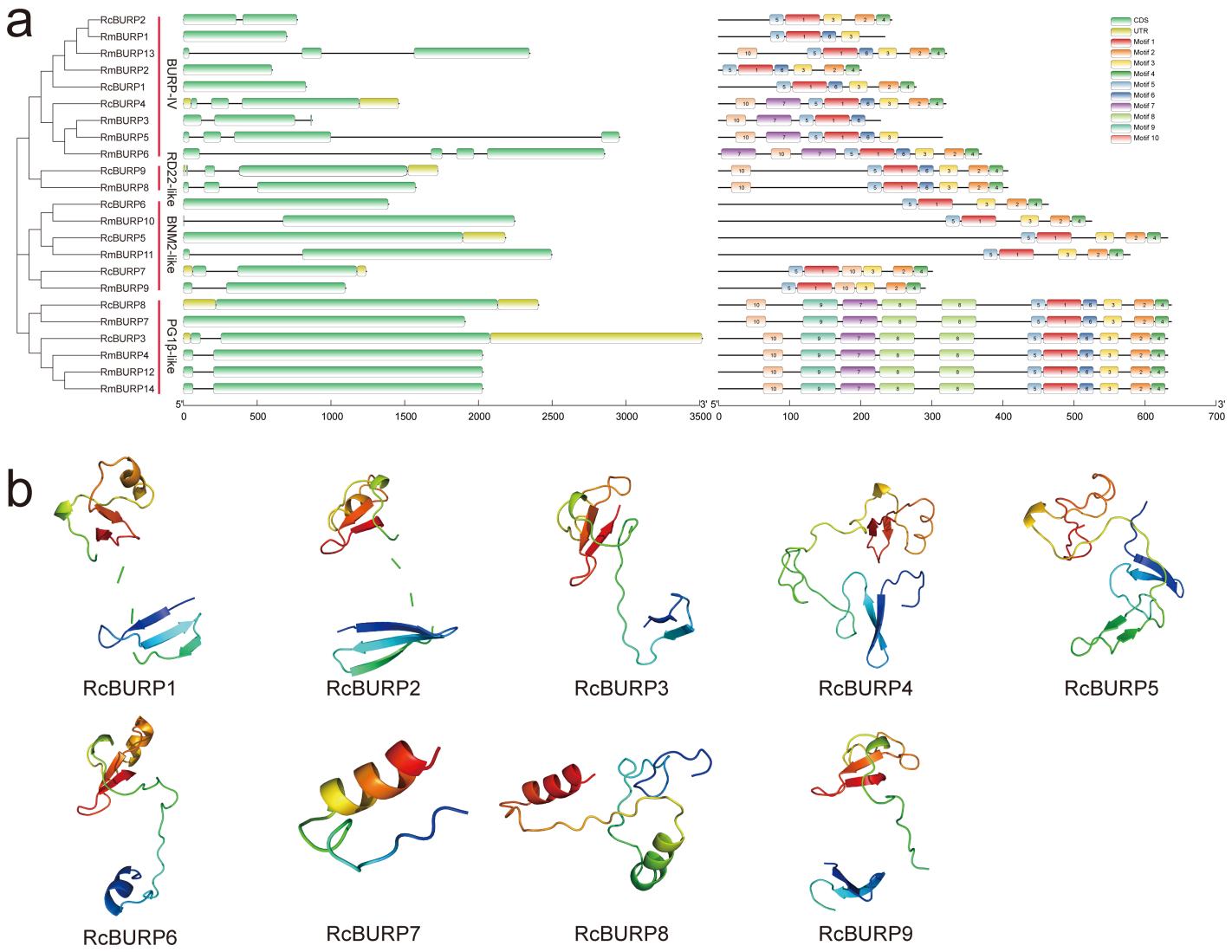

Figure 2

Phylogenetic tree and predicted three-dimensional (3D) structure of RcBURPs. a Analyses of phylogenetic relationships, gene structures, and conserved motifs of BURPs of Rosa chinensis and R. multiflora. The phylogenic tree was constructed using the neighbor-joining method and BURPs are classify according to their phylogenic relationships. The green rectangles, yellow rectangles, and black lines indicate exons, untranslated regions (UTRs), and introns, respectively. The conserved motifs in BURPs were identified by the MEME program. Predicted conserved motifs 1 to 10 are indicated with specific colors and the consensus sequences for putative motifs are shown in supplementary Fig. 1. b Homology modeling of RcBURPs. Predicted 3D structures of the nine RcBURPs were obtained by Phyre2 homology modeling.

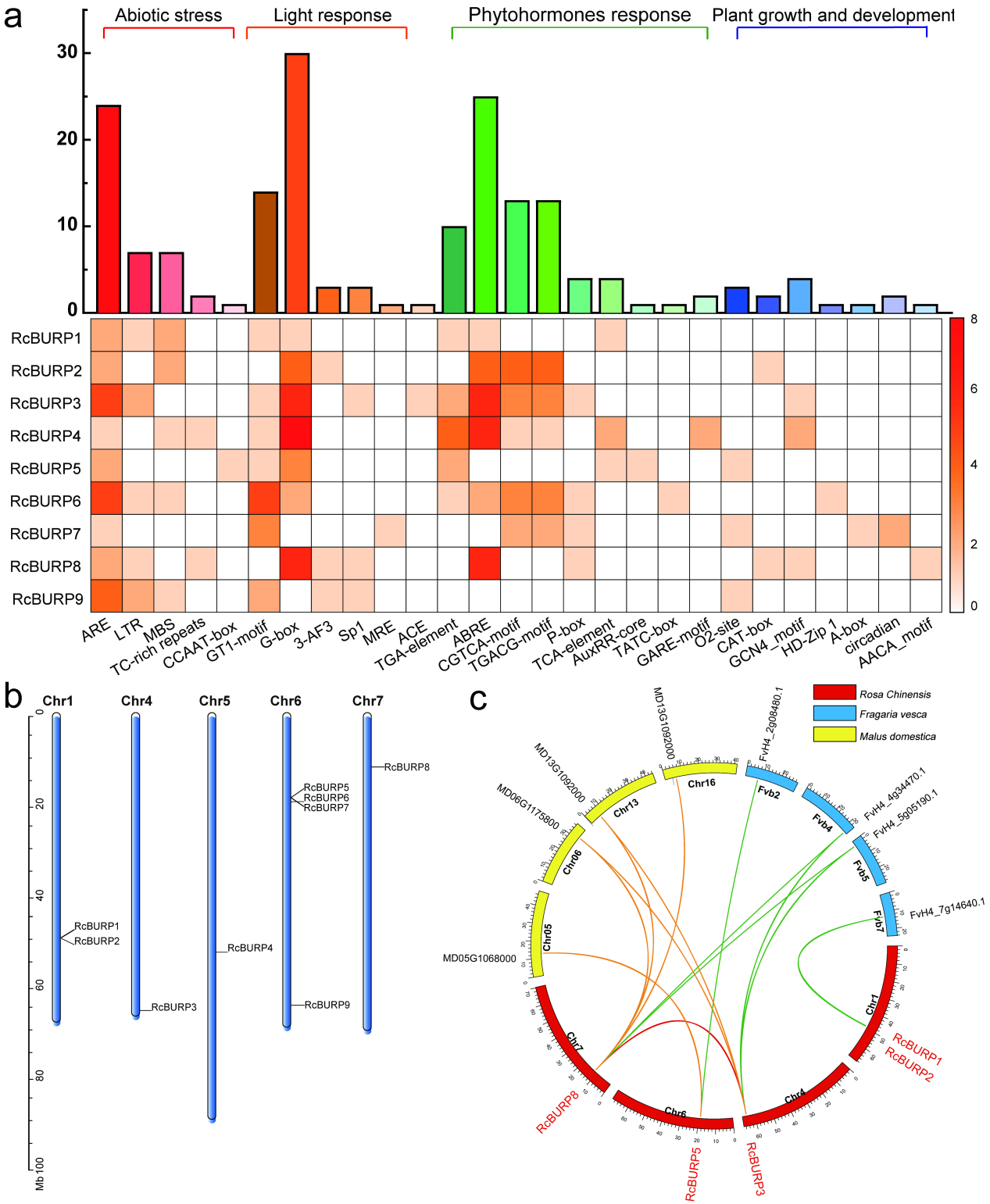

Figure 3

Cis-regulatory elements (CREs), chromosomal location, gene duplication events, and syntenic analyses of RcBURPs. a Predicted CREs in the promoter region of RcBURPs. Promoter sequences (~2000 bp) of the nine RcBURPs were examined with PlantCARE. Four different groups of CREs, including abiotic stress-, light response-, phytohormones response-, and plant growth and development-related CREs, are shown above the columns. The vertical axis represents the number of CREs in each group. b Chromosomal

distributions of RcBURPs in the rose genome. The chromosomal location of each RcBURP was determined by mapping the sequence to the rose genome. The chromosome number is indicated above each chromosome. The scale bar represents the length of the chromosomes (Mb). c Gene pairs of segmental duplication and syntenic relationships of BURPs in Rosa chinensis, Malus domestica, and Fragaria vesca. Circos was used to visualize the genome. The putative orthologous BURP genes among R. chinensis, M. domestica, and F. vesca are presented in red, yellow, and blue, respectively. The chromosome numbers and gene names are indicated around the circle.

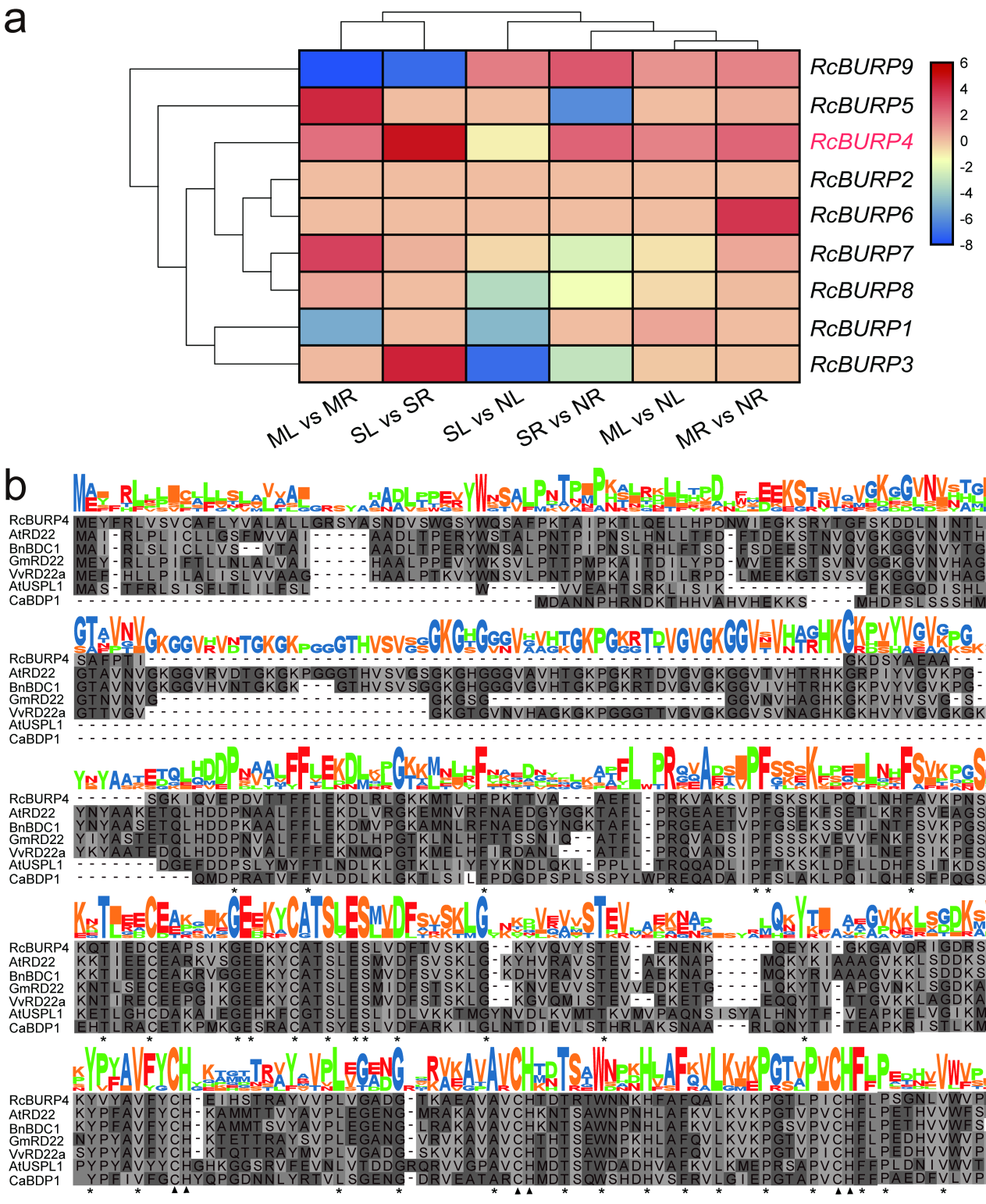

Figure 4

Expression of RcBURPs and sequence analysis of RcBURP4. a Expression profiles of RcBURPs in response to drought stress in rose leaves and roots. Hierarchical clustering of relative transcript abundance profiles (log2 fold-change) of RcBURPs. Different colors in map represent different transcript abundance values. The scale bar on the right indicates relative expression levels. Different treatments are indicated at the bottom: leaves under normal conditions (NL), leaves under mild drought stress (ML), leaves under severe drought stress (SL), roots under normal conditions (NR), roots under mild drought stress (MR), and roots under severe drought stress (SR). The individual gene names are indicated on the right. b Sequence analysis of RcBURP4 with other plant BURP proteins. The complete amino acid sequences of seven BURPs were aligned by TBtools. Sequence logos are presented above the amino acids. Asterisks and triangles below the sequence represent conserved amino acids and CH motifs, respectively. Accession numbers are as follows: RcBURP4 (MW651860), AtRD22 (At5g25610), BnBDC1 (AY293830), GmRD22 (Glyma06g08540), VvRD22a (AY634282), AtUSPL1 (At1g49320), CaBDP1 (XP_027069063.1)

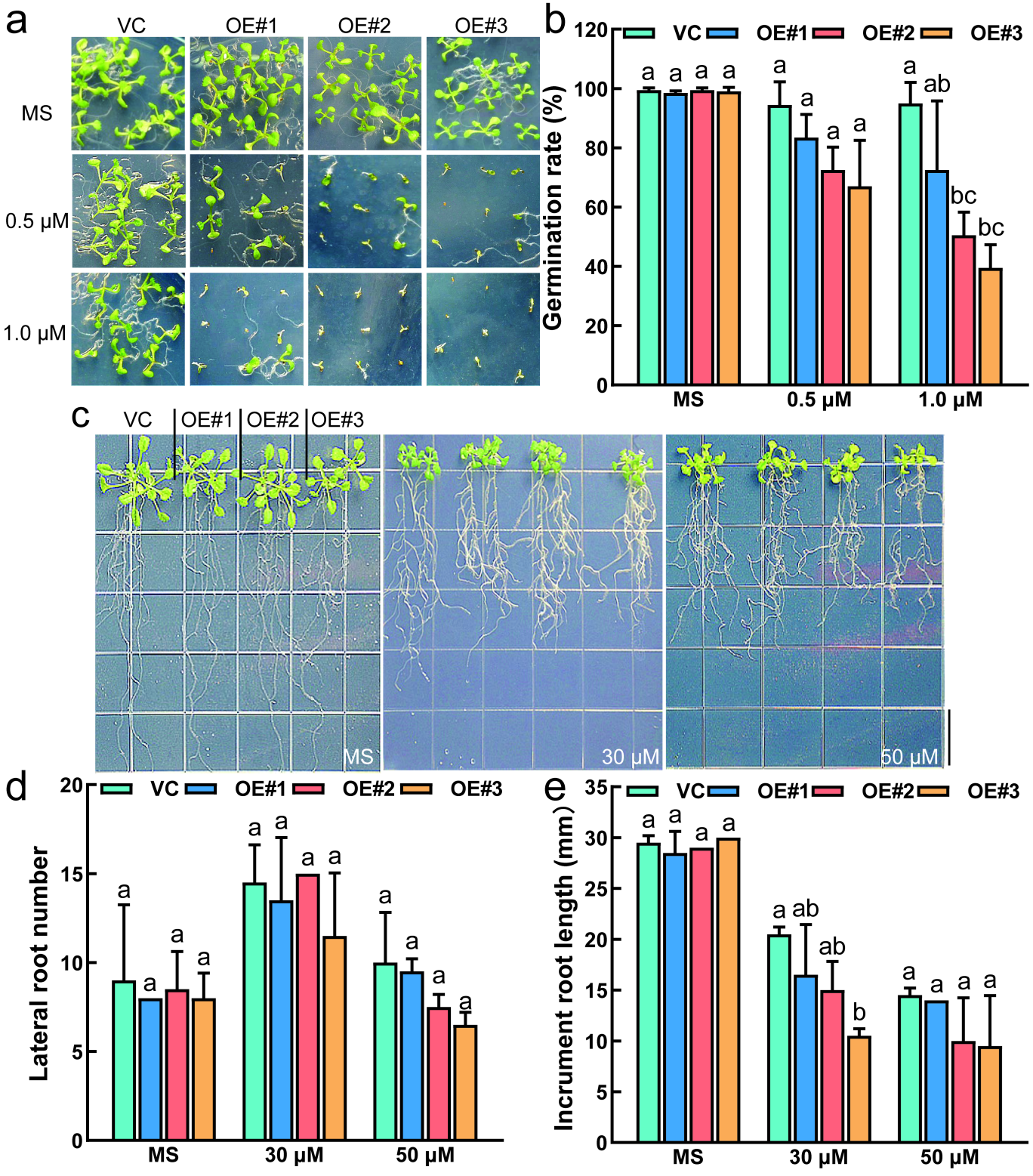

Figure 5

Overexpression of RcBURP4 improved ABA sensitivity in the transgenic Arabidopsis. a Seed germination of RcBURP4-overexpressing lines (RcBURP4-OEs) and vector control plants (VC) under various ABA concentrations. Homozygous T3 seeds of VC and RcBURP4-OEs (OE #1, 2, and 3) were grown on MS plates containing 0, 0.5, or 1.0 μM ABA, incubated at 4 °C for 3 d, and maintained at 23 °C for germination. Photographs were taken 7 d after planting. b Seed germination rates of RcBURP4-OEs and

VC under ABA treatments. c Root growth phenotypes of RcBURP4-OE and VC seedlings exposed to ABA. Lateral root numbers (d) and root length increments (e) of RcBURP4-OE and VC seedlings under various ABA concentrations. Three independent replicates were conducted, and error bars indicate standard deviations. Statistically significant differences as determined by Tukey's HSD tests ($p < 0.05$) are indicated with different letters.

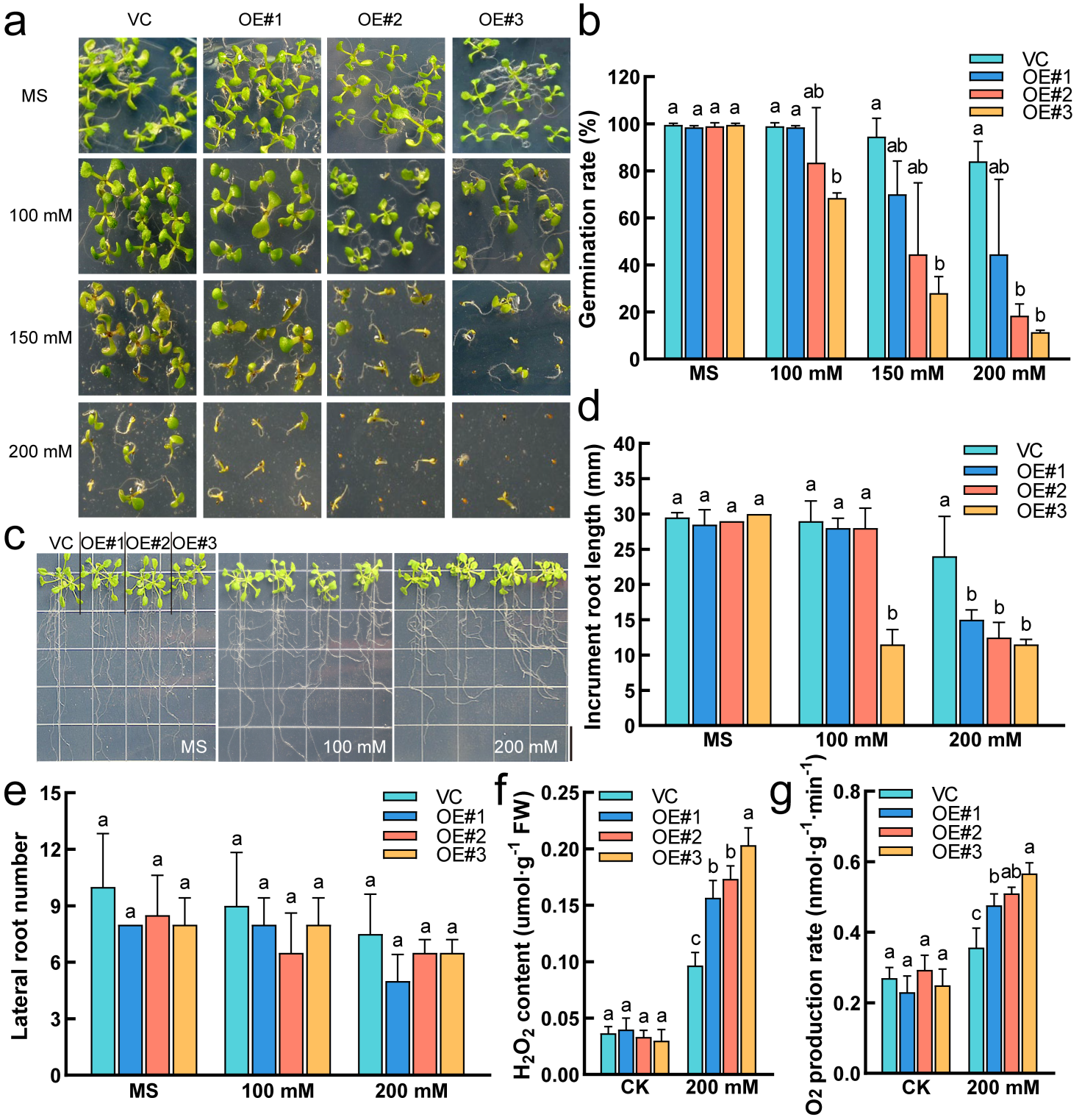

Figure 6

RcBURP4-OE plants showed reduced resistance to salt stress. a Seed germination of RcBURP4-OEs and VC under various NaCl concentrations. b Seed germination rates of RcBURP4-OEs and VC exposed to NaCl. c Root growth phenotypes of RcBURP4-OEs and VC under various NaCl concentrations. Seeds of RcBURP4-OEs and VC were grown for seven days and then transferred to MS plates supplemented with 0, 100, or 200 mM NaCl, respectively. Photographs were taken at 10 d after planting. Bars represent 1 cm. Comparisons of root length increment (d) and lateral root number (e) between RcBURP4-OEs and VC under various NaCl concentrations. Comparisons of H_2O_2 content (g) and O_2^- activity (e) of RcBURP4-OEs and VC. Three independent replicates were conducted, and error bars indicate standard deviations. Different letters above the columns indicate significant differences ($p < 0.05$) with Tukey's HSD test.

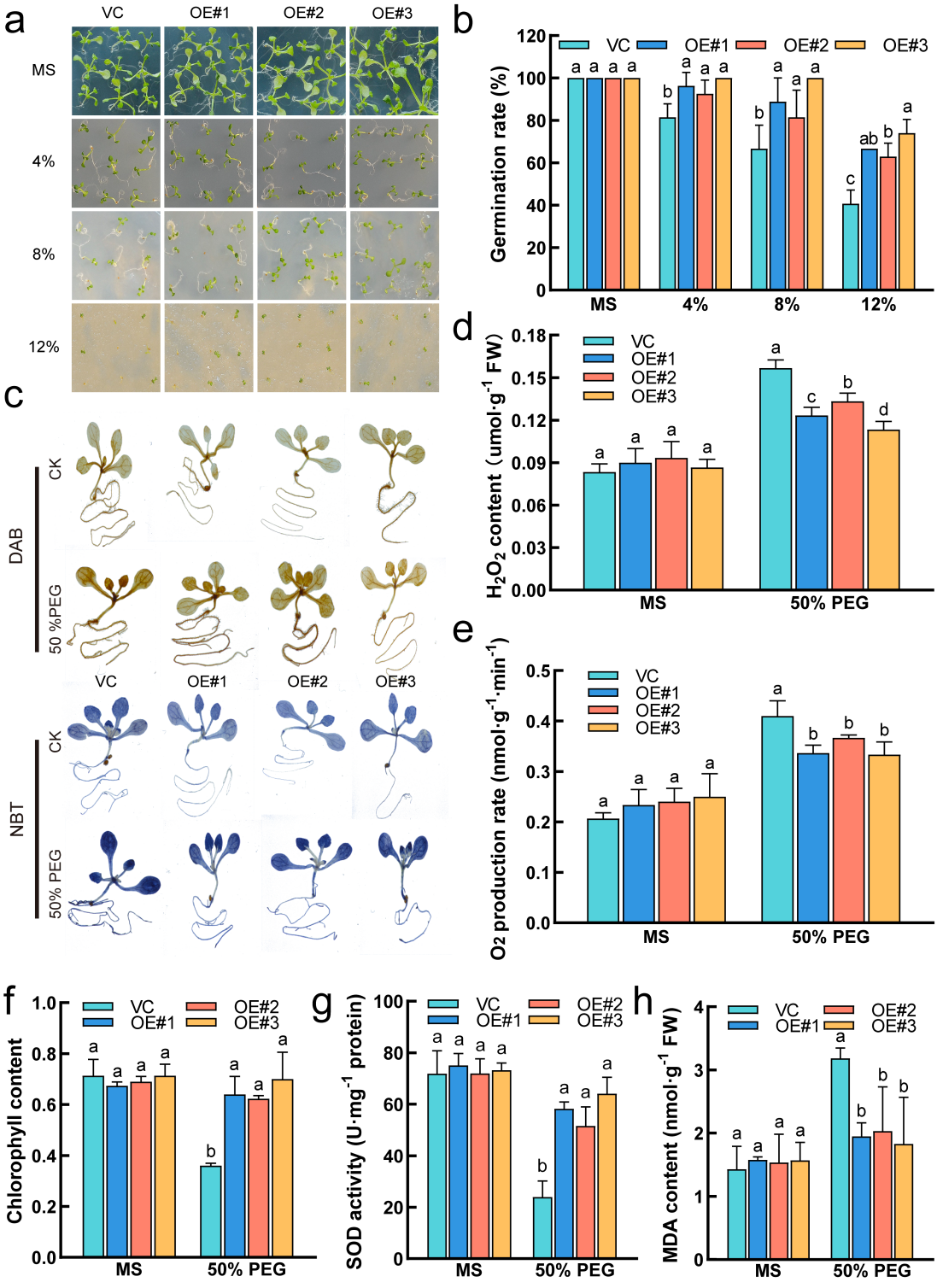

Figure 7

Effects of osmotic stress on seed germination and ROS accumulation of RcBURP4-OEs and VC under PEG treatment. a Seed germination of RcBURP4-OEs and VC under normal conditions and PEG6000 treatments. b Seed germination rates of RcBURP4-OEs and VC under various PEG6000 concentrations. c DAB and NBT staining of RcBURP4-OEs and VC. Plants were grown for 10 d prior to treatment with water (CK) or 50% PEG6000 (PEG) for 1 h. Afterwards, plants were stained with DAB and NBT solutions to

quantify H2O2 and O2- accumulation in leaves. H2O2 content (d) and O2- production rate (e) in RcBURP4-OEs and VC. Comparison of chlorophyll content (f), SOD activity (g), and MDA content (h) of RcBURP4-OEs and VC under normal conditions and 50% PEG6000 treatment. Data are presented as mean ± SD from three independent replicates. Letters above the columns indicate significant differences ($p < 0.05$) with Tukey's HSD test.

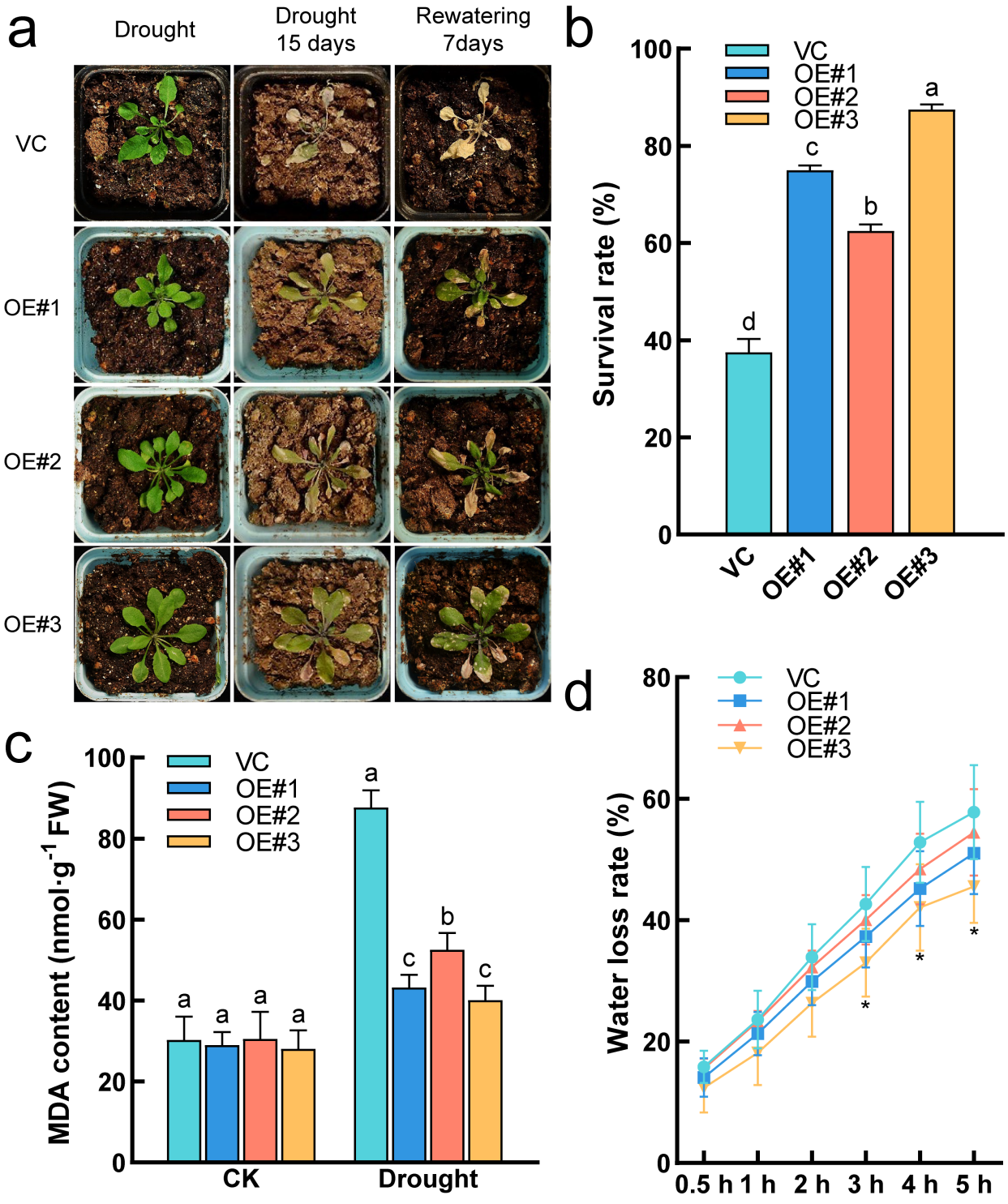

Figure 8

Overexpression of RcBURP4 conferred enhanced drought tolerance in Arabidopsis. a Whole plant status of RcBURP4-OEs and VC in a drought stress assay. Three-week-old RcBURP4-OE and VC seedlings were grown for additional 15 d without watering. The survival rate was determined 7 d after irrigation was reinitiated. b Survival rates of RcBURP4-OEs and VC under drought treatment. c MDA contents in the rosette leave of RcBURP4-OEs and VC after a 15-d drought treatment. Vertical bars represent means, and error bars represent SE based on three independent replicates. Statistically significant differences ($p < 0.05$) as determined by Tukey's HSD test are indicated by different letters. d Kinetics of water loss rate in detached leaves of RcBURP4-OEs and VC . The detached leaves from 3-week-old seedlings of RcBURP4-OEs and VC were dehydrated for 5 h. Data are presented as mean ± SE of three biological replicates. Error bars indicate SE (n=6).

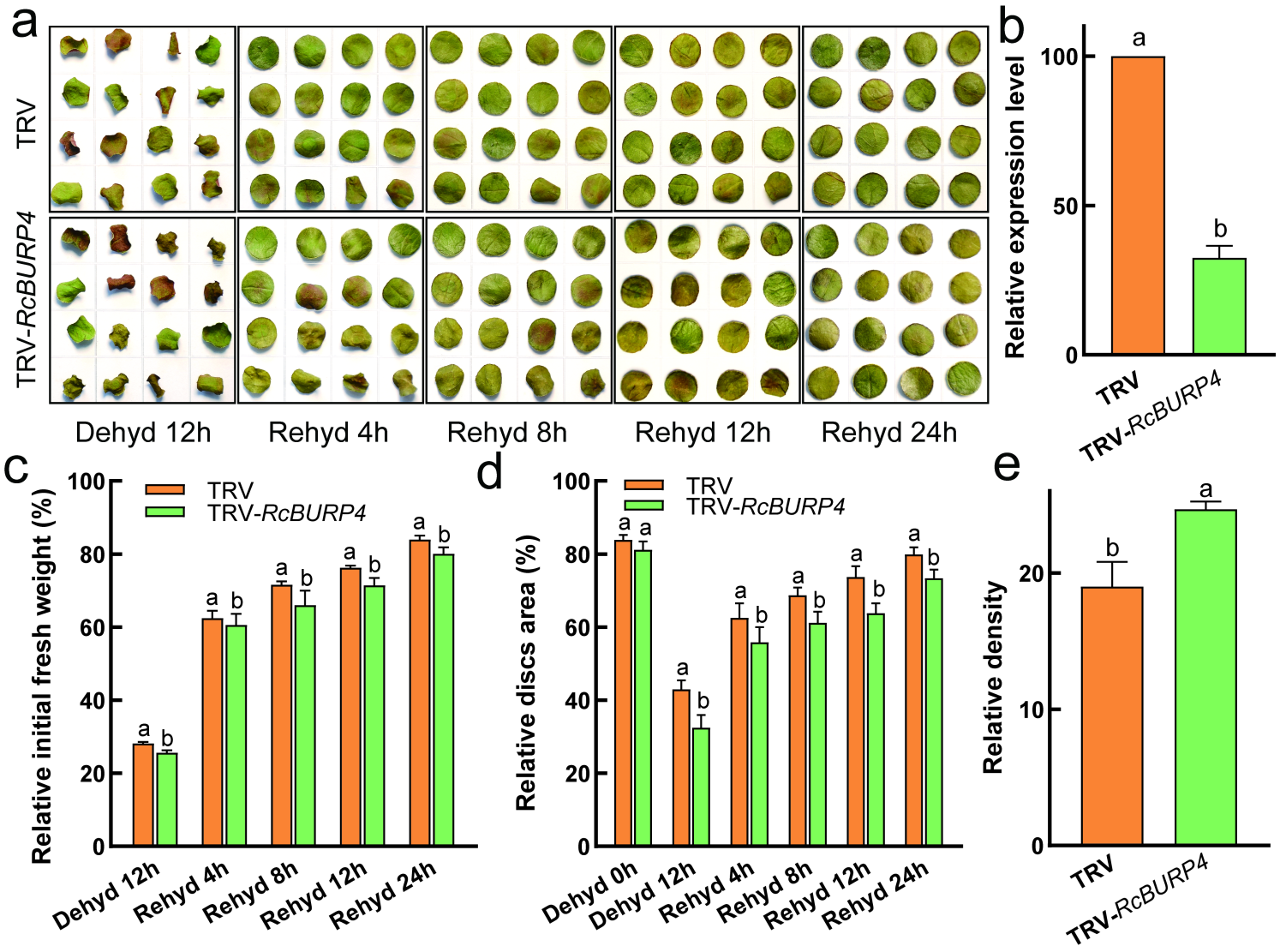

Figure 9

Silencing of RcBURP4 in rose leaf discs. a Leaf disc phenotype of RcBURP4-silenced samples and the TRV control under dehydration and rehydration conditions. Rose leaf discs were infiltrated with Agrobacterium carrying TRV controls (TRV, pTRV1 and pTRV2) or TRVs with a RcBURP4 fragment (TRV-RcBURP4, pTRV1- RcBURP4 and pTRV2-RcBURP4). After the VIGS procedure, leaf discs were dehydrated

for 12 h, and then rehydrated for 4, 8, 12, and 24 h to observe the recovery ability. B qRT-PCR analysis of RcBURP4-silenced samples and the TRV control. The discs were sampled after 12 h of rehydration to determine the VIGS efficiency by qRT-PCR. Data are presented as mean ± SD from three biological replicates. c Cell numbers of RcBURP4-silenced samples and the TRV control. Cell numbers of TRV and TRV-RcBURP4 were measured after rehydration for 12 h. Each column represents mean ± SD (n=6). Comparisons of the fresh weight (d) and disc area (e) of RcBURP4-silenced samples and the TRV control. The fresh weight and disc area of TRV and TRV-RcBURP4 leaf discs were measured after dehydration for 0 and 12 h; and rehydration for 4, 8, 12, and 24 h, respectively. Relative change refers to the value at the indicated time point versus the value at dehydration of 0 h. Error bars indicate SE (n=15). Statistically significant differences ($p < 0.05$) as determined by Tukey's HSD test are indicated with different letters.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFig.2.tif
- SupplementaryFig.1.tif
- SupplementaryTable1.xlsx
- SupplementaryTable2.xlsx
- SupplementaryTable3.xlsx
- SupplementaryTable4.xlsx