

Genome-wide identification of Kip-related protein (KRP) gene family members in eggplant and the function of *SmKRP3* under salt stress

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Abstract

Kip-related proteins (KRPs) are essential to cell division throughout their whole process of growth and development. Research on KRPs and their roles has mostly focused on *Arabidopsis thaliana*. Nonetheless, details on the structure and functions of *KRP* gene family members in eggplant are still unclear. In this study, we have discovered and analyzed five *KRP* genes in the eggplant HQ-1315 genome. Our investigation focused on determining their chromosomal position, collinearity, gene structure, conserved motif, and domain, as well as *cis*-element. The expression levels of all *KRP* genes were decreased by salt and dehydration stress, except *SmKRP3*. The expression of *SmKRP3* was significantly increased by salt stress. Transient expression assay revealed that all of the eggplant KRP proteins are located in the nucleus of epidermic cells of *Nicotiana benthamiana* leaves. Silencing of *SmKRP3* increased the sensitivity of eggplant to salt stress. This was accompanied by a significant decrease in the expression of salt stress marker genes *SmGSTU10*, *SmNCED1*, *SmDHN1*, and *SmDHNX1*. An apparent decrease in the activity of the enzymes ascorbate peroxidase (APX) and catalase (CAT). Our findings show that *SmKRP3* has a positive role in salt stress, shedding fresh light on the complete information of the *KRP* gene family members in eggplants.

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Introduction

Cells are the base unit of the plant multicellular organisms^[1]. Cell division is required for growth and development, maturity, organ morphogenesis, and stress response^[2–9]. The cell division cycle is a highly regulated and evolutionarily conserved process that is strictly controlled by multiple components, which results in DNA replication and cytoplasmic division to form two daughter cells^[10]. Cell division cycle regulation mainly depends on the cell cycle-dependent protein kinase (CDK)/cyclins complex activity. The binding of different CDKs to cyclins regulates the transition from the cell cycle G1/S phase to G2/M phase^[11]. However, the enzyme activity of CDKs was suppressed by the interaction between CDKs and Cyclin-dependent kinase inhibitor (CKI)^[12]. Based on the similarity and specificity of amino acid sequences, the *CKI* genes in mammals consist of INK4 and Kip/Cip families. The Kip/Cip family comprises three subfamilies, including p21Cip1, p27Kip1, and p57Kip2^[13]. The CKIs mainly consist of p27Kip1 and SIAMESE (SIM) gene family in plants. Due to the similar sequences in the conserved domains between p27Kip1 and Kip/Cip, the p27Kip1 in plants was named as Kip-related-proteins (KRPs)^[14]. The evidence indicated that KRPs, an inhibitor of CDKs, could negatively regulate CDKs/cyclins complex activity *via* binding to CDKs^[15].

KRP protein generally carries a conserved cyclin-dependent kinase inhibitor (CKI) domain consisting of about 50 amino acids at the C terminal, required for the binding of KRP/ICKs to CDK-cell cycle phase-specific cyclin complex (CYC)^[16]. To date, only a few studies reported that the members of *KRP* gene family in plant species were identified, such as seven *KRPs* in

Arabidopsis thaliana^[17], six *KRPs* in tomato^[18], and nine *KRPs* in soybean^[19]. Accumulating evidence has reported that KRPs played a vital role in the processes of growth and development in plants. In *Arabidopsis thaliana*, *AtKRP2* overexpression promotes trichome branching and endoreduplication^[20]. *AtKRP6* could regulate the size of rosette leaves^[21,22], gametogenesis^[23,24], germline development^[25], and cytokinesis^[26], *via* inhibiting the cell division of *Arabidopsis*. *ICK3/KRP5* is a positive regulator in *Arabidopsis thaliana* cell growth and endoreduplication^[27]. Although the functions of *KRPs* involved in plant growth and development were confirmedly demonstrated, few studies showed the functions of *KRPs* in plant response to abiotic and biotic stresses. The evidence showed that *SIKRP2* expression level was significantly up-regulated by cold stress, *SIKRP3* expression was markedly up-regulated by the treatment of drought stress in tomatoes, and silencing of *SIKRP5* enhanced the susceptibility of tomato to drought stress^[18]. In soybean, *GmKRP1a*, *GmKRP2a*, and *GmKRP4* expression levels were up-regulated by polyethylene glycol stress, and the up-regulation of *GmKRP1a*, *GmKRP2a*, *GmKRP2b*, *GmKRP4*, and *GmKRP5* expression levels treated by salt stress treatment were recorded^[19]. However, little is known about the expression of KRPs and their roles in plant response to environmental stressors as of yet.

Eggplant (*Solanum melongena*) is a popular Solanaceae vegetable cultured all around the world. The growth, development, and yield of eggplant were threatened by multiple environmental stresses, including salt, drought, high temperature, cold, or pathogenic microorganism attack^[28,29]. It helps to

breed new eggplant varieties with high environmental stress tolerance by exploring the functions of resistance-related genes. Although the knowledge of expression and functions of *KRP* gene family members in *Arabidopsis thaliana* was well known^[14,17], the information on sequences, expression, and functions of *KRPs* in eggplant has not been executed. In this study, five *KRP* gene family members (*SmKRPs*) in the eggplant HQ-1315 genome were identified, the sequences, structures and expression of *SmKRPs* in eggplant analyzed under salt and dehydration stress treatments, and the function of *SmKRP3* in eggplant response to salt stress explored. The data indicates that *SmKRP3* positively functions in eggplant against salt stress via virus-induced gene silencing (VIGS) assay.

Material and methods

Identification of *KRP* gene family members in *Solanum melongena*

The method used to identify the members of the *KRP* gene family in *Solanum melongena* was as described in previous studies^[30,31]. The amino acid sequences of *KRP* gene family members in *Arabidopsis thaliana*, which acted as the reference sequence, were obtained from The Arabidopsis Information Resource (www.arabidopsis.org) database. The TBtools software (v. 2.027)^[32], The National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) database, and the Eggplant Genome Database (<http://eggplant-hq.cn/Eggplant/home/index>; HQ-1315) were used to identify the members of *KRP* gene family in *Solanum melongena*. The *KRP* amino acid sequences from the eggplant inbred lines '67/3' v4.1 (an update version of '67/3' v3.0)^[33], '67/3' v3.0 (<https://solgenomics.net>), as well as GUIQIE-1^[34], were obtained to perform the comparative analysis of amino acid sequences of these *KRP* proteins.

Physicochemical properties analysis of *KRP* proteins in eggplant

The physicochemical properties of *KRP* proteins, including molecular weight, theoretical pI, instability index, and grand average of hydropathicity, were analyzed by searching ProtParam ExPASy (<https://web.expasy.org/protparam/>) website with amino acid sequences of *KRP* proteins in eggplant^[35].

Multiple sequence alignment and phylogenetic analysis

The amino acid sequences of *SmKRPs* from *Solanum melongena*, *Solanum lycopersicum*, *Capsicum annuum*, and *Arabidopsis thaliana* were used to perform multiple sequence alignment and phylogenetic analysis by MEGA 7.0 (v. 7.026) software^[18]. The evolutionary tree of *KRP* genes was generated by MEGA 7.0 software using the neighbor-joining (NJ) method with 1,000 bootstrap replications^[36]. The Evolview website (<https://evolgenius.info/evolview-v2/#login>) was used to modify the evolutionary tree.

Analysis of chromosomal assignment and collinearity of *SmKRPs*

The distributions of *SmKRPs* were analyzed using the Gene Location Visualize from the GTF/GFF functional module of TBtools software. Collinearity relationships of *SmKRPs* were analyzed and visualized by using the Multiple Collinearity Scan toolkit (MCScanX) functional module of TBtools software^[37].

Analysis of gene structure, *cis*-elements, conserved motif, and domains of *SmKRPs*

The gene structures of *SmKRPs* were visualized using the functional module Visualize Gene Structure (Basic) of TBtools software. The *cis*-elements within the promoters of *SmKRPs* were analyzed by searching the PlantCARE website (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>). The conserved motifs of *SmKRP* proteins were predicted by searching the MEME website (<https://meme-suite.org/meme/tools/meme>). The conserved domains of *SmKRP* proteins were analyzed by searching the SMART website (<http://smart.embl-heidelberg.de/>) with the amino acid sequences of *SmKRPs*.

Plant growth and conditions

The seeds of eggplant cultivar ML41, which exhibited middle salt tolerance, were packaged with clean gauze and then placed into the 55 °C water bath at 15 min for pre-germination. The treated seeds were transferred into tap water at room temperature overnight. The seeds were washed and then sowed in the nutrient soil for germination. The eggplant seedlings with two cotyledons were transplanted in a small plastic pot (7 cm × 7 cm) filled with nutrient soil. The seeds of *Nicotiana benthamiana* were sowed on the wet nutrient soil for germination. The seedlings of *Nicotiana benthamiana* with two cotyledons and two euphyllas were transplanted in the small plastic pot (7 cm × 7 cm) with nutrient soil. The eggplant and *Nicotiana benthamiana* seedlings were placed in the illumination incubator with the condition of 25 °C, photoperiod 16 h light/8 h dark, and 60% relative humidity.

Salt and dehydration stresses treatment

The plants of eggplant ML41 with 4–6 leaves were gently pulled out from the nutrient soil. The roots were washed with tap water and then cultivated in the Hoagland nutrient solution for 2 days for root restoration. For salt stress treatment, the roots of the eggplant were soaked in 200 mM NaCl solution. The root samples were taken at 0, 2, 6, 12, 24, and 48 h and directly placed in liquid nitrogen. For dehydration stress, the surface of eggplant roots was dried with filter paper and then placed on a table. The root samples were harvested under the condition of liquid nitrogen at the time points of 0, 0.5, 1, 3, 6, and 9 h post-treatment.

Vector construction

For subcellular localization assay, the full-length codon sequence (CDS) of *SmKRPs* were respectively amplified by PCR assay and cloned into the plant overexpression vector pBinGFP2 (containing a GFP protein tag) by using ClonExpress II One Step Cloning Kit (C112-01, Vazyme, China). For the VIGS assay, the specific DNA fragment of *SmKRP3* was amplified and cloned into the entry pDONR207 plasmid by BP reaction and then transferred into the pTRV2 vector by LR reaction. The primer pairs sequences used for vector construction in this study are listed in Supplemental Table S1.

Plant total RNA extraction, cDNA synthesis, real-time fluorescent quantitative PCR analysis

For total RNA extraction, the roots of eggplant were ground into powder by tissue crusher under liquid nitrogen conditions. The plant cells were broken by lysis buffer TRIpure Reagent (RN0102, Aidlab Biotechnologies Co., Ltd, China). The total RNA was extracted by tri-chloromethane and then sedimented by

isopropanol. The concentration of purified total RNA was measured by NanoPhotometer (NP80, Implen, Germany). An agarose gel electrophoresis assay was performed to assess whether total RNA degradation occurs. The cDNA synthesis was carried out following the protocol of HiScript III RT SuperMix for qPCR (+gDNA wiper) (R323-01, Vazyme, China) kit. For the detection of target genes' expression, real-time fluorescent quantitative PCR (RT-qPCR) analysis was performed following the specification of ChamQ Universal SYBR qPCR Master Mix (Q711-02, Vazyme, China). *SmActin* (Smechr1100649) was regarded as the reference gene to standardize the target genes' expression. Three biological repeats were utilized. The target genes' expression was calculated by using $2^{-\Delta\Delta C_t}$ method^[38]. The primer pairs sequences used in this study are listed in [Supplemental Table S1](#).

Agrobacterium tumefaciens cultivation and infiltration as well as subcellular localization analysis of *SmKRP* proteins

Agrobacterium tumefaciens strain GV3101 cells carrying 35S:*GFP-SmKRPs* or 35S:*GFP* (acted as a control) constructs grew in the liquid Luria-Bertani (LB) medium with 50 µg/mL kanamycin and 50 µg/mL rifampicin under the condition of 28 °C, 200 rpm overnight. *Agrobacterium* culture was centrifuged under 28 °C, 8,000 rpm for 10 min, and gathered the bacterium cells. The *Agrobacterium* cells were resuspended by infiltration buffer (10 mM MES, 10 mM MgCl₂, 200 µM acetosyringone, pH = 5.4) to adjust the OD₆₀₀ to 0.8. *Agrobacterium* GV3101 cells harboring containing 35S:*GFP-SmKRPs* or 35S:*GFP* constructs were incubated in the shaker under the condition of 28 °C, 60 rpm for 1 h. The bacterium was injected into the leaves of *Nicotiana benthamiana* using a disposable sterile syringe without a needle.

VIGS assay

Agrobacterium GV3101 cells containing TRV1, TRV2:*SmPDS* (encoded a phytoene desaturase, which was used as a marker to prove whether the virus-induced gene silencing assay successful), TRV2:*SmKRP3*, or TRV2:*00* constructs were respectively cultivated in the liquid LB medium. The GV3101 cells were gathered by centrifugation. The OD₆₀₀ of bacterium solution was adjusted to 0.8 by using an infiltration buffer. The GV3101 cells carrying TRV1 constructs were respectively mixed with the GV3101 cells containing TRV2:*SmPDS*, TRV2:*SmKRP3*, or TRV2:*00* constructs at a 1:1 ratio. The mixtures were incubated in the shaker at 28 °C, 60 rpm for 1 h, and then injected into the cotyledon of eggplant seedlings with 2–3 leaves. The infiltrated eggplant seedlings were placed in the illumination incubator at 20 °C without light for 48 h. The eggplant seedlings grew in the illumination incubator at 25 °C, photoperiod 16 h light/ 8 h dark for 3 weeks until the leaves of the plants infiltrated with TRV:*SmPDS* exhibited photobleaching. For survival rate statistics, each biological repeat including *SmKRP3*-silenced and control contained eight plants, which were treated with 200 mM NaCl solution. After 4 d, the healthy plants and the plants with yellow or wilt leaves of eggplants in each biological repeat were counted to calculate the survival rate.

Measurement of physiological index

The physiological indexes, including APX and CAT enzyme activity, were measured as described in previous studies^[31,39].

Results

Identification and physicochemical property analysis of *KRP* proteins in eggplant

According to previous studies, six *KRP* members were identified in the eggplant '67/3' genome (v3.0)^[18,40]. However, only five *KRP* genes were identified, including *SmKRP1-5* in the eggplant HQ-1315 genome. The amino acid sequences of *KRP* proteins in eggplant '67/3' v4.1, GUIQIE-1, and the HQ-1315 genome were further investigated. The numbers and amino acid sequences of *KRP* members in eggplant '67/3' v4.1 and v3.0 genome did not change ([Supplemental Fig. S1a](#)). In the GUIQIE-1 genome, six *KRP* members were identified, and five transcripts of EGP26265 and two transcripts of EGP17106 were observed ([Supplemental Table S2](#)). It was found that five *KRP* members in the eggplant '67/3' (v4.1 and v3.0) and GUIQIE-1 genome had corresponding homologs in eggplant HQ-1315 genome. However, the amino acid sequence of *KRP* protein (SMEL4.1_09g013240.1 and SMEL_002g167200) in eggplant '67/3' (v4.1 and v3.0) and EGP31387.1 in GUIQIE-1 exhibited some difference comparing to its homolog (Smechr0202706) in eggplant HQ-1315 ([Supplemental Fig. S1a](#)). In addition, the results of conserved domain prediction showed that SMEL4.1_09g013240.1, SMEL_002g167200, and EGP31387.1 protein respectively contained a conserved CDI domain, while Smechr0202706 protein did not carry a conserved CDI domain ([Supplemental Fig. S1b](#)). This may be the reason for the difference in the number of *KRP* members between in eggplant '67/3', GUIQIE-1, and HQ-1315. In addition, *KRP* genes were further identified in other Solanaceae plants including six *KRP* genes in potato, six *KRP* genes in tobacco, six *KRP* genes in pepper, and six *KRP* genes in tomato ([Supplemental Table S3](#)). The physicochemical properties of five *SmKRPs* in eggplant HQ-1315 were further analyzed. As shown in [Table 1](#), *SmKRPs* encoded 157~222 amino acids with 474~669 base pairs (bp). The molecular weight, theoretical pI, instability index, and grand average of hydropathicity of *SmKRP* proteins were 18,579.92~24,529.1 Da, 5.11~9.84, 40.16~62.55, and -1.162~-0.834, respectively ([Table 1](#)).

Phylogenetic analysis of *KRP* genes from eggplant, tomato, pepper, and *Arabidopsis thaliana*

To analyze the phylogenetic relationship of *SmKRPs* with its homologs from the other plant species, we generated the evolutionary tree of *SmKRPs* with the *KRP* gene family members from *Arabidopsis thaliana*, tomato (*Solanum lycopersicum*), and pepper (*Capsicum annuum*) using MEGA 7.0 software. We found that the *KRP* genes from these four plant species were divided into three subgroups, including class I, class II, and class III ([Fig. 1](#)). In class I, *SmKRP5*, and *SIKRP5* were grouped into the same branch, implying that the sequence and function of *SmKRP5* may be similar to *SIKRP5*. In class II, there were three *SmKRPs*, including *SmKRP1*, *SmKRP2*, and *SmKRP3*. In class III, *SmKRP4* had a high sequence similar to *SIKRP6*.

Analysis of chromosomal location, collinearity relationship, gene structure, conserved domain and motif, and *cis*-elements of *SmKRP* members

Next, the chromosomal location of *SmKRPs* were analyzed. The results showed that five *SmKRPs* were located on four chromosomes including Chr. 1, Chr. 3, Chr. 5, and Chr. 9 ([Fig. 2a](#)).

Table 1. Physicochemical properties of the *KRP* family genes in eggplant.

Gene name	Gene ID	CDS length (bp)	Protein length (aa)	Molecular weight (Da)	Theoretical pI	Instability index	Grand average of hydropathicity
<i>SmKRP1</i>	Smechr0902520.1	636	211	23,426.48	9.47	45.52	−0.889
<i>SmKRP2</i>	Smechr0901526.1	483	160	18,779.21	9.84	62.55	−0.834
<i>SmKRP3</i>	Smechr0502598.1	669	222	24,471.22	9.28	50.26	−1.078
<i>SmKRP4</i>	Smechr0100331.1	663	220	24,529.1	5.11	40.16	−0.862
<i>SmKRP5</i>	Smechr0300499.1	474	157	18,579.92	6.98	62.29	−1.162

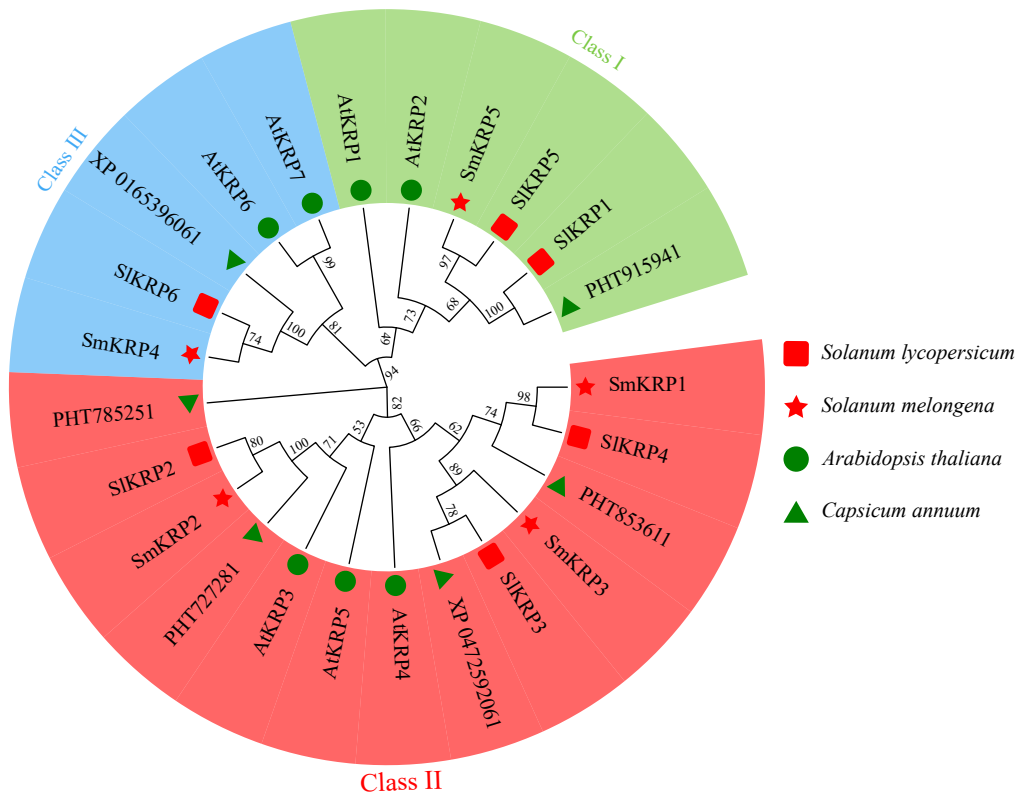


Fig. 1 Phylogenetic relationship analysis of *SmKRPs* with its homologs from *Arabidopsis thaliana*, *Solanum lycopersicum*, and *Capsicum annuum* by constructing an evolutionary tree. The *KRP* proteins were distributed into three classes, which is distinguished by different colors.

Both *SmKRP1* and *SmKRP2* are located on Chr. 9, while *SmKRP3*, *SmKRP4*, and *SmKRP5* are located on Chr. 5, Chr. 1, and Chr. 3, respectively. In addition, we also investigated the collinearity relationship among five *SmKRPs*. We analyzed the homology and sequence similarity of the *KRP* members between eggplant and tomato. The *KRP* members *SmKRP1*~*SmKRP5* shared 84%, 64%, 70%, 81%, and 43% sequence similarities with the *KRP* members *SIKRP4*, *SIKRP2*, *SIKRP3*, *SIKRP6*, and *SIKRP1* in tomato, respectively (Supplemental Table S4). Interestingly, it was found that the locations of *SmKRP1* and *SmKRP2* on the eggplant Chr. 9 were similar to the locations of their homologs *SIKRP4* and *SIKRP2* on the tomato Chr. 9 (Fig. 2a), suggesting that these two groups of *KRP* homologous exhibit similarities in evolution. A collinearity relationship was found between *SmKRP1* and *SmKRP3* (Fig. 2b), suggesting that a duplicated segment occurred between *SmKRP1* and *SmKRP3*. Moreover, the collinearity relationship of *KRP* members in eggplant, *Arabidopsis thaliana*, and tomato were also analyzed. However, the collinearity relationship among these *KRP* members was not observed (Supplemental Fig. S2).

The phylogenetic relationship among *SmKRPs* were analyzed. There is a high sequence similarity between *SmKRP1* and *SmKRP3*, and *SmKRP4* and *SmKRP5*, respectively (Fig. 3a). The gene structures of *SmKRPs* were also analyzed. According to the phylogenetic relationship among *SmKRPs*, the *KRP* members with higher sequence similarity have the same exon number. *SmKRP1*–*3* have three exons, while *SmKRP4* and *SmKRP5* contain four exons (Fig. 3b). The conserved domain in the amino acid sequences of *SmKRP* proteins were analyzed by searching the SMART website and found that all the *SmKRP* proteins exhibited a conserved CDI domain at C terminal (Fig. 3c). Moreover, 10 conserved motifs (motif 1–10) of *SmKRP* proteins were identified by searching the Multiple Em for Motif Elicitation (MEME) website with amino acid sequences (Fig. 3d). The amino acid sequence length of these conserved motifs ranged from 6 to 47 aa. Motif 1 within these five *SmKRPs* was the CDI domain.

As shown in Fig. 3e, multiple *cis*-elements related to phyto-hormone response elements, transcription factor binding elements, and stress response elements were observed within these five *SmKRPs* promoters (Supplemental Table S5). The

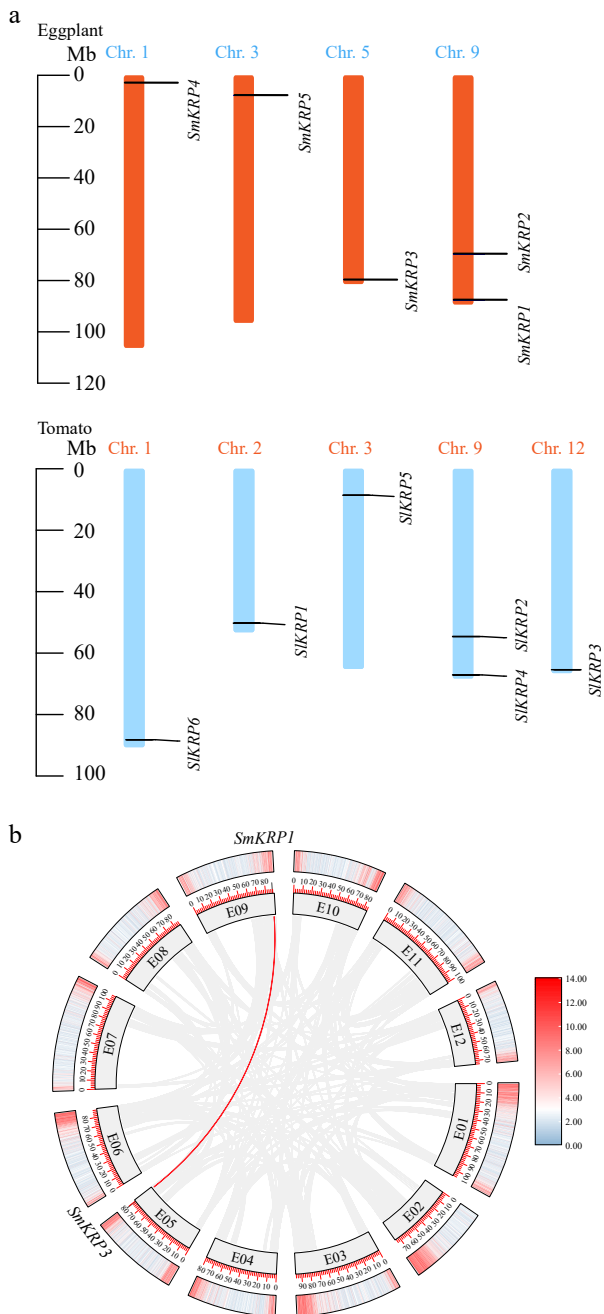


Fig. 2 (a) Chromosomal assignment of KRP members in eggplant and tomato, (b) the duplication of *SmKRPs* on eggplant chromosomes. The Chr. 1, Chr. 3, Chr. 5, Chr. 9, and Chr. 12 in (a) as well as E01~E12 in (b) represent the chromosome number.

phytohormone response elements consisted of abscisic acid (SA) responsive element TCA-element, jasmonic acid methyl ester (MeJA) responsive element TGACG-motif, abscisic acid (ABA) responsive element ABRE, ethylene-responsive element ERE, auxin-responsive element TGA-element or TGA-box, and gibberellin responsive element GARE-motif or P-box within the promoters of *SmKRPs*. The transcription factor binding elements, including MYB transcription factor binding element MYB, MYC transcription factor binding element MYC, bZIP transcription factor binding element G-box, and WRKY transcription factor binding element W-box, were identified in the

promoters of *SmKRPs*. In addition, we also found that the *SmKRPs* promoters contained some elements related to stress response, including the stress response element STRE, *cis*-acting element involved in defense and stress responsiveness TC-rich repeats, and *cis*-acting regulatory element essential for the anaerobic induction ARE.

Analysis of expression profiles of *SmKRPs* under salt and dehydration stress treatment

Based on the analysis of the mRNA sequencing result of eggplant roots under salt stress treatment, it was found that salt stress could only induce the up-regulation of *SmKRPs3* expression (Supplemental Fig. S3). To verify this result, the expression of *SmKRPs* in eggplant roots treated with 200 mM NaCl stress was detected by RT-qPCR assay. Salt stress could induce significant up-regulation of *SmKRPs3* at 6 h post-treatment. The expression of other *SmKRPs* exhibited a down-regulated tendency under salt stress treatment (Fig. 4a). The expression profiles of *SmKRPs* under dehydration stress treatment were also tested. It was found that the expression levels of all *SmKRPs* displayed a down-regulated trend in the condition of dehydration stress treatment (Fig. 4b), implying that *SmKRPs* may play a negative role in eggplant response to dehydration stress.

Analysis of tissues specific expression of *SmKRPs*

It is informative to understand the role of *SmKRPs* in different biological processes in eggplant by analyzing the tissue-specific expression of *SmKRPs*. An RT-qPCR assay was performed to examine the expression of *SmKRPs* in diverse tissues from various developmental stages of eggplants. It was observed that a higher expression of *SmKRPs1*, *SmKRPs2*, and *SmKRPs3* exhibited in the root (RT) than in the young leaf (YL) and stem (ST), while the expression of both *SmKRPs4* and *SmKRPs5* in YL were higher than that in the RT and ST in the seedlings. In mature plants, the highest expression levels of *SmKRPs1*, *SmKRPs2*, and *SmKRPs3* were recorded in the fruit (FRT) compared to the other tissues. The highest expression levels of both *SmKRPs4* and *SmKRPs5* were observed in the YL (Fig. 5).

Subcellular localization analysis of *SmKRPs* proteins

Next, the subcellular localization of *SmKRPs* proteins was investigated. Firstly, *SmKRPs* proteins' subcellular localization was predicted by searching the Plant-mPloc (www.csbio.sjtu.edu.cn/bioinf/plant-multi) website with their amino acid sequences. All *SmKRPs* proteins were predicted to be located in the nucleus (Supplemental Fig. S4a). The nuclear localization signal (NLS) within amino acid sequences of *SmKRPs* proteins were further analyzed by searching the INSP (www.csbio.sjtu.edu.cn/bioinf/INSP) website. Shown in Supplemental Fig. S4b, one NLS in the amino acid sequence of *SmKRPs2* protein was observed, while two NLS were contained in the other four *SmKRPs* protein amino acid sequences. To further confirm whether *SmKRPs* proteins are located in the nucleus, subcellular localization of *SmKRPs* proteins in the epidemic cells of *Nicotiana benthamiana* leaves was investigated via *Agrobacterium*-mediated transient infiltration. The full-length CDS of *SmKRPs* were cloned into the destination vector pBinGFP2 (Fig. 6a). The laser scanning confocal microscope was used to observe the fluorescence signals. We observed that the green fluorescence signal of all *SmKRPs*-GFP proteins occurred in the nucleus. The

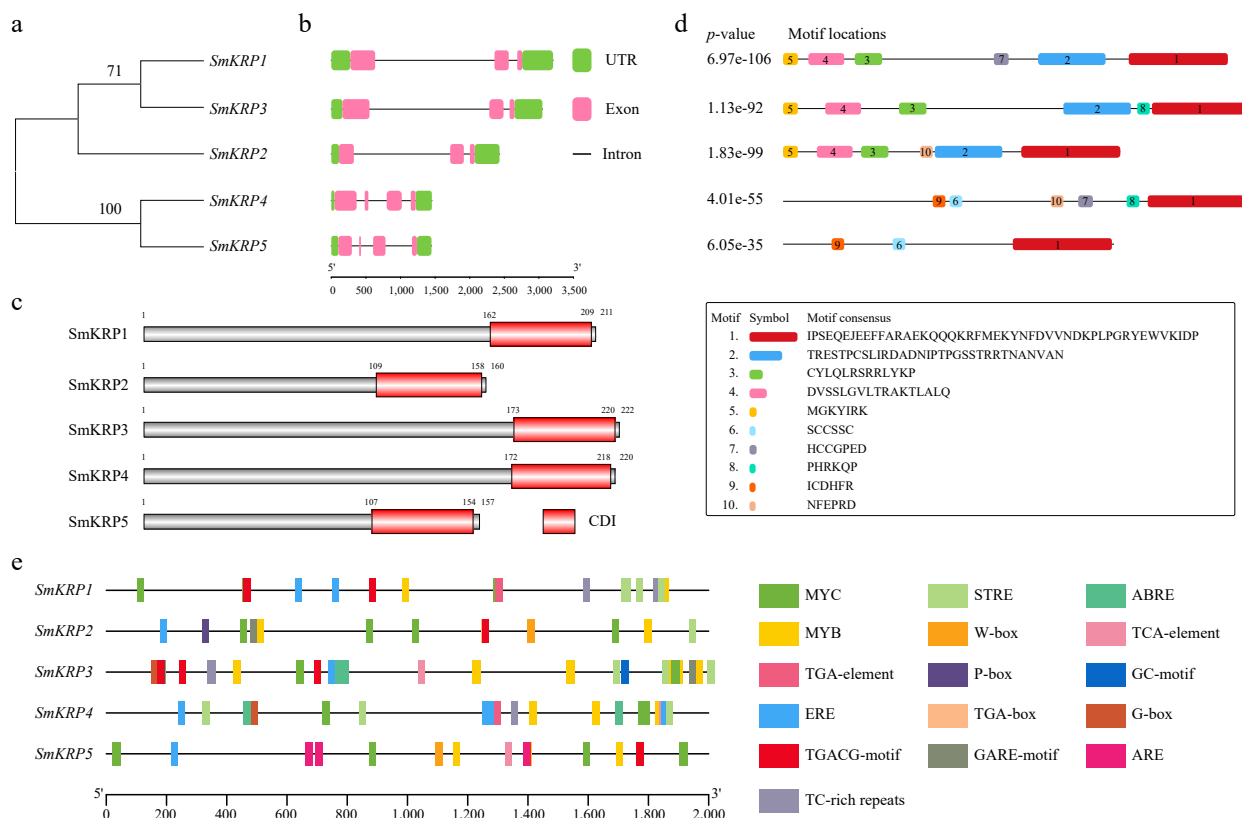


Fig. 3 Analysis of gene structure, conserved domains and motifs of *SmKRPs*. (a) Phylogenetic relationship analysis among *SmKRPs*. (b) Distribution of exon, intron, and UTR of *SmKRPs*. Green box, pink box and black line represent UTR, exon, and intron, respectively. (c) Conserved domains analysis of *SmKRP* proteins. The red box represents conserved domain CDI. (d) Distribution of conserved motifs within *SmKRP* proteins. The 1–10 motifs were identified by searching MEME website, and were distinguished by different color boxes. The motif 1 is CDI domain. (e) Analysis of *cis*-elements within the promoters of *SmKRPs*. The *cis*-elements were predicted by searching the PlantCARE website, and were differentiated by different colored boxes.

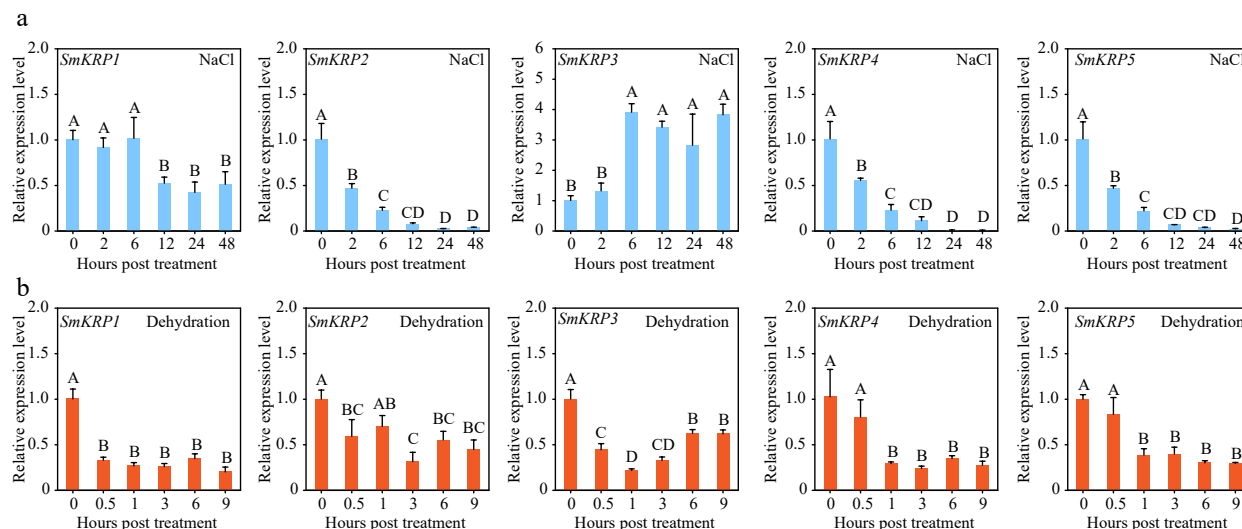


Fig. 4 Expression profile analysis of *SmKRPs*. Expression levels of *SmKRPs* were detected in eggplant roots treated with (a) NaCl or (b) dehydration stress by RT-qPCR assay. Three biological repeats were applied to calculate the mean \pm standard deviation. Different upper letters represent highly significant differences, as performed by Fisher's protected LSD test ($p < 0.01$).

green fluorescence signal expressed by an empty vector appeared in the whole cell (Fig. 6b), suggesting that all *SmKRP* proteins are located in the nucleus of the epidermic cells of *Nicotiana benthamiana* leaves.

Analysis of amino acid sequence multiple alignment and phylogenetic relationship of *SmKRP3*

Due to the significant up-regulation of *SmKRP3* expression under salt stress treatment, we explored the function of

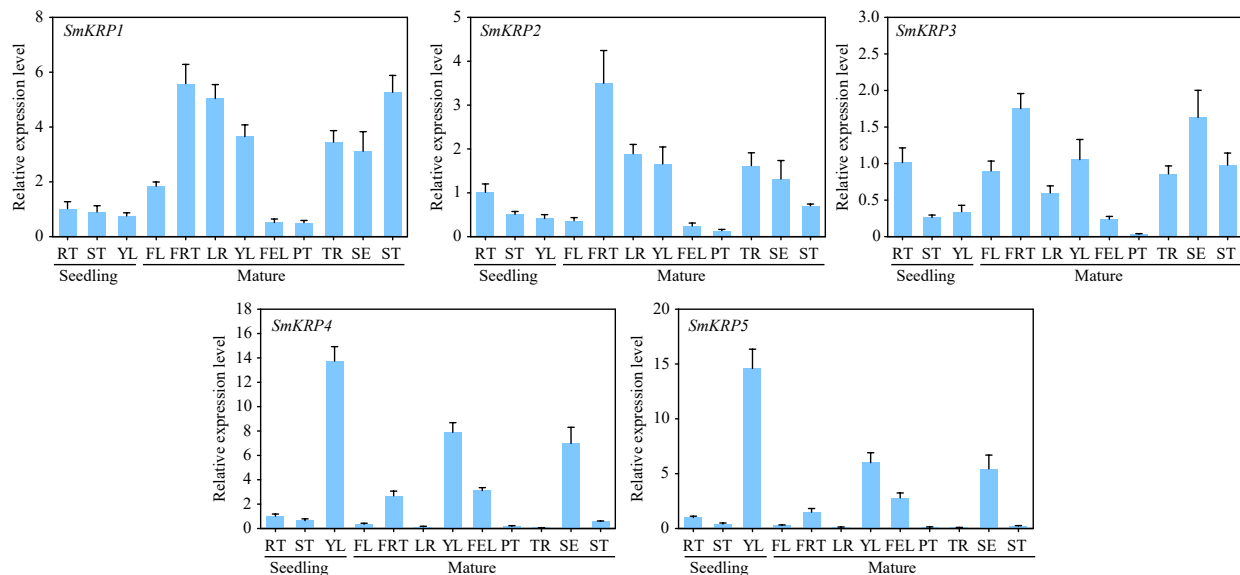


Fig. 5 Analysis of tissue specific expression of *SmKRPs*. The relative expression levels of *SmKRPs* in the different tissues from seedlings including root (RT), stem (ST), and young leaf (YL) and from mature plants including flower (FL), fruit (FRT), lateral root (LR), YL, full expand leaf (FEL), petiole (PT), taproot (TR), sepal (SE), and ST by RT-qPCR assay. Three biological repeats were applied to calculate the mean \pm standard deviation. Different uppercase letters represent highly significant differences, as performed by Student's *t*-test ($p < 0.01$).

SmKRP3 in eggplant against salt stress. Firstly, we analyzed the multiple alignment of the *SmKRP3* amino acid sequence with its homologs from the other plant species. We found that *SmKRP3* amino acid sequence shares 71%, 70%, 66%, 54%, 51%, 49%, 45%, 44%, 43%, 43%, 39%, and 37% sequence similarities with its homologs from the selected plant species including *Solanum tuberosum* hypothetical protein KY285_009599 (KAH0733892.1), *Solanum lycopersicum* cyclin-dependent kinase inhibitor 4-like (NP_001304938.1), *Solanum dulcamara* cyclin-dependent kinase inhibitor 4-like (XP_055813395.1), *Nicotiana tabacum* cyclin-dependent kinase inhibitor 5-like (XP_016513903.1), *Lycium ferocissimum* cyclin-dependent kinase inhibitor 4-like (XP_059286725.1), *Capsicum annuum* cyclin-dependent kinase inhibitor 4 (XP_047259206.1), *Ipomoea nil* cyclin-dependent kinase inhibitor 3-like (XP_019156941.1), *Gossypium hirsutum* cyclin-dependent kinase inhibitor 5 (XP_016732133.1), *Prunus persica* cyclin-dependent kinase inhibitor 5 (ALV85618.1), *Arabidopsis thaliana* inhibitor/interactor with cyclin-dependent kinase (NP_199693.1), *Triticum aestivum* cyclin-dependent kinase inhibitor 4-like isoform X2 (XP_044449154.1), and *Oryza sativa Japonica Group* cyclin-dependent kinase inhibitor 4 (NP_001390949.1), respectively (Supplemental Fig. S5a). Based on the amino acid sequences of *SmKRP3* homologs above, an evolutionary tree was constructed to analyze the phylogenetic relationship of these homologs. *SmKRP3* exhibited a closest phylogenetic relationship with *Solanum dulcamara* cyclin-dependent kinase inhibitor 4-like (Supplemental Fig. S5b).

Silencing of *SmKRP3* enhances the susceptibility of eggplant to salt stress

Additionally, the impact of *SmKRP3* silencing on eggplant tolerance to salt stress was evaluated using a VIGS assay. Compared to the control eggplants, *SmKRP3* expression level was significantly down-regulated in the roots of *SmKRP3*-silenced (TRV:*SmKRP3*) eggplants, and the increased regulation of *SmKRP3* expression by salt stress treatment was inhibited in

the *SmKRP3*-silenced eggplants compared to the TRV:00 control eggplants (Fig. 7a). In addition, it was also found that the *SmKRP3* expression level in the leaves of *SmKRP3*-silenced eggplants was observably down-regulated comparing to the control plants (Supplemental Fig. S6), suggesting that silencing of *SmKRP3* was successful. The *SmKRP3*-silenced and control eggplants were treated with 200 mM NaCl solution. After 24 h, *SmKRP3*-silenced eggplants exhibited more obvious wilt symptom than that of control plants (Fig. 7b). The lower survival rate was recorded in the *SmKRP3*-silenced eggplants comparing to the control under salt stress treatment after 4 d (Fig. 7c), revealing that silencing of *SmKRP3* enhanced eggplant susceptibility to salt stress. Moreover, silencing of *SmKRP3* induced overt down-regulation of expression levels of salt stress defense-related marker genes including *SmGSTU10*^[41], *SmNCED1*^[28], *SmDHN1*^[30], and *SmDHNX1*^[30] under salt stress treatment (Fig. 7d). *SmKRP3*-silenced and control eggplants treated with salt stress showed physiological indices, including APX and CAT enzyme activity. An obvious decrease of APX and CAT enzyme activities were observed in the roots of *SmKRP3*-silenced eggplants compared to the control plants (Fig. 7e). These data revealed that *SmKRP3* positively regulates salt stress tolerance in eggplants.

Discussion

Plant needs to create new organs *via* rapid cell division and differentiation to repair the damage caused by multiple abiotic and biotic stresses^[42,43]. Acted as an inhibitor of CDKs activity, KRPs play a vital role in regulating the cell cycle process^[44]. So far, the *KRP* genes have been identified in certain plant species, such as *Arabidopsis thaliana*^[17], tomato^[18], and soybean^[19], and mainly function in the plant's growth and development processes. However, the information of *KRP* gene family members in plants and their functions in plant response to abiotic stresses were largely unknown. Herein, we identified

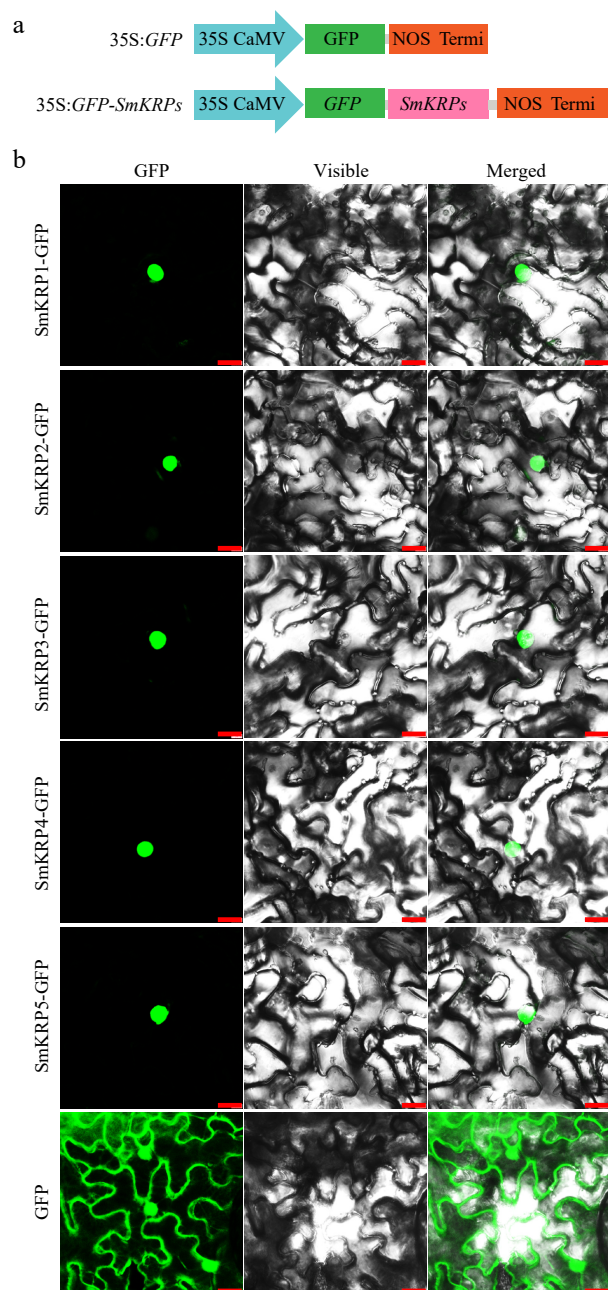


Fig. 6 Subcellular localization of *SmKRP* proteins. (a) Diagrammatic drawing of recombinant vector structures of 35S::GFP-*SmKRPs* or empty vector 35S::GFP. (b) Subcellular localization of *SmKRP* proteins in the epidermic cells of *Nicotiana benthamiana* leaves. Bar = 25 μm.

five *KRP* genes in the eggplant genome, further analyzed the sequences, structures, and expression of *SmKRPs*, and explored the functions of *SmKRP3* in eggplant response to salt stress.

In the present study, five *KRP* members in the eggplant HQ-1315 genome were identified, named *SmKRP1*–*5*. Interestingly, a previous study reported six *KRP* members in eggplant '67/3' (v3.0 and v4.1) genome^[18]. Six *KRP* members in the genome of eggplant variety GUIQIE-1 were also identified (Supplemental Table S2). This difference in the number of *KRP* members is due to the distinction of amino acid sequences and conserved domain between SMEL_002g167200 or SMEL4.1_09g013240.1

protein in eggplant '67/3' (v3.0 and v4.1), EGP31387.1 protein in GUIQIE-1, and its homolog Smechr0202706 protein in eggplant HQ-1315 (Supplemental Fig. S1). The deficiency of the conserved CDI domain of the Smechr0202706 protein may be likely due to genetic evolution or mutations in eggplant HQ-1315. We also found that there was no change in the number of *KRP* members in Solanaceae plants such as six *KRP* genes in potatoes, six *KRP* genes in tobacco, six *KRP* genes in pepper, and six *KRP* genes in tomato (Supplemental Table S3), suggesting that *KRP* gene family in Solanaceae probably did not expand during evolution. We next generated an evolutionary tree of *SmKRPs* with its homologs from *Arabidopsis thaliana*, tomato, and pepper to analyze their phylogenetic relationship. Five *SmKRPs* were classified into three classes (Fig. 1), consistent with the *Arabidopsis thaliana* report^[17]. In addition, it was found that *SmKRP1* is more closely related to *SmKRP3* among the *SmKRPs* by analyzing the phylogenetic relationship of *SmKRPs* (Fig. 3a), implying that segment duplication may occur between *SmKRP1* and *SmKRP3*. The result of chromosomal assignment analysis revealed that *SmKRPs* located on four chromosomes, including Chr. 1, 3, 5, and 9 (Fig. 2a). The inter-chromosome segment duplication and tandem duplication are important ways of genetic variation and species evolution in plants^[45]. The collinearity relationship between *SmKRP1* and *SmKRP3* was observed, suggesting that a duplicated segment indeed exhibited between *SmKRP1* and *SmKRP3* (Fig. 2b). Previous study showed that *SIKRP1* and *SIKRP3*, *SIKRP1* and *SIKRP5*, *SIKRP1* and *SIKRP6*, as well as *SIKRP3* and *SIKRP4* exhibit inter-chromosomal fragment replication in tomato^[18]. At the same time, only *SmKRP1* and *SmKRP3* occur in inter-chromosome segment duplication in eggplant, suggesting that *SmKRPs* may be more conservative in structures and functions in eggplant. However, we did not observe the collinearity relationship among the *KRP* members in eggplant, *Arabidopsis thaliana*, and tomato (Supplemental Fig. S2), implying that *KRP* genes in these three plant species may have evolved independently. The results of gene structures and conserved motifs analysis showed that the similar gene structures and motifs were observed among the closely related genes such as *SmKRP1*, *SmKRP2*, and *SmKRP3* (Fig. 3b, d). Multiple *cis*-elements including phytohormone response, transcription factor binding, and stress response related elements were contained within the promoters of *SmKRPs* (Fig. 3e), suggesting that *SmKRPs* expression levels were regulated by multiple phytohormone and transcription factor components in different biological processes.

Previous studies revealed that *KRP* genes mainly function in the processes of growth and development in plants^[19,21,22,46–48]. However, a few studies reported that *KRP* genes were involved in the plant's response to abiotic stresses. In tomato, only a few *SIKRP* expression levels were up-regulated by abiotic stresses. Drought and cold stresses could only up-regulate the expression of *SIKRP3* and *SIKRP2*, respectively^[18]. Salt stress could up-regulate *GmKRP2b*, *GmKRP4*, and *GmKRP5* expression^[19]. A similar expression pattern of *KRPs* in this study was observed in that only *SmKRP3* expression level was up-regulated by salt stress. In contrast, a declining trend of *SmKRPs* expression was recorded under dehydration stress treatment (Fig. 4a, b). The result of tissues specific expression of *SmKRPs* in the diverse tissues from different developmental stages of eggplants revealed that *SmKRP1*–*3* have higher expression levels in RT in the seedlings

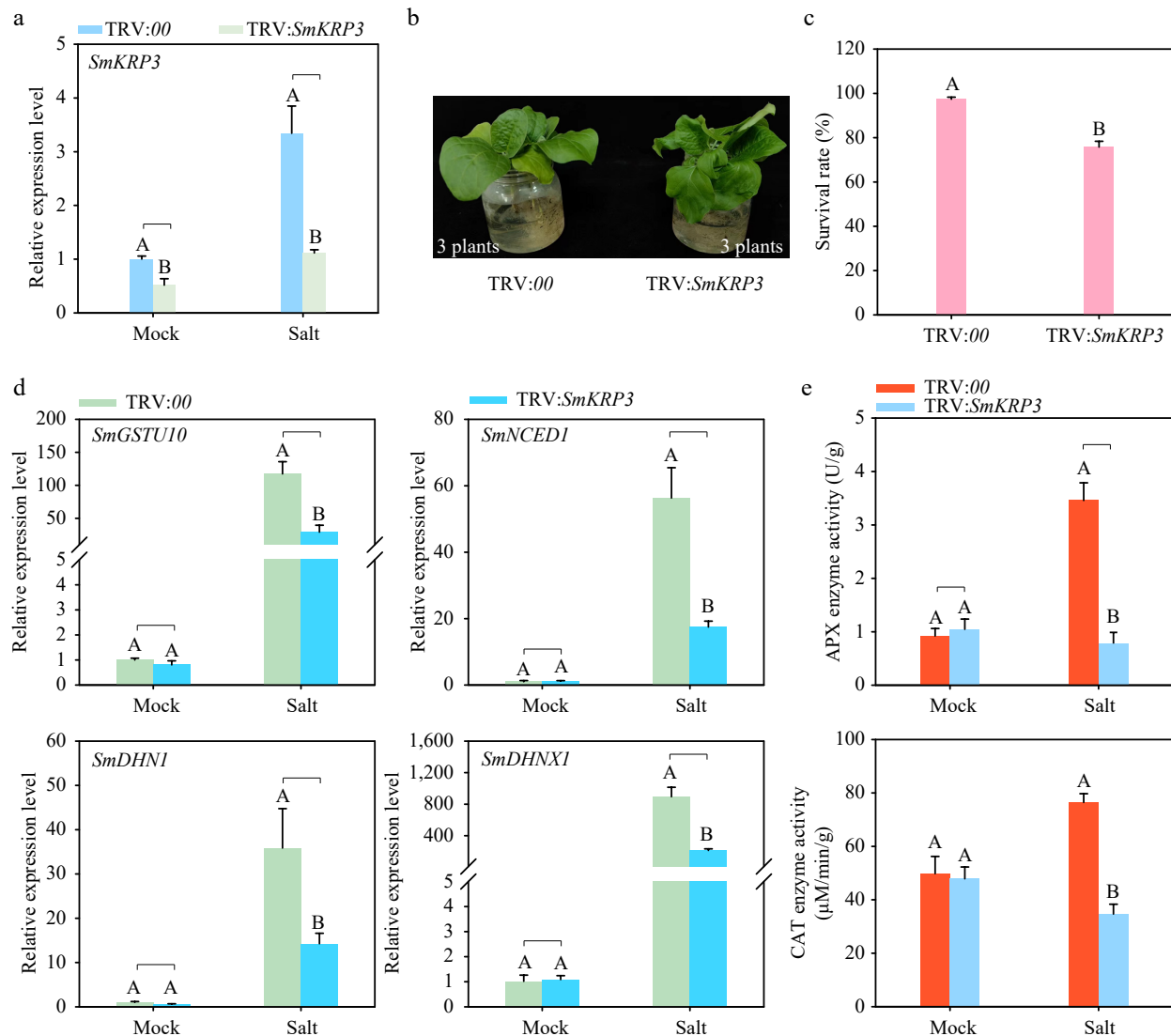


Fig. 7 Silencing of *SmKRP3* enhances susceptibility of eggplant to salt stress. (a) Detection of silencing efficiency of *SmKRP3*. (b) Phenotype analysis of *SmKRP3*-silenced or control eggplants under salt stress treatment after 24 h post treatment. (c) Survival rate analysis of *SmKRP3*-silenced or control eggplants under salt stress treatment at 4 d post salt stress treatment. (d) Detection of expression levels of salt stress defense related marker genes *SmGSTU10*, *SmNCED1*, *SmDHN1*, and *SmDHNX1* in the roots of *SmKRP3*-silenced or control eggplants at 24 h post salt stress treatment. (e) Measurements of APX and CAT enzyme activities in *SmKRP3*-silenced or control eggplant roots at 48 h post salt stress treatment. Three biological repeats were applied to calculate the mean \pm standard deviation. Different uppercase letters represent highly significant differences, as performed by Student's *t*-test ($p < 0.01$).

and in FRT in the mature plants, while *SmKRP4* and *SmKRP5* have higher expression levels in YL both in the seedlings and mature plants (Fig. 5), suggesting that *SmKRPs* mainly function in the growth and development in root, leaf, and fruit. Subsequently, subcellular localization of *SmKRP* proteins was investigated in epidermic cells of *Nicotiana benthamiana* leaves, and it was found that all *SmKRP* proteins located in the nucleus (Fig. 6b), which was similar to a previous study^[18]. To investigate the function of *SmKRPs* in eggplant response to abiotic stresses, the function of *SmKRP3* in eggplant response to salt stress was explored as *SmKRP3* expression was up-regulated by salt stress treatment. Based on the VISG assay, we found that silencing of *SmKRP3* enhanced the susceptibility of eggplants to salt stress (Fig. 7b) and significantly down-regulated the expression levels of salt stress defense-related genes *SmGSTU10*, *SmNCED1*,

SmNDH1, and *SmNDHX1* (Fig. 7d), and decreased APX and CAT enzyme activities (Fig. 7e), indicating that *SmKRP3* positively functions in eggplant response to salt stress. Abiotic stresses such as salt, drought stress could cause oxidation reaction and DNA damage, which resulted in cell cycle retention^[49]. The cell damage caused by abiotic stresses was alleviated by the interaction between CDK and KRP to maintain cell cycle process to generate new cells^[49]. Therefore, *SmKRP3* may be involved in the process of regulating the cell cycle to promote plant cell division, leading to alleviate the damage to eggplant caused by salt stress and increase tolerance of eggplant to salt stress.

Conclusions

In this study, five *KRP* gene family members were identified in the eggplant genome located on the four chromosomes and

divided them into three classes by phylogenetic relationship analysis. A collinearity relationship between *SmKRP1* and *SmKRP3* was exhibited. The study on gene structures, conserved motifs and domains, and *cis*-elements within *SmKRPs* promoters revealed the evolutionally conserved function of *SmKRPs*. The nucleus locations of *SmKRP* proteins were observed, and the high expression of *SmKRPs* in young leaf (YL), fruit (FRT), and root (RT). In addition, except for the up-regulation of *SmKRP3* under salt stress treatment, the other *SmKRPs* expression displayed unchanged or declining trends under salt or dehydration stress treatment. Silencing of *SmKRP3* enhanced the susceptibility of eggplant to salt stress, suggesting that *SmKRP3* positively regulates the salt stress tolerance in eggplants. The reported data provides new insight into the *KRP* gene family of eggplant and the function of *SmKRPs* in eggplant response to abiotic stresses.

Author contributions

The authors confirm contribution to the paper as follows: study conception and design: Shen L, Yang X; data collection: Shen L, Yang S, Xia X; analysis and interpretation of results: Shen L, Nie W; draft manuscript preparation: Shen L; manuscript revision: Shen L, Nie W, Yang X. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflict of interest

The authors declare that they have no conflict of interest.

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