Functional identification of CCR1 gene in apple (Malus halliana) demonstrates that it enhances saline-alkali stress tolerance

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Abstract

Background

Lignin is a complex aromatic polymer that plays an important biological role in maintaining plant structure and defending plants against biotic and abiotic stresses. Cinnamoyl-CoA reductase (CCR) is a key enzyme involved in the lignin synthesis-specific pathway and regulates lignin biosynthesis and accumulation.

Methods

Based on transcriptome data, *MhCCR1*, which was significantly induced by saline-alkali stress, was cloned from *Malus halliana*. The physicochemical properties, evolutionary relationships and *cis*-acting elements were analyzed. Subsequently, the tolerance of overexpressed *MhCCR1* in *Arabidopsis thaliana*, tobacco and apple calli to saline-alkali stress was verified by genetic transformation. And yeast two-hybridization technique was applied to screen and validate the interacting proteins.

Results

We found that overexpression of *MhCCR1* enhanced the tolerance of *A. thaliana*, tobacco and apple calli under saline-alkali stress, and caused a variety of physiological and biochemical changes. As compared to the wild type, the transgenic plants showed better growth, higher lignin, chlorophyll and proline contents, lower conductivity and MDA content, and significant increase in antioxidant enzyme activities (SOD, POD, CAT) in the transgenic lines under stress condition. In addition, expression of saline-alkali stress-related genes in overexpressed *A. thaliana* were also higher than in WT, including the antioxidant genes, the Na^+ transporter genes, and the H^+-ATPase genes, while expression of the K^+ transporter genes displayed opposite changes. Meanwhile, the expression levels of genes related to lignin synthesis, *AtPAL1*, *AtCOMT*, *AtC4H*, *At4CL1*, and *AtCCOAOMT*, were also significantly up-regulated. At last, the Y2H experiment confirmed the interaction between *MhCCR1* and *MhMYB4*, *MhMYB1R1*, *MhHXK*, and *MhbZIP23* proteins.

Conclusions

These results suggest that *MhCCR1* may play a positive regulatory role in saline-alkali tolerance of transgenic lines by regulating the lignin content, osmoregulatory substances, chlorophyll content, antioxidant enzyme activities, and genes related to saline-alkali stress, thus providing excellent resistance genes for the stress-responsive regulatory network of apples, and providing a theoretical basis for the cultivation of saline and alkali resistant apple varieties.

Background

With increasing population and deterioration of the natural environment, soil salinization-alkalization has become a major constraint on global agricultural crop production [1, 2]. The stress effects of soil

alinization-alkalization on plants include the effects of salt stress and alkali stress [3, 4], which together lead to more severe nutrient ion imbalances, lower osmoregulatory capacity, lower root vigor and photosynthetic function, inhibition of antioxidant system, massive accumulation of ROS and more severe plant growth inhibition [5]. In order to maintain ROS homeostasis and mitigate saline-alkali stress injury, an enzyme scavenging system has evolved in plants to scavenge ROS [6, 7]. In addition, in order to adapt to growth and development under stress conditions, plants also regulate the accumulation of secondary metabolites [8, 9], such as lignin, flavonoids and carotenoids, increase the osmotic potential in the body to absorb water, adjust the degree of lignification in the body, ultimately enhance nutrient transport or reduce water dissipation [10].

Lignin is the second most abundant biopolymer in plant secondary cell walls after cellulose [11], and exhibits important roles in several aspects of plant growth, development and adversity stress [12]. The continuous exploration of plant response to saline-alkali stress has revealed that the adjustment of lignification is also one of the important mechanisms in stress[13]. Alamgir Hossain et al.[14] in wheat found that aluminum salt stress significantly promoted H_2O_2 accumulation, leading to higher membrane lipid peroxidation, while a high accumulation of lignin was observed. In *A. thaliana*, overexpression of PAL and CAD promoted lignin accumulation in vascular tissues and improved adaptation to salt stress [13]. Similarly, overexpression of SOD improved tolerance to salt stress in *A. thaliana*, presumably because the accumulation of lignin content caused an increase in secondary cell wall synthesis [15].

It is now widely recognized that the process of lignin biosynthesis can be divided into three stages: mangiferolic acid, phenylpropanoid metabolism and lignin synthesis, and lignin biosynthesis requires a series of enzymes [16]. CinnamoylCoA reductase (CCR) is the first rate-limiting enzyme that catalyzes lignin synthesis [17], and it can catalyze a series of hydroxycinnamoylCoA reduction reactions produced by the phenylpropane metabolic pathway to generate the corresponding cinnamaldehyde, and CCR plays a dominant role in lignin biosynthesis (Additional file 1: Fig. S1). Studies on the CCR gene and its encoded proteins have been carried out in a variety of plants, including *A. thaliana* [18], rice (*Oryza sativa* L.) [19], tobacco, and maize (*Zea mays* L.) [20]. CCR was induced to be expressed by low temperature, droughts, high temperature as well as salt stress [21]. The overexpression of *BpCCR* increased salt resistance in *Betula alba*, while the repressor expression lines were sensitive to salt stress. The MYB-like transcription factors *MdMYB46* and *BpNAC012* regulated the expression of a number of genes including CCR, increased lignin accumulation and improved plant salt tolerance [21, 22]. The rice genes *OsCCR17* and *OsCCR21* showed a significant increase in gene expression after salt stress [23]. Currently, most studies on CCR have focused on the effects of lignin biosynthesis pathway, but the functional characterization and mechanism of CCR under saline-alkali stress in apple have not been reported.

Malus halliana is an apple rootstock native to the Hexi Corridor in Gansu Province [24]. In this experiment, based on the transcriptome data of our group under saline-alkal stress (Additional file 1: Fig. S2) [25], the CCR1 gene was screened to cope with stress and cloned it using *M. halliana*. The function of *MhCCR1* was verified by heterologous transformation into *A. thaliana*, tobacco, and homologous transformation into apple calli under saline-alkali stress conditions, and the mechanism of action was reflected in four

aspects: ion homeostasis, osmoregulation, antioxidant system, and pH homeostasis. This study provides a theoretical basis for further understanding the mechanism of saline-alkali tolerance in fruit and variety improvement, and also provides a useful reference for studying the response of CCR1 gene under abiotic stresses in fruit trees.

Materials and methods

Plant materials and treatments

The materials used in this study were *M. halliana* subculture seedlings (Provided by Fruit Tree Tissue Culture Lab 601, College of Horticulture, Gansu Agricultural University), wild-type *A. thaliana* accession Columbia (Col-0) and 'Wanglin' apple calli (Donated by Xiaofei Wang Laboratory of Shandong Agricultural University).

M. halliana was subcultured at 30-day intervals on Murashige and Skoog (MS) solid medium containing 0.5 mg/L 6-BA and 0.1 mg/L NAA at 25°C with a 16 h light/ 8 h dark period. During subgeneration, a 3-4cm stem segment of tissue-cultured *M. halliana* seedlings was excised under sterile conditions and subsequently inserted into the subculture medium. *M. halliana* rooting medium was 1/2 MS + 30 g/L Sucrose + 8 g/L Agar + 0.4 mg/L NAA, pH 5.8–6.0.

Wild-type *A. thaliana* (Col-0) seeds were extracted with 75% ethanol for 5 min, then soaked with 2.6% NaClO for 10 min, and finally washed with ddH_2O for 3–4 times. After cleaning, *A. thaliana* seeds were spread in MS medium, placed in 4℃ refrigerator vernalization for 3 days, and then cultured in an incubator at 26℃ with a light cycle of 16 h/8 h. After 30 days, *A. thaliana* seedlings were transferred to a plastic tub (7*7cm) containing a substrate (substrate: vermiculite = 3:1) and continued to be cultured in incubator. Infecting inflorescences by Agrobacterium-mediated inflorescence method at flowering time, cutting off fruit pods before infection, infecting once every 3–5 days, a total of 3–4 times. After the infection was completed, the seeds were collected and screened on Kan-resistant medium for 3 generations to obtain homozygous transgenic seeds.

Tobacco seeds were subjected to the same treatment as *A. thaliana*. Following a 30-day cultivation period on MS medium, stem segments measuring 3–4 cm in length were excised from tissue-cultured tobacco seedlings under sterile conditions. Subsequently they were transferred into fresh MS medium every 30 days. The culture conditions were 25℃ with a light cycle of 16 h/8 h.

'Wanglin' apple calli were subcultured at a 20-day interval on MS medium that contained 1.5 mg/L 2,4-D and 0.4 mg/L 6-BA at 25°C in the dark. Under saline-alkali stress, tobacco was grown on normal MS medium or saline-alkali medium supplemented with NaCl and $NaHCO_3$. Similarly, 'Wanglin' apple calli grew on normal MS subculture medium supplemented with 1.5 mg/L 2,4-D and 0.4 mg/L 6-BA or saline-alkali medium supplemented with NaCl and $NaHCO_3$.

Saline-alkali stress treatment of M. halliana seedlings

After 30 days of subculture, *M. halliana* seedlings was placed on rooting medium. When the seedlings grew to eight true leaves, healthy seedlings with consistent growth were selected and moved to a plastic box containing 1/2 Hongland's solution (pH 5.8 ± 0.2) [26] for 7 day preculture, and then incubated in Hongland's solution for another 7 days with five plants in each bowl. The oxygen was continuously oxygenated with an electric oxygen pump, and the nutrient solution was replaced every 7 days. Plants were grown at 26 ℃ with a 16h/8h light/dark cycle, 6000 lx of light and 85%/80% of relative humidity. After 2 weeks, the seedlings were treated with nutrient solution containing 100 mM 1:1 NaCl: $NaHCO_3$ (pH 8.0). Seedlings grown in nutrient solution served as controls. Three biological replicates were independently performed, and each treatment contained five plants in one biological replicate. Samples were taken at five time points (0, 6, 12, 24, 48 and 72 hours respectively).

Methods

Cloning of MhCCR1 gene and Quantitative real-time PCR

0.1 g of *M. halliana* leaves were measured and total RNA of the samples was extracted by Trizol method. TaKaRa's PrimeScriptTM RT reagent Kit with gDNA Eraser (Perfect Real Time) was performed for reverse transcription. The CDS sequence of *MhCCR1* was searched in the apple genome database, and DNAMAN software to design specifc primers (Additional file 1: Table S1), then PCR amplifcation was performed. Reaction procedure: pre-denaturation at 94 ℃ for 5 min; denaturation at 94 ℃ for 30 s, annealing at 58 ℃ for 30 s, extension at 72 ℃ for 90 s, 42 cycles; extension at 72 ℃ for 10 min. Finally, it was transformed into *E.coli*, identifed positive single colony, and transformed into agrobacterium tumefaciens GV3101 for genetic transformation by freeze-thaw method. At the same time, the sequences were obtained through the NCBI database, Real-time PCR primer pairs are listed in Table S1. Real-time PCR was performed using cDNA of *M. halliana* plantlets as the template. GAPDH was used as a reference for quantitative real-time PCR. Three replicates were performed for each sample. Finally, the data were calculated using $2^{-\Delta\Delta Ct}$ method, and the difference was analyzed by Duncan test of single-factor ANOVA ($P < 0.05$).

Bioinformatics analysis of MhCCR1 gene

Protein sequences homologous of *MhCCR1* were identifed in other species using NCBI database. ProtParam (https://web.expasy.org/protparam/) website was used to analyze the physicochemical properties of the protein. DNAMAN software was used to compare the amino acid sequences of the protein. MEGA-X software was used to construct a phylogenetic tree by the neighbor-joining method (NJ) [27]. PlantCARE (https:///webtools/plantcare/html/) website was applied to predict the cis-acting elements on the *MhCCR1* promoter.

Agrobacterium-mediated transformation of A. thaliana, tobacco and apple calli

Committed to the approach of Hu et al. [28], we obtained transgenic *MhCCR1 A. thaliana* seeds by means of genetic transformation. The seeds of *A. thaliana* were first treated with 75% ethanol for 5 min, then

with 26% sodium hypochlorite (NaClO) for 10 min, and finally washed with deionized water (ddH_2O) for 4–5 times. Then, they were seeded on MS medium containing 30 mg/L Kan, the resistant plants were screened by PCR to obtain heterozygous transgenic plants, and after three successive generations of screening, the homozygous transgenic plants of T3 generation were obtained.

The main veins tobacco leaves were removed by infection with agrobacterium tumefaciens for 8 min. The leaves were placed on pre-culture medium (MS medium + 3 mg/L 6-BA + 0.4 mg/L NAA) under dark conditions for 2–3 days. After that, the tobacco leaves was transferred to the medium containing 250 mg/L cephalosporin and 30 mg/L kan resistance for screening and culture to obtain stable growth resistant tobacco. When the buds grew to about 1.5 cm, they were cut and transferred to rooting medium for culture. The regenerated shoot DNA was extracted and identifed by PCR [25].

Infection of apple calli was based on the method of Hu et al [29]. The calli of subculture for about 15 days was infected by agrobacterium tumefaciens. Calli of the same culture state were immersed in infection solution with an OD value of 0.6 to 0.8 cultured in the dark (220 r/min) for 15–20 min, then filtered. The calli was cultured in darkness on solid MS medium for 2 days. After that, the calli was evenly distributed on 250 mg/L cephalosporin and 30 mg/L kan resistant medium for about 30 days until the transgenic calli was obtained. They were screened for about 30 days on the plate until a transgenic callus was obtained. DNA was extracted and detected by real-time quantitative PCR.

Construction and self-activation detection of pGBKT7-*MhCCR1* bait expression vector

To identify the proteins that interact with *MhCCR1*, Matchmaker was used ™GoldYeast Two Hybrid System was used for library screening analysis. In order to select suitable bait for double hybridization screening, a bait self-activation test was conducted on *MhCCR1*. According to the instructions of the ClonExpress II One Step Cloning Kit, the *MhCCR1* gene coding region (CDS) fragment after gel recovery was subjected to PCR reaction using the cDNA of *M. halliana* as a template, and the target fragment was recovered by gel recovery. At the same time, using EcoR I and BamH I double enzyme digestion bait vector pGBKT7 (BD) after homologous recombination, and then transformed into Trans5α receptor cells, after the positive clone screening, sent a single clone to the company sequencing, sequencing accurate to obtain the recombinant plasmid BD-*MhCCR1*, which was amplified and cultured for later use. These recombinant plasmids were transformed into yeast strain Y_2HGold and coated on SD/-Leu/-Trp (DDO) and SD/-Trp/-Leu/-Ade/-His/(QDO/X) media plates containing X-α-Gal, respectively, at 30°C, and incubated inverted for 3 days, and the colony growth was observed and recorded to determine whether there was transcriptional activity or not. Additionally, BD + AD was used as negative control.

Construction of a Y2H cDNA library and screening of *MhCCR1* protein

Yeast two-hybrid cDNA text was constructed using the Clone Miner™ II cDNA Library Construction Kit (Invitrogen, USA) for subsequent Y2H cotransformation. The pGADT7 library plasmid was co-transformed

with 6 μg BD-*MhCCR1* bait plasmid into Y2H Gold receptor cells. The cells were first coated on SD/-Leu/-Trp (DDO) solid medium and cultured in inverted mode at 30℃ for 3–5 d. The primary screening was completed when the single clone grew to 1–2 mm. Then the positive clones on the DDO plate were picked and transferred to SD/-Leu/-Trp/-His/-Ade/X-α-Gal(QDO/X) solid medium for re-screening, and then placed in an inverted incubator at 30℃ for 3–5 d. After picking the PCR products with positive clones > 500 bp, the positive clones were sent to the company for sequencing, and the candidate intercalating proteins were analyzed by comparison at NCBI Blastx. Candidate intercalating proteins containing the correct ORFs were selected, primers were designed according to their sequences (Additional file 1: Table S1), cloned into the AD vector, and co-transformed with BD-*MhCCR1* in Y2H Gold receptor cells for rotary validation.

Saline-alkali stress treatment of transgenic A. thaliana, tobacco and apple calli and determination of related indexes

The seeds of wild type and T3 generation homozygous transgenic lines of *A. thaliana* were sterilized, then vernalized at 4℃ for 3 days, and finally seeded on MS medium. After 3days, the seedlings were transferred to MS and MS + 100mmol/L (NaCl + $NaHCO_3$) medium, respectively, and cultured in incubator. After 20 days, observe the phenotype and measure the indexes. The WT tobacco and transgenic tobacco were cultured for 14 days under normal conditions (MS medium), and then transferred to MS and MS + 100 mmol/L (NaCl + $NaHCO_3$) medium for 20 days. The phenotypes were observed and the indexes were determined. The WT calli and transgenic calli were cultured for 15 days under normal conditions (the aforementioned medium for 'Wanglin' apple calli), then transferred to the medium containing MS and MS + 100 mmol/L (NaCl + $NaHCO_3$) for 20 days, and then the relevant indexes were determined.

Determination of chlorophyll contents refer to Cheng [30]. Lignin content was determined with reference to Foster et al.[31]. For DAB staining, leaf samples were immersed in 50 mM DAB solution (Solarbio, China) for 12 or 24 h and then decolorized in 95% [v/v] ethanol until the color turned white. For NBT staining, root tips or leaf samples were immersed in 50 mM NBT solution (Creek Huizhi, China) for 4 h and then decolorized in 95% [v/v] ethanol until the color turned white. The proline content was determined by Ferreira Junior et al. [32]. The contents of MDA and relative conductivity (REC) were determined by thiobarbituric acid method [31]. The SOD, POD, and CAT activities were measured on a spectrophotometer using kits from Suzhou Keming Biological Co.,Ltd. Relative conductivity was measured by conductivity method(DDS-307). Three replicates were tested for each line.

Statistical analyses

Treatment effects were assessed by analysis of variance and means were compared using the Duncan's test ($P < 0.05$). Statistical analyses were performed in SPSS version 22.0 (IBM, Armonk, NY, USA), and figures were prepared using Origin 8.0 software (Origin Lab, Hampton, MA, USA).

Results

Analysis of the MhCCR1 gene

Taking the cDNA of *M. halliana* seedlings as a template, a 1020 bp band of *MhCCR1* was obtained (Additional file 1: Fig. S3a). Sequence analysis showed that it was *MhCCR1*, which was forwardly ligated to the expression vector pRI 101 (Additional file 1: Fig. S3b)

Analysis of physical and chemical properties of MhCCR1 gene

The physical and chemical properties showed that the molecular weight of *CCR1* gene was 37.176 kDa, encoding 339 amino acids. The isoelectric point is 6.02, which is acidic protein; the positively and negatively charged residues were 36 and 41, respectively. The lipid coefficient is 86.81, indicating poor lipid solubility; and the average hydrophilicity is -0.240, indicating a hydrophilic protein. The instability coefficient is 32.71, indicating that the protein is stable (a coefficient greater than 40 is an unstable protein), which indicates that the protein encoded by *MhCCR1* is an stable acidic hydrophilic protein (Additional file 1: Table S2).

Protein sequence analysis of MhCCR1 gene

The *CCR1* protein sequences of 15 species were downloaded from the NCBI and analyzed by multiple sequence comparison. The amino acid sequence was performed by DNAMAN software, and the results showed that the *CCR1* protein of *M. halliana* and other species presented certain differences at the C-terminal and N-terminal (Additional file 1: Fig. S4). A conserved motif of KNWYCYGK is present in most of the helical acid sequences of all plant CCR proteins, and it is hypothesized that it may be the catalytic site of CCR and the binding region of its cofactor NADPH. The sequences of *MhCCR1* protein and CCR1 protein from other species were selected, and the phylogenetic tree was constructed by neighbor joining (NJ) with MEGA-X software (Additional file 1: Fig. S5). The results showed that *MhCCR1* was closely related to *Malus sylvestris* (XP_050135366.1), clustered into a subfamily, and is distantly related to all other species

Analysis of *cis*-acting elements of *MhCCR1* promoter

Analysis of *cis*-acting elements on *MhCCR1* promoter (Additional file 1: Table S3) revealed the presence of several hormone-related elements such as ABRE related to ABA, CGTCA-Motif related to MeJA, TATC-box related to gibberellin and AuxRR-core related to auxin. In addition, it also contained a variety of abiotic stress response elements, such as drought response element MBS, light response element G-box and GT1-motif, anaerobic induction response element ARE. In summary, these indicates that *MhCCR1* can respond to a variety of external signals such as drought, light and hormone, and participate in a series of biological processes to regulate the growth and development of plants.

Response of *MhCCR1* to saline-alkali in *M. halliana* seedlings

qRT-PCR was used to detect the expression level of *MhCCR1* in the leaves of *M. halliana* under saline-alkali stress at different time periods. As shown in the Fig. 1, after 48h of saline–alkali stress treatment, the expression level of *MhCCR1* gene in leaves of *M. halliana* seedlings was relatively high, which was 17.89-fold that of the control (0 days). It shows that *MhCCR1* gene can respond to stress and may play an important role in saline–alkali stress.

Screening and identification of transgenic *A. thaliana*, tobacco and overexpressed apple calli

The expression levels of *MhCCR1* in transgenic *A. thaliana*, tobacco and overexpressed apple calli were detected by qRT-PCR. Compared with WT plants, the expression levels of *MhCCR1* in transgenic *A. thaliana*, tobacco and overexpressed apple calli displayed higher values, indicating that *MhCCR1* was over-expression (OE) in *A. thalia*na, tobacco and apple calli (Fig. 2a). Wild type (WT) and transgenic material DNA were used as templates for PCR amplification, and then the transgenic material was identified at the DNA level. The results were shown as follows (Fig. 2b-d): when primers were used as primers, PCR product fragments of WT and transgenic material both had bands; when PRI-*MhCCR1* primers were used for amplification, PCR products of transgenic material had clear bands, while WT had no bands, indicating that the transformation of PRI-*MhCCR1* vector was successful.

Resistance of transgenic *MhCCR1 A. thaliana* under saline-alkali stress

To determine whether *MhCCR1* plays a role in response to saline-alkali stress, three transgenic *A. thaliana* lines and the WT controls were cultured under normal and saline–alkali stress (100mM 1:1 NaCl: $NaHCO_3$) for 20 days, respectively. As shown in Fig. 3, the overexpressed strain and the WT control *A. thaliana* grew well under normal conditions, but the growth of the WT and *MhCCR1*-OE strains was affected to different degrees under stress (Fig. 3a). In contrast, *MhCCR1*-OE *A. thaliana* plants grew well with less chlorosis and longer root systems (Fig. 3b). *A. thaliana* leaves were stained with DAB and NBT, respectively (Fig. 3c-d), and detect changes in reactive oxygen species in *MhCCR1*-OE and WT *A. thaliana* under normal or saline treatment conditions. Darker blue and yellow-brown colors indicate greater accumulation of O_2^- and H_2O_2. Under normal conditions, no substantial differences were observed between NBT and DAB staining; However, the depth of leaf color in the three *MhCCR1*-OE transgenic lines was considerably lower than that of the WT plants under saline-alkali treatment, suggesting that the transgenic material significantly mitigated the accumulation of ROS. Under saline-alkali treatment, the relative conductivity (Fig. 4a) and MDA (Fig. 4b) of the three *MhCCR1*-OE strains were also lower than that of WT, while the proline (Fig. 4c) and chlorophyll contents (Fig. 4g) were higher than that of WT, but there was no difference between *MhCCR1*-OE and WT under normal conditions. In addition, the SOD, POD and CAT contents (Fig. 4d-f) of the three *MhCCR1*-OE strains were much higher than that of the control. At last, the lignin content (Fig. 4h-i) in leaves and roots of *MhCCR1*-OE strains increased under stress, and the increase was more in roots.

Resistance of transgenic MhCCR1 tobacco under salinealkali stress

Three transgenic tobacco lines and the WT control were grown for 30 days under normal conditions, then shifted to MS liquid medium (no saline-alkali stress, CK) and MS liquid medium containing 100mM 1:1 NaCl: $NaHCO_3$ (saline-alkali stress,T) by filter paper bridge method for 3d. As shown in Fig. 5a, both transgenic and WT control tobacco grew vigorously in CK. However, the growth of WT and *MhCCR1*-OE tobacco was inhibited under saline-alkali stress, but the degree of chlorosis of WT tobacco was significantly more severe compared to transgenic lines, which was consistent with the treatment effect of *A. thaliana*. Meanwhile, root length and root number were also reduced in both WT and *MhCCR1*-OE tobacco seedlings under saline-alkali stress, but the root length and root number of WT seedlings were significantly shorter than those of *MhCCR1*-OE seedlings. In addition, the REC (Fig. 5b) and MDA contents (Fig. 5c) of the three transgenic lines were significantly lower than those of the WT control, and the activities and expression levels of SOD, POD and CAT (Fig. 5e-g) were much higher than those of the WT control. Similarly, the proline (Fig. 5d), chlorophyll (Fig. 5h) and lignin contents (Fig. 5i-j) were significantly higher than those of the WT control, indicating that the ectopic expression of CCR1 in transgenic tobacco can cope with saline-alkali stresses and improve its tolerance to the stresses.

Morphological characteristics and physiological indices of overexpressed MhCCR1 gene in apple calli under saline-alkali stress

To further investigate the function of *MhCCR1* under saline-alkali stress, we selected WT and three normally growing transgenic apple calli (OE-1,4,7), and set up apple calli grown on normal conditions and saline-alkali stress conditions, respectively. As shown in Fig. 6, there was no significant difference in the growth status of the *MhCCR1*-OE and WT apple calli under normal conditions (CK). However, under saline-alkali conditions, the *MhCCR1*-OE and WT apple calli immensely vary in growth state with overexpressed calli showing better growth compared to WT calli. The contents of Pro and MDA and the activities of SOD, POD and CAT were similar to those of tobacco and *A. thaliana* (Fig. 6b-f).

Expression levels of genes related to lignin pathway and saline-alkali stress

To further investigate the role of *MhCCR1* in the signaling pathway of saline stress, qRT-PCR was used to detect the expression levels of overexpressed *MhCCR1* in *A. thaliana* for genes related to saline stress and lignin synthesis. As shown in Fig. 7, the expression levels of antioxidant enzyme genes *AtSOD*, *AtPOD*, and *AtCAT* in WT and overexpressed *A. thaliana* increased significantly under stress, and the elevation of antioxidant genes in overexpressed *A. thaliana* was significantly greater than that in WT. The expression levels of H^+-ATPase genes (*AtAHA2* and *AtAHA3*) and Na^+ transporters (*AtSOS1*, *AtALT3*, *AtCAX5*) were also significantly increased in WT and overexpressed *A. thaliana* under stress, with higher values in overexpressed lines compared to WT. The expression of K^+ transporter genes (*AtSKOR*, *AtNSCCs*, and *AtNHX4*) was reduced under saline-alkali stress compared to WT. Meanwhile, the expression levels of the key genes for lignin synthesis (*AtPAL*, *AtCOMT*, *AtCAD*, *AtC4H*, and *At4CL*) was significantly increased in overexpressing *MhCCR1* lines under saline-alkali stress compared with wild-type

A. thaliana, and the most pronounced change was observed in *AtCAD*, with a 12-fold increase in the expression level. The above results indicate that overexpression of *MhCCR1* in *A. thaliana* can regulate the enhanced saline-alkali tolerance of plants by increasing the expression of antioxidant enzymes and Na^+/H^+ transporter genes, decreasing the expression of K^+ transporter genes, and increasing the expression of genes of the lignin synthesis pathway.

Yeast two-hybrid screening for MhCCR1-interacting proteins

The constructed pGBKT7-*MhCCR1* bait vector needs to be subjected to self-activation assay before screening the library. The results of the self-activation assay showed that BD empty vector and BD-*MhCCR1* + AD grew on SD/-Leu/-Trp (DDO), and did not grow on SD/-Leu/-Trp/-His/-Ade/X-α-Gal (QDO/X), which indicated that the BD-*MhCCR1* plasmid was successfully transfected into yeast strains and did not have self-activating activity, and it could be used for subsequent screening assays (Fig. 8b, first two lines).

The BD-*MhCCR1* bait plasmid and the constructed Y2H library plasmid were co-transformed into Y2H Gold yeast receptor cells, and the transformed products were coated on SD/-Leu/-Trp (DDO) plates and cultured for 2–3 d. A total of 50 yeast clones were obtained in the initial screening, and then the positive clones on SD/-Leu/-Trp (DDO) screening plates were picked and transferred to the SD/-Leu/-Trp/-His/-Ade/X-α-Gal (QDO/X) screening plates, a total of 35 blue clones were selected, cloned, and sequenced (Fig. 8a). Seven candidate proteins that may interact with *MhCCR1* were ultimately screened, including *MhMYB4*, *MhMYB1R1*, *MhbZIP23*, *MhSOS2*, *MhDIN*, *MhHXK*, and *MhNAC1*. To further validate the interactions between *MhCCR1* and the candidate proteins, the ORF sequences of *MhMYB4*, *MhMYB1R1*, *MhbZIP23*, *MhSOS2*, *MhDIN*, *MhHXK*, and *MhNAC1* were homologously cloned from the cDNA of *M.halliana*. The prey protein was constituted by homologous recombinant ligation of pGADT7 (AD) vector, and then the prey protein and BD-*MhCCR1* were cotransformed into yeast receptor cells for reciprocal validation. The cotransformed yeasts all grew normally on DDO medium, but on QDO/X medium BD-*MhCCR1* + AD-*MhMYB4*, BD-*MhCCR1* + AD-*MhMYB1R1*, BD-*MhCCR1* + AD-*MhbZIP23* and BD-*MhCCR1* + AD-*MhHXK* showed blue yeast colonies, and the blue colonies were decreasing with increasing dilution, suggesting that *MhCCR1* interacts with *MhMYB4*, *MhMYB1R1*, *MhHXK*, and *MhbZIP23* proteins, but not with *MhSOS2*, *MhDIN*, and *MhNAC1* proteins. did not interact(Fig. 8b).

Discussion

Among various abiotic stresses, excessive saline-alkali is one of the major abiotic stresses that inhibits plant growth, and at least 20% of the world's arable land are affected by increased soil salinization-alkalization [33]. During the evolutionary process of coping with stress over a long period of time, plants have evolved a series of physiological and molecular mechanisms for saline-alkali tolerance to adapt to growth and development under stress conditions. Cell wall thickening is an important response to saline-alkali stress in plants [34], and the expression of cell wall-related genes is altered to cope with the stress when the plant is exposed to saline-alkali environments [35]. Therefore, mining genes that regulate plant

stress tolerance to improve plant resistance to abiotic stresses at the molecular level has important research value and broad application prospects.

Lignin, as an essential component of the cell wall in all vascular plant cells, is extensively involved in plant growth and developmental processes, increasing the mechanical strength of the plant body to enhance the resistance to stress [36]. The lignification of plant cell wall was enhanced under different environmental stresses, and the root lignification and cell wall coagulation of vascular and xylem tissues were affected by saline-alkali stress [37]. Lignification is a dynamic process that is tightly regulated at different levels during normal development and in response to different stresses [38]. Treatment of salt-sensitive and salt-tolerant poplars under salt stress by Janz et al. found that enhanced lignin biosynthesis had a positive effect on plant salt tolerance [39], which was also shown that high activation of lignifying enzymes in clover (*Trifoliumrepens* L.) under water deficit stress conditions [40]. In addition, the enhancement of secondary metabolism is also an important mechanism to cope with saline-alkali stress. Genes responsible for secondary metabolism, such as PAL, CAD, and GST1 genes, as well as their corresponding enzyme activities, are induced when wild barley is subjected to salt stress, resulting in an increase in lignification and the accumulation of secondary metabolites, which improves the osmotic potential of the plant [41]. As the first key enzyme in lignin biosynthesis, CCR protects plants from oxidative damage and is effective against abiotic stresses. Currently, several studies have shown that the relationship between CCR gene expression and lignin accumulation influences plant response to stress [42]. In chrysanthemum, the highest expression of CCR genes was found in stems and leaves, and the expression varied with salt treatment time [43]. Yu's study found that the *SmCCR1* gene was significantly induced in willow under Cd stress, increasing the lignin content of transgenic poplar calli tissues and enhancing tolerance to Cd [44]. In addition, the study of *C. albicans* seedlings by Sameer et al. demonstrated increased lignification of stems in drought-treated samples, and the corresponding accumulation of CCR proteins was higher in samples treated with salt stress than those treated with control [21]. However, the response of CCR1 to stress has not been reported in apple. In this experiment, *MhCCR1* was bioinformatically analyzed and genetically transformed in *M. halliana* to verify whether and how it responds to saline-alkali stress.

In order to verify the function of CCR1 under saline-alkali stress, which was screened out the transcriptome database, and its biological information was analyzed. Multiple sequence comparisons showed that *MhCCR1* showed high sequence identity with other species at the amino acid level. Phylogenetic tree analysis showed that *MhCCR1* of *M. halliana* was closely related to *Malus sylvestris* and had the highest homology, probably because they belong to rosaceae plant and they have similar evolutionary process and biological functions. The function and regulation of the gene are largely determined by the cis-regulatory elements in the promoter region, and the *MhCCR1* promoter contains response elements for abiotic stresses such as drought, MeJA, light, and ABA, indicating that *MhCCR1* may respond to a variety of abiotic stresses. The presence of defense and stress response elements in the promoter region of *MhCCR1* suggests that it may be directly regulated by stress-induced transcription factors.

We also obtained *MhCCR1* transgenic *A. thaliana*, tobacco, and overexpressed apple calli tissues and observed their phenotypes under saline-alkali stress and identified relevant indicators. The transgenic *A. thaliana* and tobacco lines had longer rhizome lengths, which favored plant tolerance to saline-alkali stress. Similarly, transgenic plants exhibited lower leaf chlorosis and higher chlorophyll content under stress conditions. Saline-alkali stress leads to the production of reactive oxygen species (ROS), and in order to visualize the accumulation of ROS in leaves more visually, we stained *A. thaliana* leaves with DAB and NBT [45]. The results of both staining showed that the color depth of WT *A. thaliana* under stress was higher than that of the three *MhCCR1*-OE transgenic lines. In contrast, the leaf color depth of the three *MhCCR1*-OE lines was considerably lower than that of the WT plants. However, under normal growth conditions, there was no significant difference between the WT and *MhCCR1*-OE leaf coloring results. These results suggest that *MhCCR1* may play a positive regulatory role in saline-alkali tolerance in apple. In this experiment, REC and MDA contents of all transgenic materials were significantly lower than those of WT under normal and saline-alkali conditions [46], and REC and MDA were higher in both WT and transgenic materials under stress conditions compared with normal treatments [47]. It indicated that both WT and transgenic plants would cause greater permeability of plant membranes and more cell membrane lipid peroxidation under saline-alkali conditions, but overexpressed plants were relatively less damaged under stress. The proline content of osmoregulators is one of the important indicators of plant stress tolerance [48]. In this study, the Pro content of the *MhCCR1*-OE strain was significantly higher than that of the WT strain, indicating that the transgenic material could increase the content of osmoregulators more effectively to alleviate saline-alkali stress. Under abiotic stress, plants rapidly accumulate antioxidant enzymes, such as POD, SOD, and CAT, to scavenge reactive oxygen species and protect the organism from damage [49]. In this study, the activities of the three enzymes in the *MhCCR1*-OE strain were significantly higher than those of the WT, which indicated that the ability of transgenic materials to protect the system was increased compared to the WT, which could be attributed to increase the activity of antioxidant enzymes, thus increasing the tolerance of the plant to stress. Similar results were obtained by Yildiz et al. in their study of the antioxidant system of *Fragaria ananassa* [50].

The qRT-PCR analysis showed that the expression levels of antioxidant genes *AtSOD*, *AtPOD*, and *AtCAT* in WT and *MhCCR1*-OE *A. thaliana* tended to increase under saline-alkali stress. These results suggest that overexpressed plants have a higher scavenging capacity for ROS reactive oxygen species than WT, which acts mainly through antioxidant enzymes. Under saline-alkali stress, vacuolar membrane Na^+/H^+ reverse transporters participate in the regulation of cytoplasmic Na^+ concentration and pH, segregating Na^+ and K^+ into vacuoles and playing a crucial role in plant salt stress response [51]. In this experiment, the expression levels of *AtAHA2* and *AtAHA3* significantly increased under saline-alkali stress [52]. We speculate that the *MhCCR1* gene affects root pH and alleviates high pH damage by increasing the expression level of AHA family genes. Na^+ transporters can reduce the harm of saline-alkali stress by squeezing sodium ions out of cells. In this experiment, the expression levels of Na^+ transporters (*AtCAX5*, *AtSOS1*, and *AtALT3*) were significantly increased under saline-alkali stress [53, 54]. Under stress conditions, the expression levels of *AtSKOR*, *AtNSCCs*, and *AtHKT1* in WT cells were higher than those in overexpressing K^+ transporters. These results indicate that overexpressed plants increase Na^+ efflux

under saline-alkali stress, inhibit K^+ efflux, reduce K^+ segregation in vacuoles, and increase intracellular Na^+/K^+ [55]. In addition, the expression levels of lignin synthesis pathway genes (*AtPAL1*, *AtCOMT*, *AtCAD*, *AtC4H*, and *At4CL*) were determined in WT and transgenic *A. thaliana* in order to validate the effects of *MhCCR1* expression on other genes related to the lignin pathway. The results showed that the expression of lignin synthesis pathway-related genes in the transgenic lines all increased significantly under stress conditions, and the changes in CAD genes downstream of CCR were the most obvious, and we hypothesized that the expression of CCR had a direct effect on CAD.

Interactions between proteins are important for elucidating intracellular signaling. In this study, the yeast two-hybrid assay showed that the apple *MhCCR1* protein is not self-activating, and seven candidate proteins were screened for their interactions with *MhCCR1*, and it was demonstrated that *MhCCR1* interacts with *MhMYB4*, *MhMYB1R1*, *MhHXK*, and *MhbZIP23*, and that their interactions play an important role in salinity tolerance in plants. However, the Y2H assay is only an in vitro validation of the interactions, and their functions and mechanisms of action in plants need to be further explored.

Conclusion

In summary, transgenic *MhCCR1* gene *A. thaliana*, tobacco, and overexpressed apple calli tissues could respond to and increase resistance to saline-alkali stress, and revealed its mechanism of action under saline-alkali stress in four aspects: reactive oxygen system, ion homeostasis, osmotic regulation, and lignin synthesis. Specifically, it could improve the scavenging efficiency of ROS, protect the membrane integrity, promote the efflux of Na^+, and inhibit the efflux of K^+. Meanwhile, *MhCCR1* could improve saline-alkali tolerance by increasing the expression of genes related to the lignin synthesis pathway and the accumulation of lignin content. At last, it has been demonstrated that *MhCCR1* interacts with *MhMYB4*, *MhMYB1R1*, *MhHXK*, and *MhbZIP23* proteins. Therefore, *MhCCR1* has an up-regulatory role in stress, which provides a direction for further research on other functions of *MhCCR1* and a theoretical basis for breeding apple rootstocks with effective saline-alkali tolerance.

Abbreviations

CCR Cinnamoyl-CoA reductase

ROS Reactive oxygen species

MS Murashige and Skoog

CAT Catalase

POD Peroxidase

SOD Superoxide Dismutase

APX Ascorbate peroxidase

Cef	Cefotaxime
Amp	Ampicillin
Kan	Kanamycin
ddH_2O	Distilled and deionized water
MDA	Malondialdehyde
Mh	Malus halliana
NAA	Naphthalene acetic acid
OE	Overexpression
WT	Wild type
PA	proanthocyanidin
Pro	proline
qRT-PCR	Quantitative Reverse Transcription-PCR
REC	Relative conductivity
Rif	Rifampicin
DAB	Diaminobenzidine
NBT	Nitrotetrazolium Blue chloride
Y2H	Yeast Two-Hybrid
BD	pGBKT7
AD	pGADT7

Declarations

Author Contribution

XW and YXW designed the research. XW and WXW performed the experiments. ZXZ, JLL and STL performed the data analysis and interpretation. ZXZ and YXW prepared the figures and tables. XW wrote the manuscript. All authors read, commented on and approved the manuscript.

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Figures

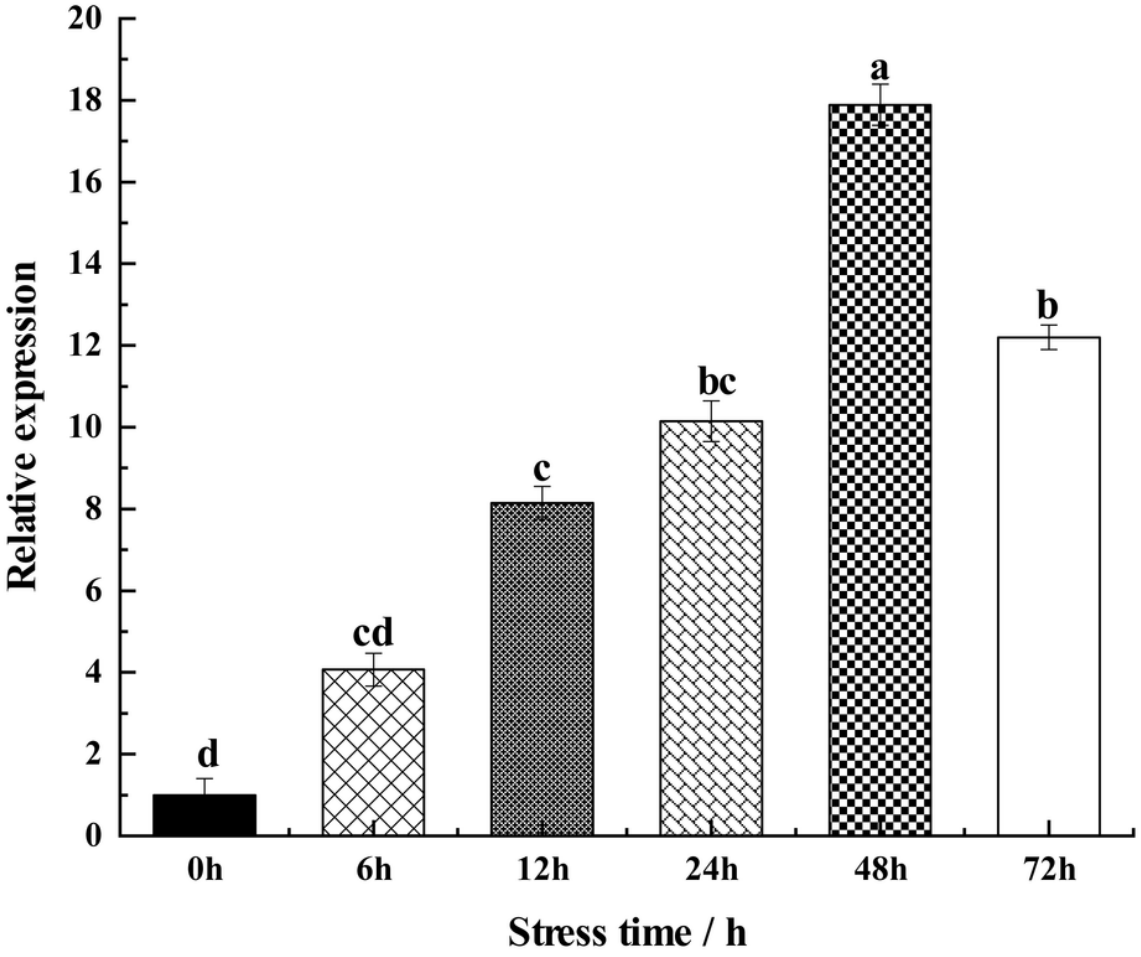

Figure 1

Expression levels of *CCR1* gene in *M. halliana* seedlings on saline-alkali stress at 0, 6, 12, 24, 48 and 72h, respectively. Data are means of three replicates with SE. Values not followed by the same letter indicate significant differences between treatments, according to Duncan method of single-factor ANOVA ($P < 0.05$). The same below.

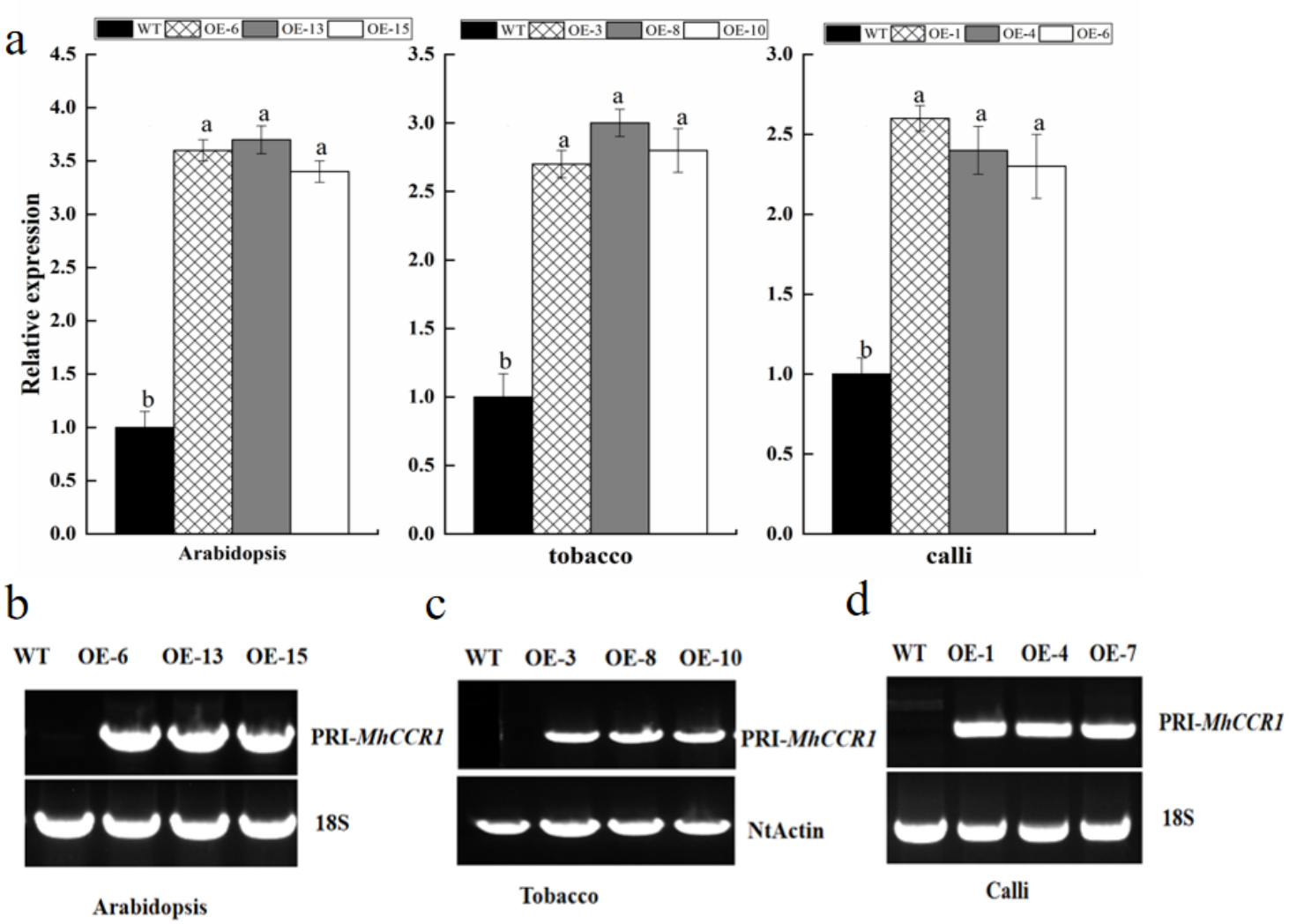

Figure 2

Identification of transgenic materials. **a**RNA level identification. **b** DNA level identification of *A. thaliana*. **c** DNA level identification of tobacco. **d** DNA level identification of calli.

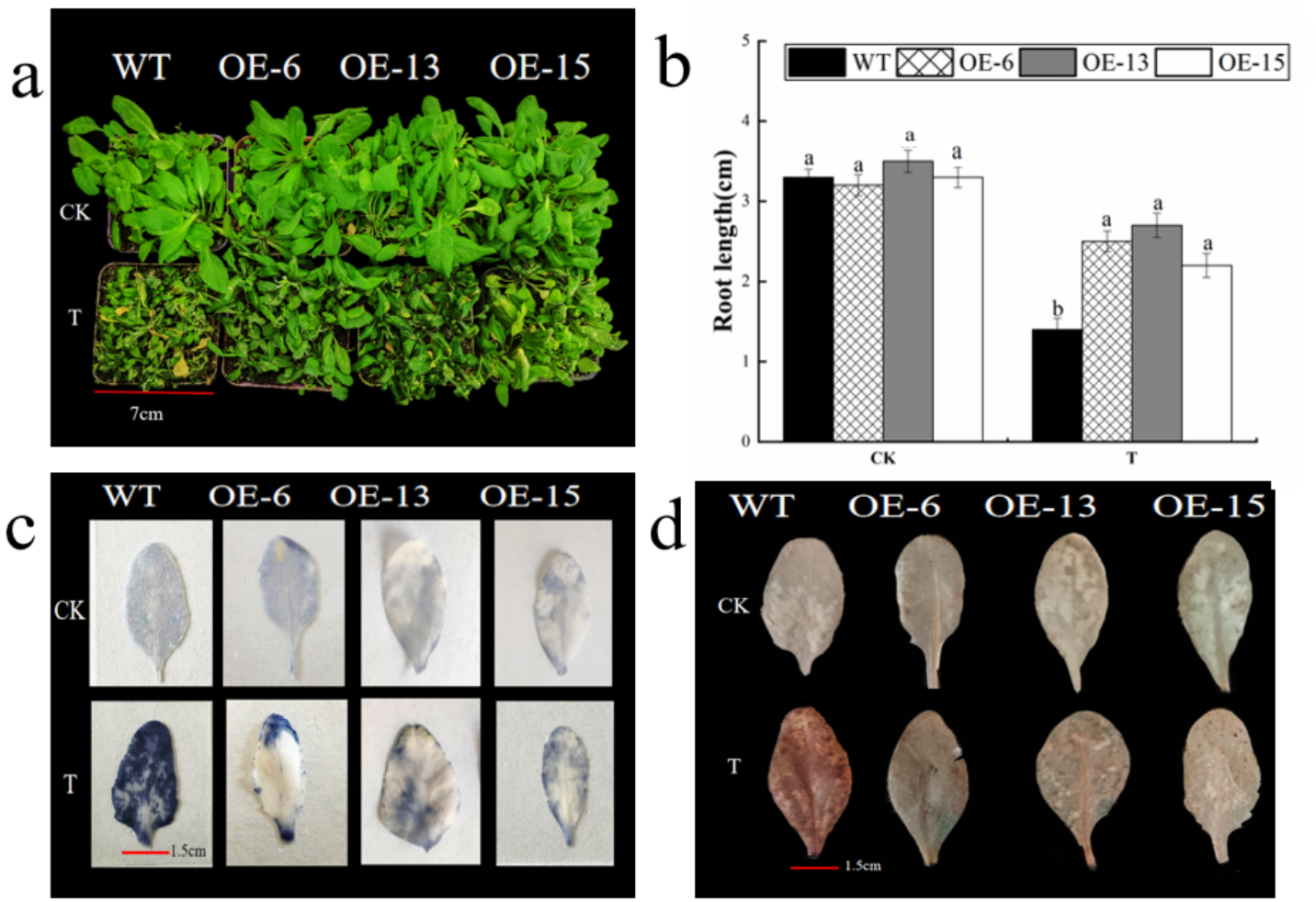

Figure 3

The phenotype, root length, NBT and DAB staining of *MhCCR1*-OE and wild-type (WT) *A.thaliana* under normal conditions (CK) and saline - alkali stress (T). **a** The phenotypes. **b** Root length. **c** NBT staining. **d** DAB staining.

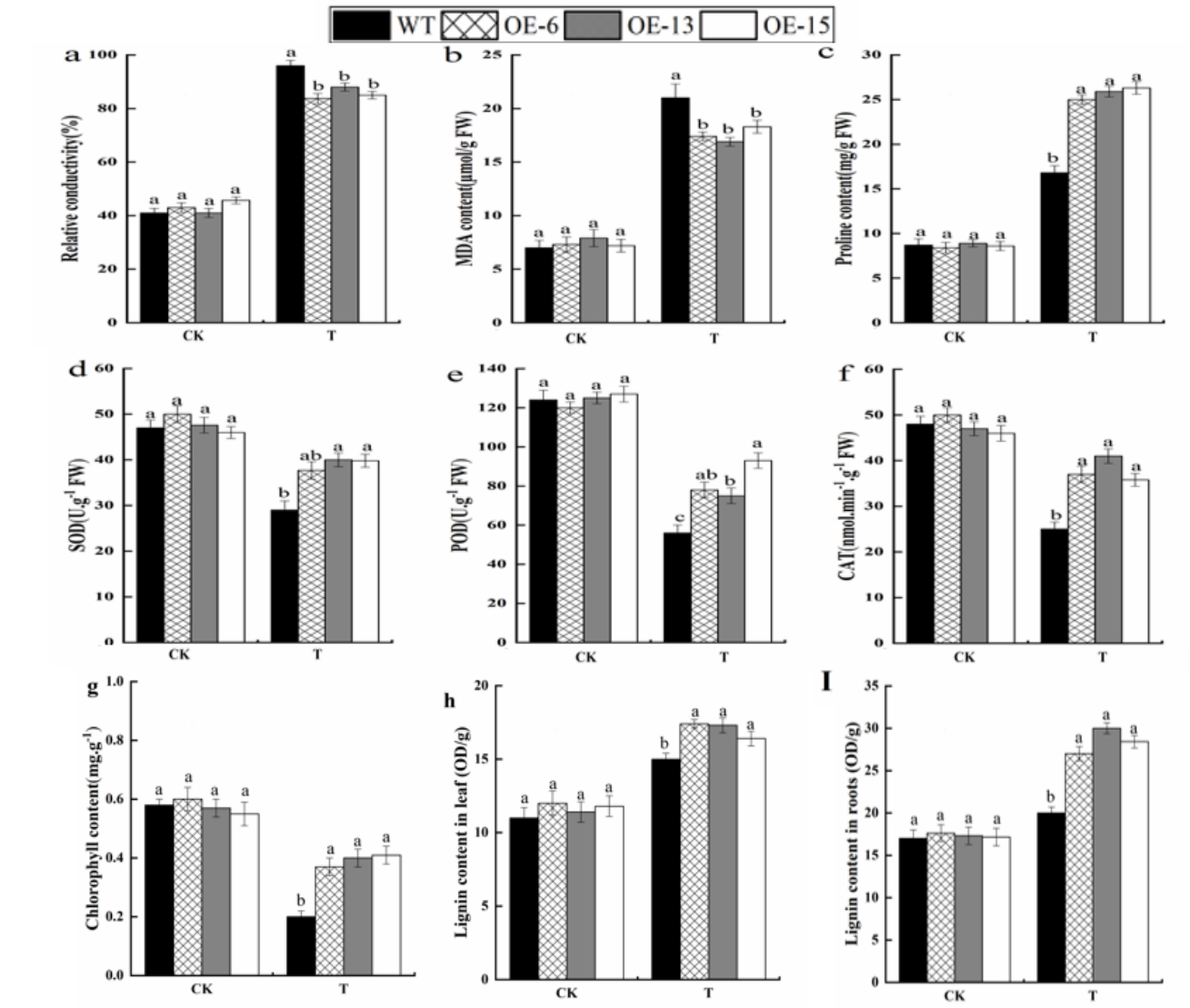

Figure 4

Physiological indices of *MhCCR1-OE* and WT *A.thaliana* under normal conditions (CK) and saline - alkali stress (T). **a** Relative conductivity. **b**MDA content. **c** Pro content. **d** SOD activity. **e** POD activity. **f** CAT activity. **g** chlorophyll content. **h** lignin content in leaf. **i** lignin content in roots.

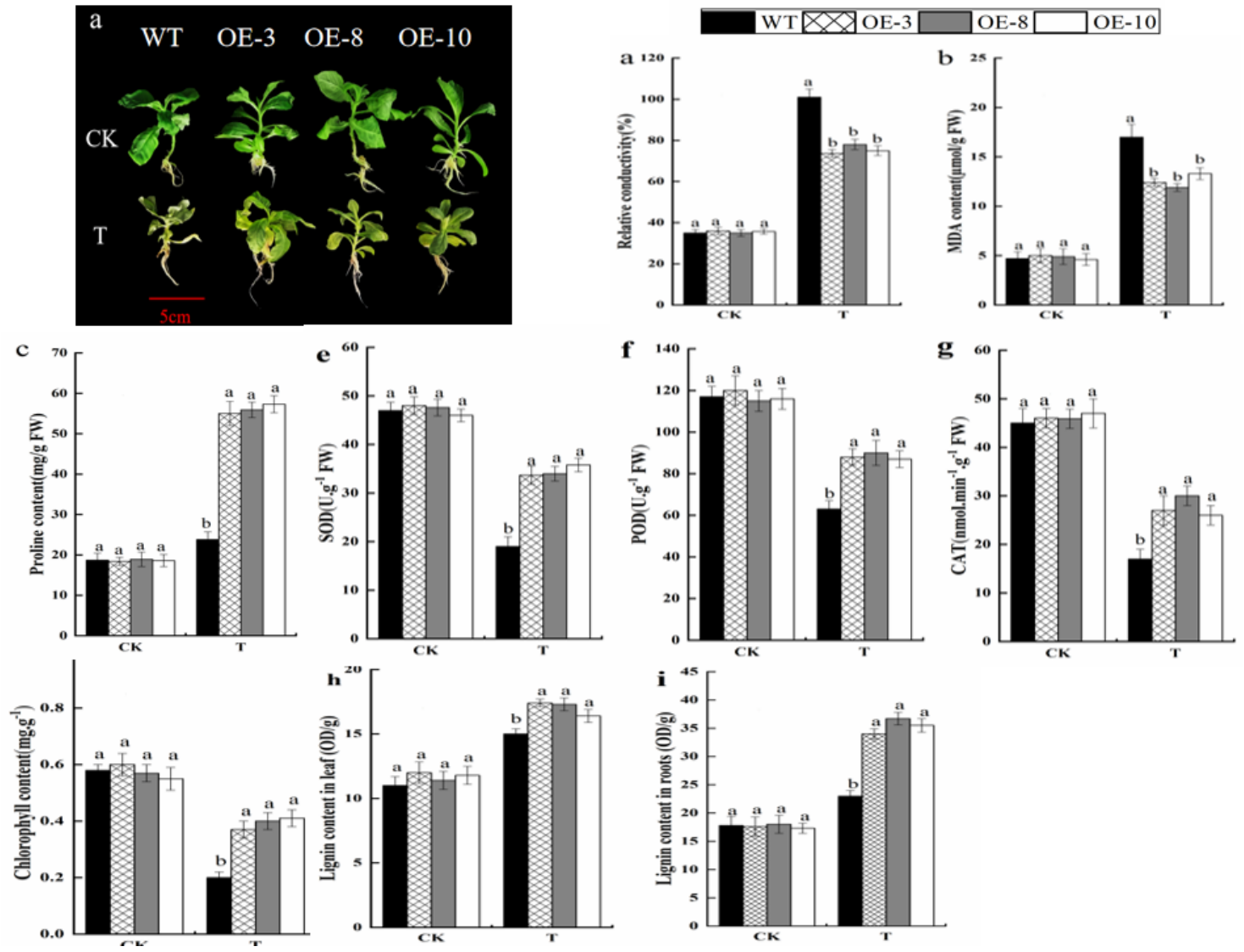

Figure 5

Overexpression of *MhCCR1* improves saline-alkali resistance in tobacco. **a** The phenotypes of *MhCCR1*-OE and WT tobacco under normal conditions (CK) and saline-alkali stress (T). **b** Relative conductivity. **c** MDA content. **d** Pro content. **e** SOD activity. **f** POD activity. **g** CAT activity. **h** chlorophyll content. **i** lignin content in leaf. **j** lignin content in roots.

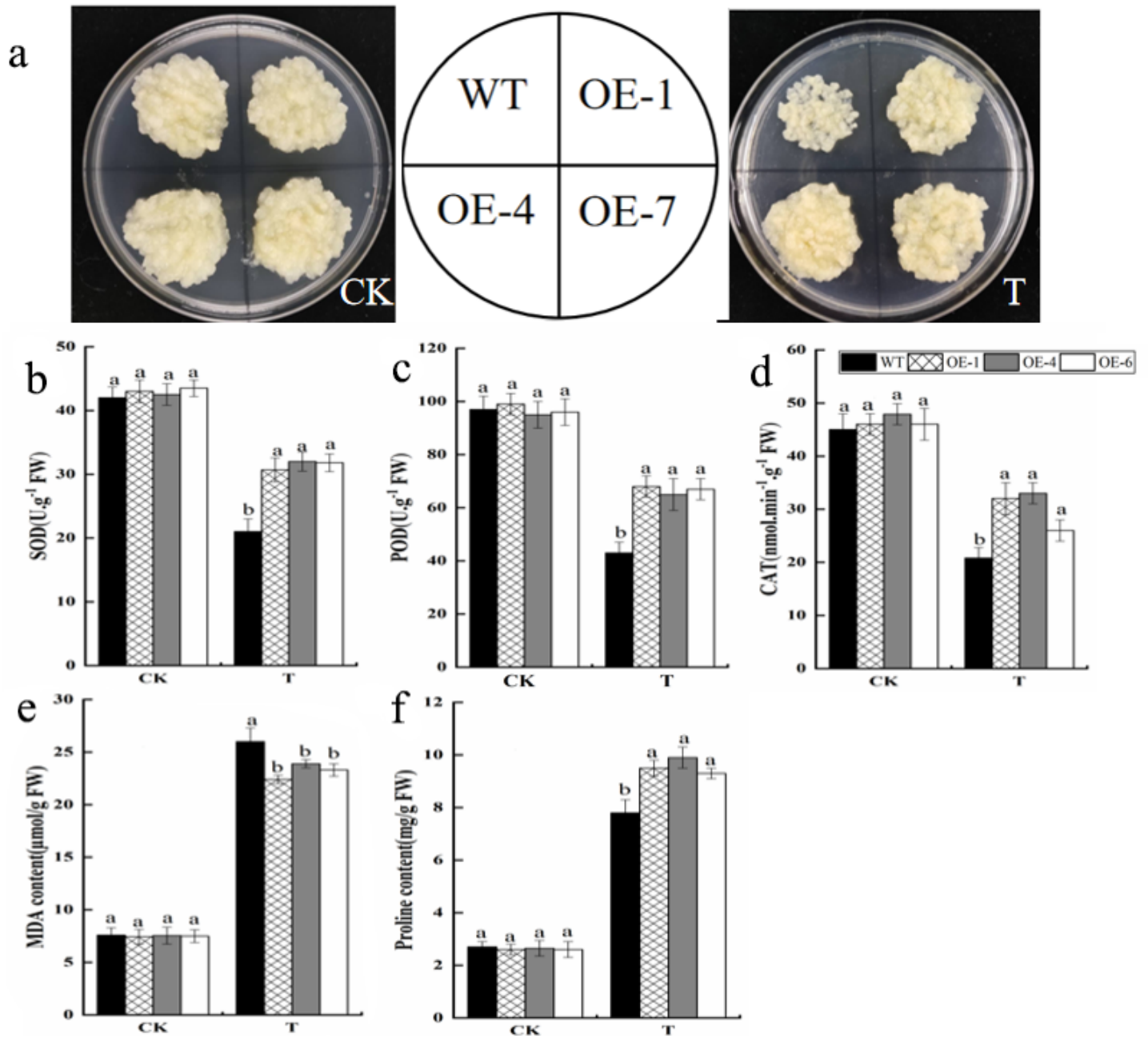

Figure 6

Overexpression of *MhCCR1* improves saline-alkali resistance in apple calli. **a** The phenotypes of *MhCCR1*-OE and WT apple calli under normal conditions (CK) and saline-alkali stress (T). **b** SOD activity. **c** POD activity. **d**CAT activity **e** MDA content. **f** Pro content.

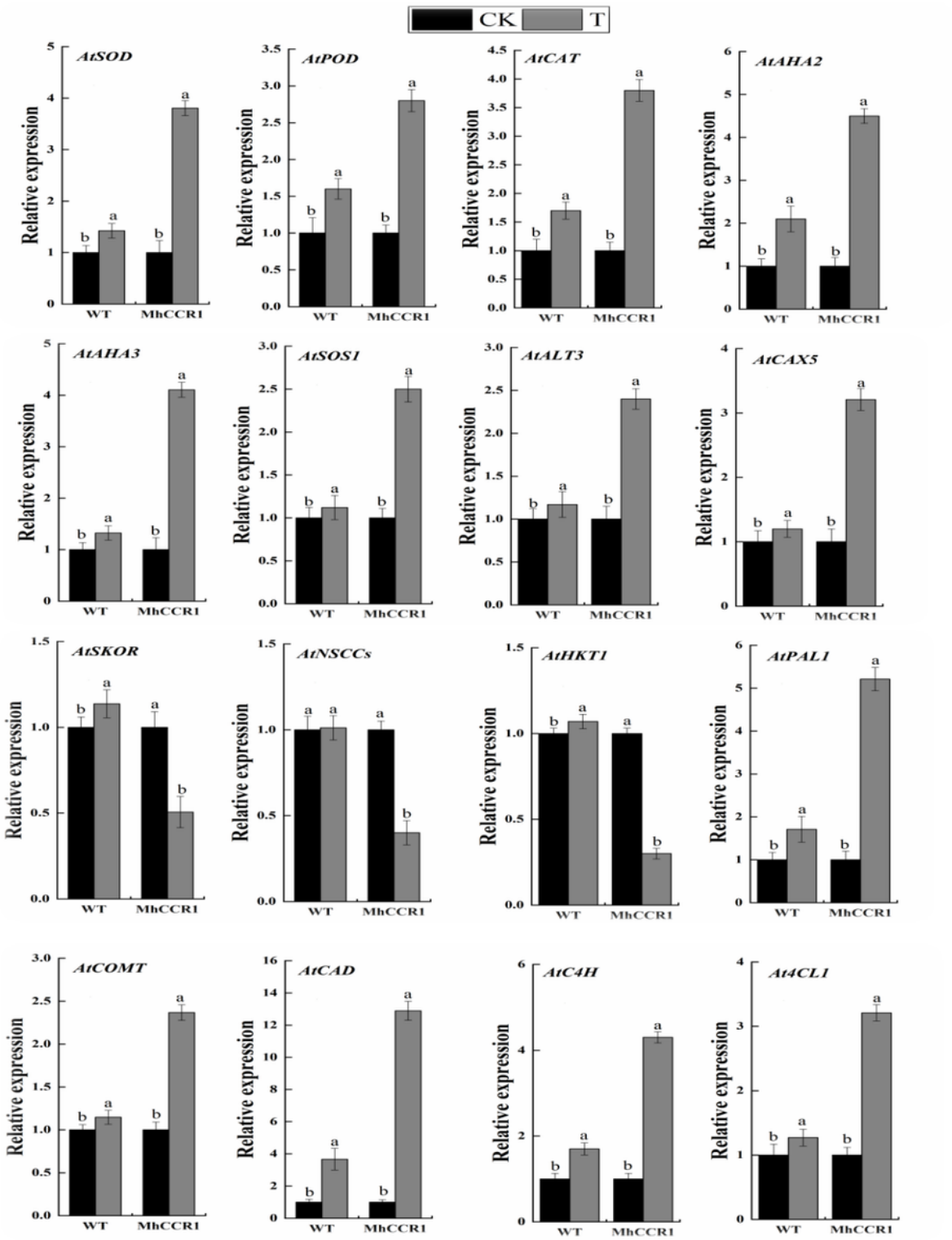

Figure 7

Expression levels of WT and overexpressed *A. thaliana* (CCR1) lignin pathway and saline-alkali stress response genes under normal conditions (CK) and salinity stress (T). Significance using Student's test: values not followed by the same letter indicate significant differences between treatments.

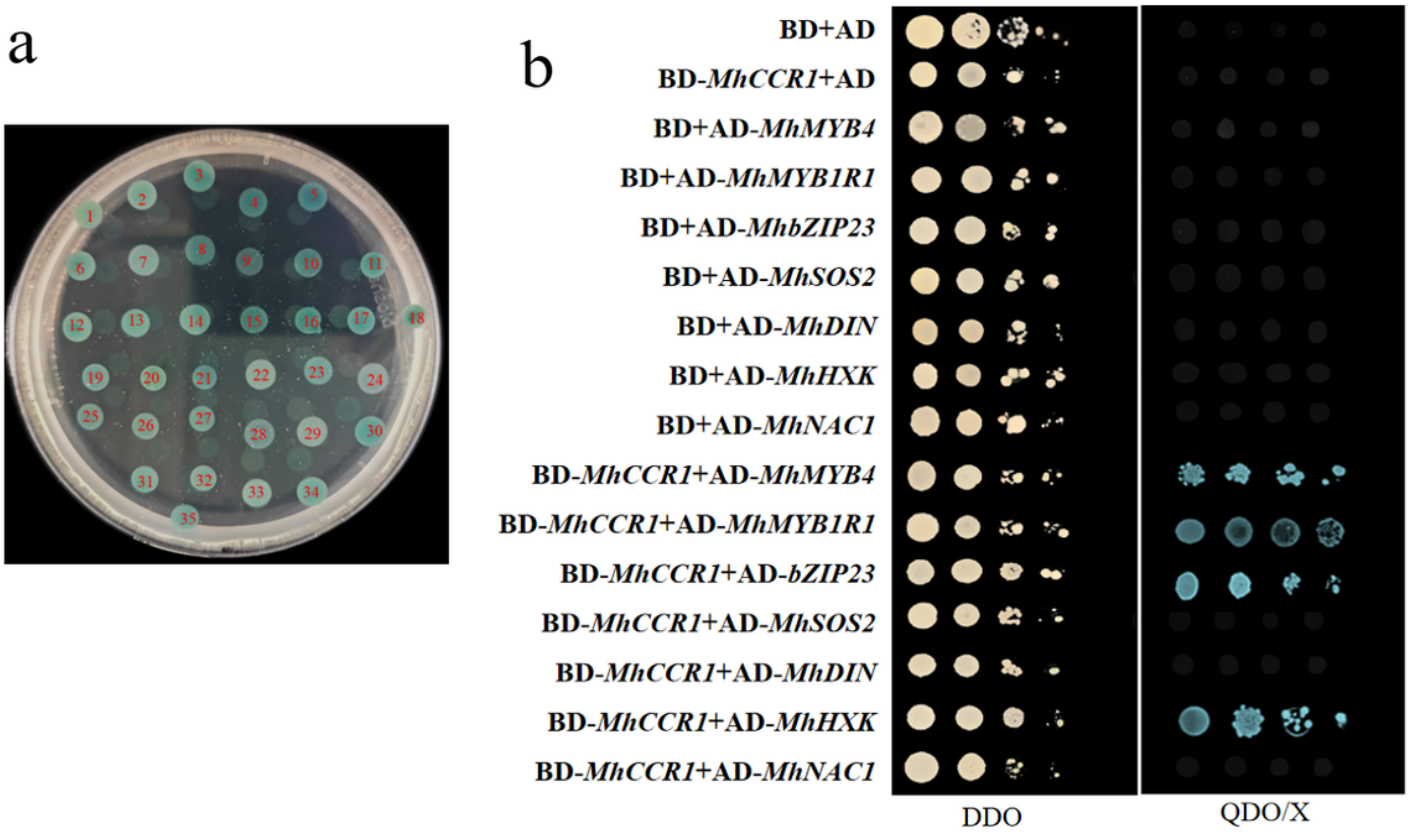

Figure 8

Yeast two-hybrid screening for *MhCCR1*-interacting proteins. **a** Screening of positive clones on plates. **b** *MhCCR1*has no transcriptional activation activity and interacts with *MhMYB4*, *MhMYB1R1*, *MhHXK*, and *MhbZIP23.* DDO and QDO/X represent SD/-Leu/-Trp and SD/-Leu/-Trp/-His/-Ade/X-α-Gal medium, respectively.

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