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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. We obtained transgenic materials by heterologous transformation of *MhCCR1* into *Arabidopsis thaliana* and tobacco, as well as homologous transformation into apple callus, and characterized their saline-alkali stress resistance by a series of physiological and biochemical experiments. 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.

Keywords Lignin synthesis, CCR1, Saline-alkali stress, *Malus halliana*, Y2H

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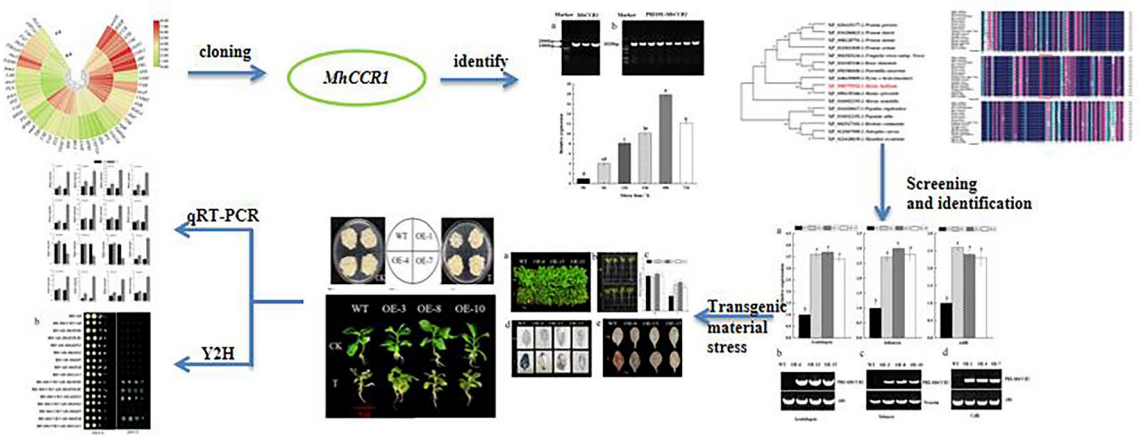
Graphical Abstract



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 salinization-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]. 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]. Cinnamoyl-CoA reductase (CCR) is the first rate-limiting enzyme that catalyzes lignin synthesis [17], and it can catalyze a series of hydroxycinnamoyl-CoA 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 study, we screened and cloned CCR1, a gene related to the lignin synthesis pathway significantly induced by saline-alkali stress, from *M. halliana* by transcriptome analysis. (Additional file 1: Fig. S2) [25]. The function of *MhCCR1* was verified by transformation into *A. thaliana*, tobacco, and apple calli. This study provides a theoretical basis for further understanding the mechanism of saline-alkali tolerance in fruit and variety improvement.

Materials and methods

Plant materials and treatments

Malus halliana histocultured seedlings were used as experimental materials for gene expression analysis. After being placed on rooting medium for 4 weeks, the rooted seedlings were transplanted into plastic pots (20.0 cm × 20.0 cm, organic matter: vermiculite = 3:1) with 5 plants per pot for uniform management and regular observation. Wild-type (WT) *A. thaliana* was Columbia type (Col-0), and tobacco and 'Wanglin' apple calli were donated by Xiaofei Wang Laboratory of Shandong Agricultural University and kept in our laboratory.

Malus halliana subculture medium was MS + 30 g/L Sucrose + 8 g/L Agar + 0.5 mg/L 6-BA + 0.1 mg/L NAA, pH 5.8—6.0 and subgeneration was performed every 30 days. *Malus halliana* rooting medium was 1/2 MS + 30 g/L Sucrose + 8 g/L Agar + 1 mg/mL IBA + 1 mg/mL IAA, pH 5.8—6.0. Tobacco solid medium was MS + 30 g/L sucrose + 8 g/L agar, pH 5.8–6.0, and the culture was succeeded every 30 days. The culture conditions were 25 ℃ with a light cycle of 16 h/8 h. Transgenic *A. thaliana* was screened on Kan-containing MS medium to the T3 generation. '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 $NaCl + NaHCO_3$. 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 $NaCl + NaHCO_3$.

Saline-alkali stress treatment of *M. halliana* seedlings

For gene expression analysis, *M. halliana* seedlings were precultured on 1/2 Hoagland's solution for 15 days when they reached eight true leaves [26]. After 15 days of incubation, the leaves were treated with a nutrient solution containing 100 mM 1:1 $NaCl:NaHCO_3$ (pH 8.0) for saline-alkali stress and collected at 0, 6 h, 12 h, 24 h, 48 h, and 72 h, respectively. Each treatment had one biological replication with five plants, and three biological replicates were carried out independently.

Cloning of *MhCCR1* gene and quantitative real-time PCR

Malus halliana leaves weighing 0.1 g were measured, and the Trizol technique was used to extract the samples' total RNA. Reverse transcription was carried out using TaKaRa's PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time). After searching the apple genome database for the *MhCCR1* CDS sequence, specific primers were designed using DNAMAN software (Additional file 1: Table S1), and PCR amplification was carried out. The reaction protocol consists of the following steps: 42 cycles of pre-denaturation at 94 ℃ for 5 min, denaturation at 94 ℃ for 30 s, annealing at 58 ℃ for 30 s, and extension at 72 ℃ for 90 s. PCR products were electrophoresed on 1.25% agarose gel and recovered the target band, ligated with pMD19-T cloning vector, then transformed into *E. coli* DH5α and selected positive clones for sequencing. The correctly sequenced *MhCCR1* plasmid was extracted, digested with *Sma I* and *Kpn I* restriction enzymes, and ligated to pRI101 expression vector. Finally, it was transformed into *E. coli*, identified positive single colony, and transformed into agrobacterium tumefaciens GV3101 for genetic transformation by freeze–thaw method. Simultaneously, the sequences were acquired via the NCBI database. Additional file 1: Table S1 contains a set of primer pairs for real-time PCR. The cDNA of *M. halliana* plantlets was used as a template for real-time PCR. GAPDH was used as a reference for quantitative real-time PCR. For every sample, three replicates were made. Ultimately, the data were computed using the $2^{-\Delta\Delta Ct}$ method, and the Duncan test of single-factor ANOVA ($P < 0.05$) was used to assess the difference.

Bioinformatics analysis of *MhCCR1* gene

Using the NCBI database, protein sequences similar to *MhCCR1* were identified in additional species. The ProtParam website (https://web.expasy.org/protparam/) was utilized to examine the protein's physicochemical characteristics. Protein amino acid sequences were compared using DNAMAN software. A phylogenetic tree was created using the neighbor-joining (NJ) method with MEGA-X software [27]. The PlantCARE (http://bioinformatics.psb.ugent.be/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

Transgenic *A. thaliana* was obtained using inflorescence infestation according to Hu et al. [28]. Then they were sown on MS medium containing 30 mg/L Kan and screened for resistant plants by PCR to obtain

heterozygous transgenic plants, and homozygous transgenic plants were obtained in the T3 generation after three successive generations of screening.

Tobacco was infected using the method of Wang et al. [6], and transgenic tobacco was cultured in medium containing 250 mg/L cephalosporin and 30 mg/L Kan. The regenerated shoot DNA was extracted and identified by PCR.

Infection of apple calli was based on the method of Hu et al. [29]. The overexpressed calli was evenly distributed on 250 mg/L cephalosporin and 30 mg/L Kan medium three to five times to obtain stable resistant calli. DNA was extracted and detected by qRT-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 Y2HGold and coated on SD/-Leu/-Trp (DDO) and SD/-Trp/-Leu/-Ade/-His/ (QDO/X) medium plates containing X-α-Gal, respectively, at 30 ℃, 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 co-transformation. 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 days. 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 days. 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

After sterilizing, the seeds of the wild type and homozygous transgenic lines of *A. thaliana* from the T3 generation were vernalized at 4 °C for three days before being sown on MS medium. Following three days, the seedlings were cultivated in an incubator on MS and MS + 100 mmol/L ($NaCl + NaHCO_3$) medium, respectively. Measure the indicators and examine the phenotypic after 20 days. After being cultivated in MS medium for 14 days, both WT and transgenic tobacco were moved to MS and MS + 100 mmol/L ($NaCl + NaHCO_3$) medium for a further 20 days. The indexes were established and the phenotypes were noted. After 15 days of normal culture in the medium used for the 'Wanglin' apple calli, the WT and transgenic calli were moved 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 Wang 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. 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

Statistical significance analysis of all data was performed using SPSS software (version 22.0; SPSS, IBM, Armonk,

NY, USA), with one-way ANOVA and Duncan's multiple range test ($P < 0.05$), and figures were prepared using Origin 8.0 software (Origin Lab, Hampton, MA, USA).The results of the one-way ANOVA are labeled using letters.

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).

As shown in Additional file 1: Table S2, the CCR1 gene has a molecular weight of 37.176 kDa and encodes 339 amino acids. It has an isoelectric point of 6.02 and is an acidic protein. The positive and negative charges are 36 and 41, respectively, and the average hydrophilicity is − 0.240, making it a hydrophilic protein. The coefficient of instability was 32.71, indicating that the protein is stable (coefficient greater than 40 is considered unstable), which suggests that the protein encoded by *MhCCR1* is a stable acidic hydrophilic protein.

Protein sequence analysis of *MhCCR1 gene*

Sequence alignment was performed on the amino acid sequences encoded by *MhCCR1* were compared with those of other species, which showed high similarity at the N-terminal and a certain degree of difference at the C-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 distantly related to all other species.

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

Analysis of the *cis*-acting elements in the 2000 bp sequence upstream of the *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, a variety of abiotic stress-related cis-elements were identified, such as the drought-responsive element MBS, the light-responsive elements G-box and GT1-motif, and the anaerobic-inducible response element ARE. Taken together, these suggested that *MhCCR1* may play a key role in responding to adversity stresses.

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

In order to clarify the expression level of *MhCCR1* gene in *M. halliana* leaves under different periods of saline-alkali stress (Fig. 1), it was learned by qRT-PCR that the expression level of *MhCCR1* showed an increasing trend compared with that of the control (0 h), and reached the highest level at 48 h, which was 17.89-fold higher than that of the control (0 h). This indicates that the *MhCCR1* gene can respond to stress.

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

The expression levels of *MhCCR1* in the transgenic lines were examined by qRT-PCR. Compared with WT plants, expression of CCR1 in transgenic *A. thaliana*, tobacco and apple calli was higher, indicating CCR1 overexpression (Fig. 2a). 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 18S was used as the internal reference gene in *A. thaliana* and apple calli and *NtActin* was used as the internal reference in tobacco, 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

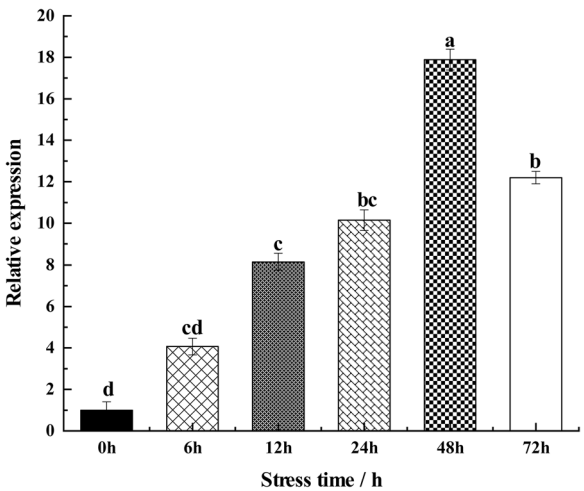


Fig. 1 Expression levels of *CCR1* gene in *M. halliana* seedlings under saline-alkali stress at 0, 6, 12, 24, 48 and 72 h, respectively. Data are means of three replicates with SE. Different letters above the bars indicated significant differences ($P < 0.05$) as assessed by one-way ANOVA and the least significant difference (LSD) test. ($P < 0.05$). The same below

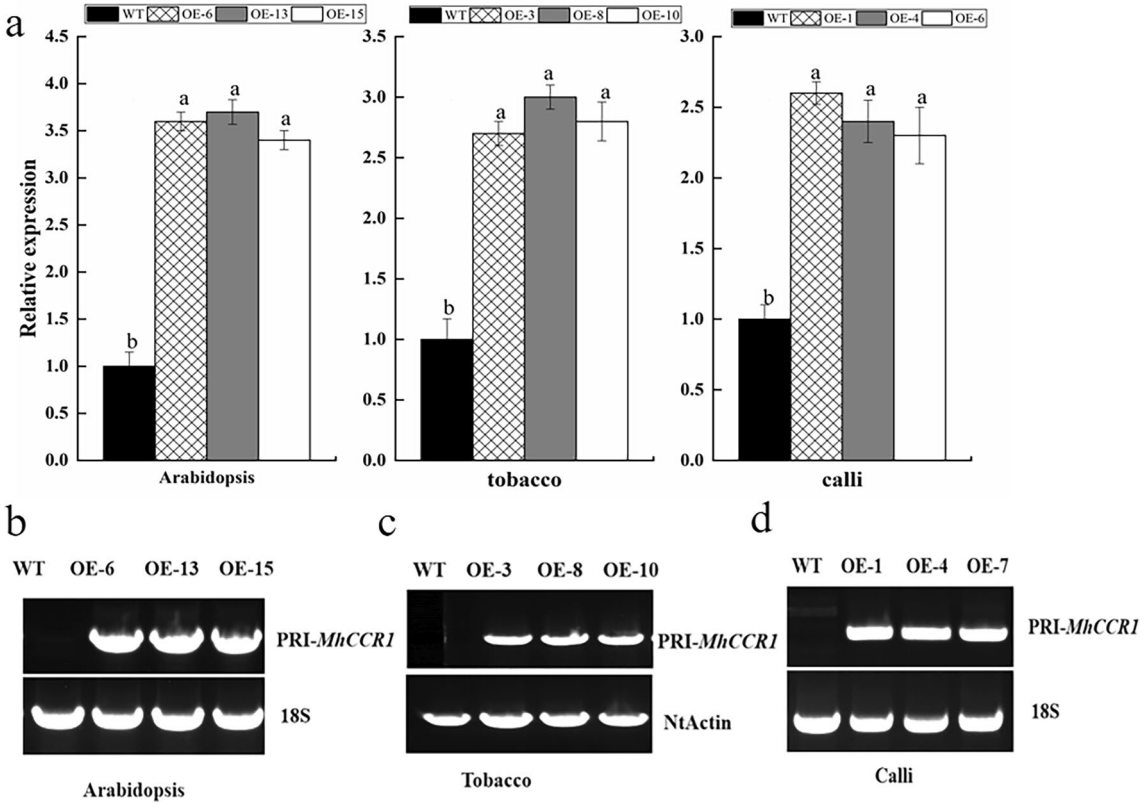


Fig.2 Identification of transgenic materials. **a** RNA level identification. **b** DNA level identification of *A. thaliana*. **c** DNA level identification of tobacco. **d** DNA level identification of calli

no bands, indicating that the transformation of PRI-*MhCCR1* vector was successful.

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

As shown in Fig. 3, the three *MhCCR1*-OE *A. thaliana* lines and the WT control were cultured under normal and saline-alkali stress (100 mM 1:1 NaCl: $NaHCO_3$) for 20 days, respectively. The WT and *MhCCR1*-OE *A. thaliana* grew well under normal conditions, with almost the same growth potential. However, the growth of both WT and *MhCCR1*-OE *A. thaliana* seedlings was affected to different degrees under saline-alkali treatment. The WT exhibited more severe shrinkage (Fig. 3a). Compared with the WT, the transgenic lines had longer root lengths and more lateral roots under saline-alkali treatment (100 mM) (Fig. 3b, c), which indicates that the transgenic plants may have a better ability to absorb water and nutrients. *A. thaliana* leaves were stained with DAB and NBT, respectively (Fig. 3d, e), 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 saline-alkali stress

As shown in Fig. 5a, both transgenic and WT tobacco grew vigorously in MS medium (CK). However, the growth of WT and *MhCCR1*-OE tobacco was inhibited under saline-alkali stress (100 mM 1:1 NaCl: $NaHCO_3$,

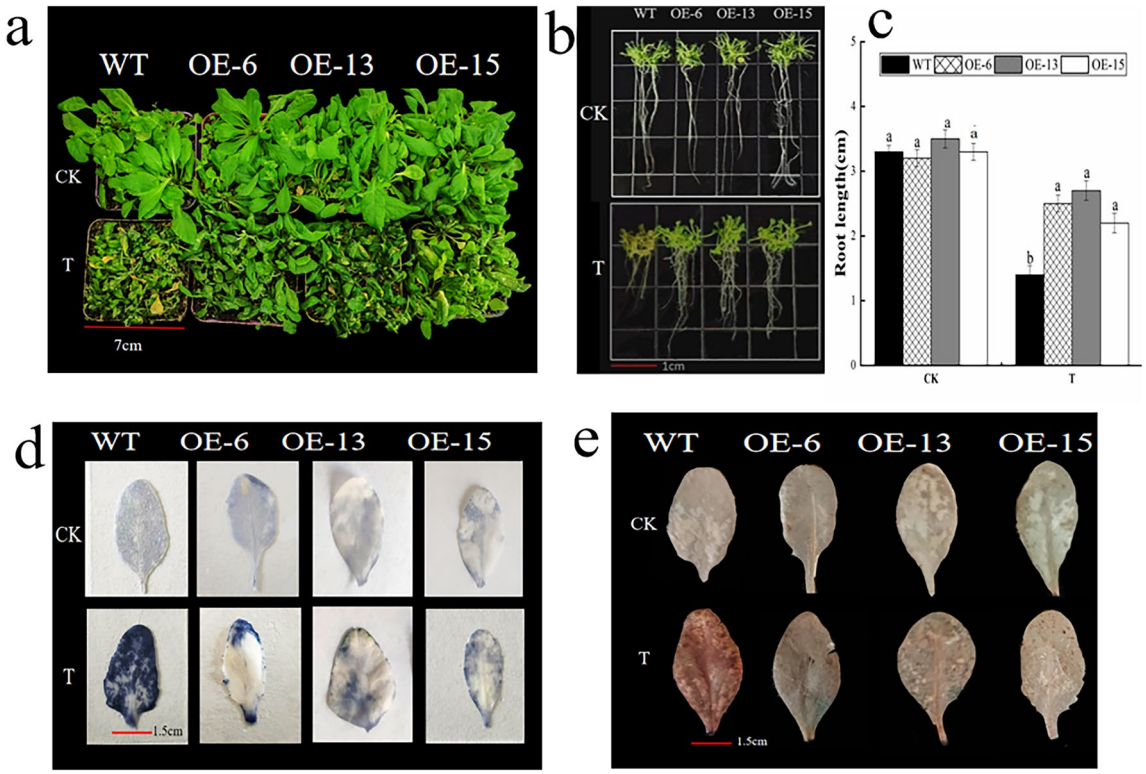


Fig. 3 The phenotype, root length, NBT and DAB staining of *MhCCR1*-OE and wild-type (WT) *A. thaliana* under normal conditions (CK) and saline—alkali stress (T). **a** The phenotypes. **b** Phenotypes of root length. **c** Root length. **d** NBT staining. **e** DAB staining

T), but the degree of chlorosis of WT tobacco was significantly more severe compared to transgenic lines, which was consistent with the effects of the *A. thaliana* treatment. In addition, the REC (Fig. 5b) and MDA (Fig. 5c) contents of the transgenic lines OE-3,8,10 were significantly lower than those of the WT control, and the activities of SOD, POD, and CAT (Fig. 5e–g) were much higher than those of the WT. Similarly, the proline (Fig. 5d), chlorophyll (Fig. 5h), and lignin (Fig. 5i, j) contents were significantly higher than those of the WT control. Based on the above results, we concluded that overexpression of *MhCCR1* enhanced the resistance of tobacco to saline-alkali stress.

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

To further investigate the function of *MhCCR1* under salinity stress, we selected WT and three transgenic apple calli (OE-1,4,7). As shown in Fig. 6, it can be clearly seen that there was no significant difference in the growth status of *MhCCR1*-OE and WT apple callus under normal conditions (CK). However, under saline-alkali stress conditions, the growth of both *MhCCR1*-OE and WT apple calli was inhibited, but the growth condition of *MhCCR1*-OE was superior to that of WT (Fig. 6a, b). The contents of Pro and MDA and the activities of SOD, POD and CAT were similar to those of tobacco and *A. thaliana* (Fig. 6c–g).

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

To further investigate the role of *MhCCR1* in the saline-alkali stress signaling pathway, qRT-PCR was used to detect gene expression in overexpressed *MhCCR1 A. thaliana*. 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

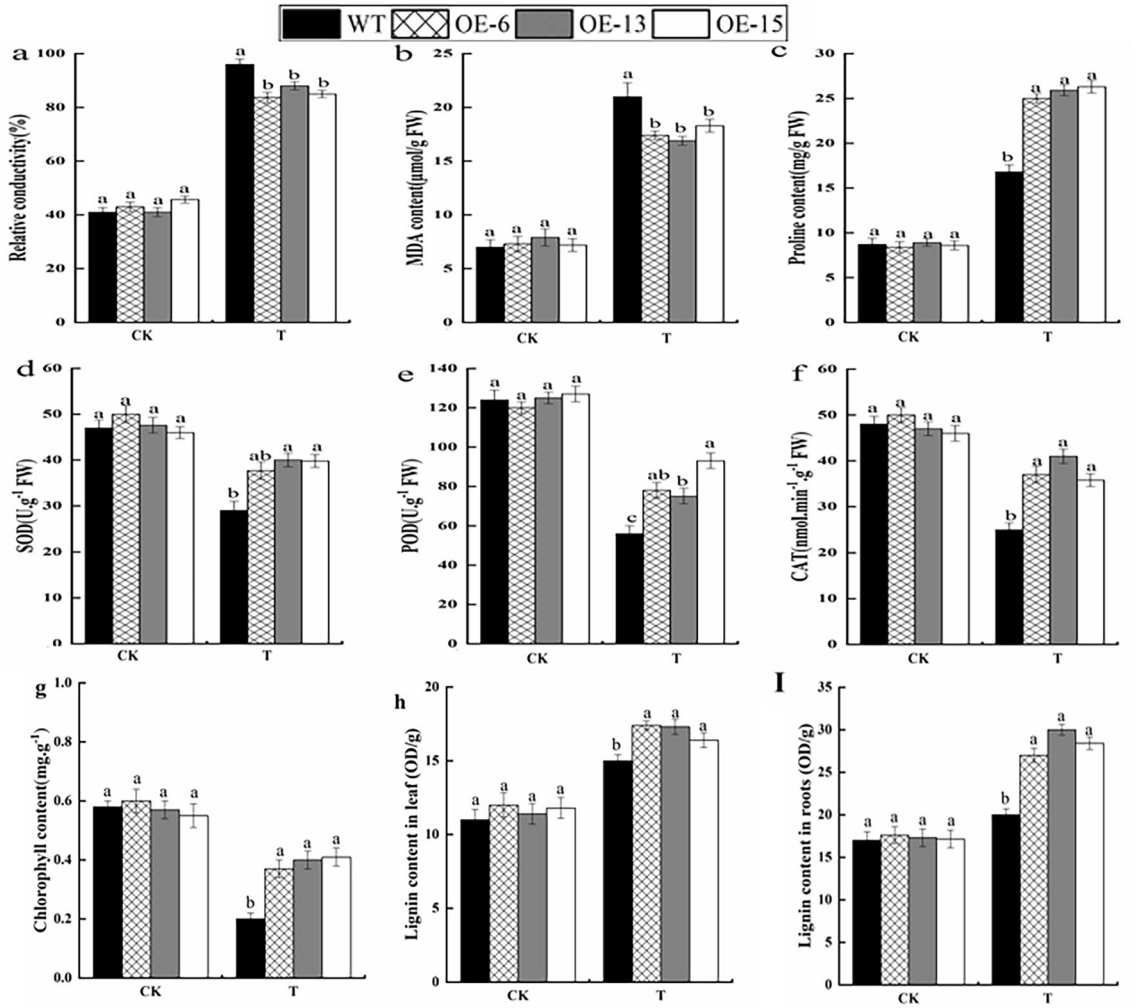


Fig. 4 Physiological indices of *MhCCR1-OE* and WT *A. thaliana* under normal conditions (CK) and saline-alkali stress (T). **a** Relative conductivity. **b** MDA content. **c** Pro content. **d** SOD activity. **e** POD activity. **f** CAT activity. **g** Chlorophyll content. **h** Lignin content in leaf. **i** Lignin content in roots

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 days. 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

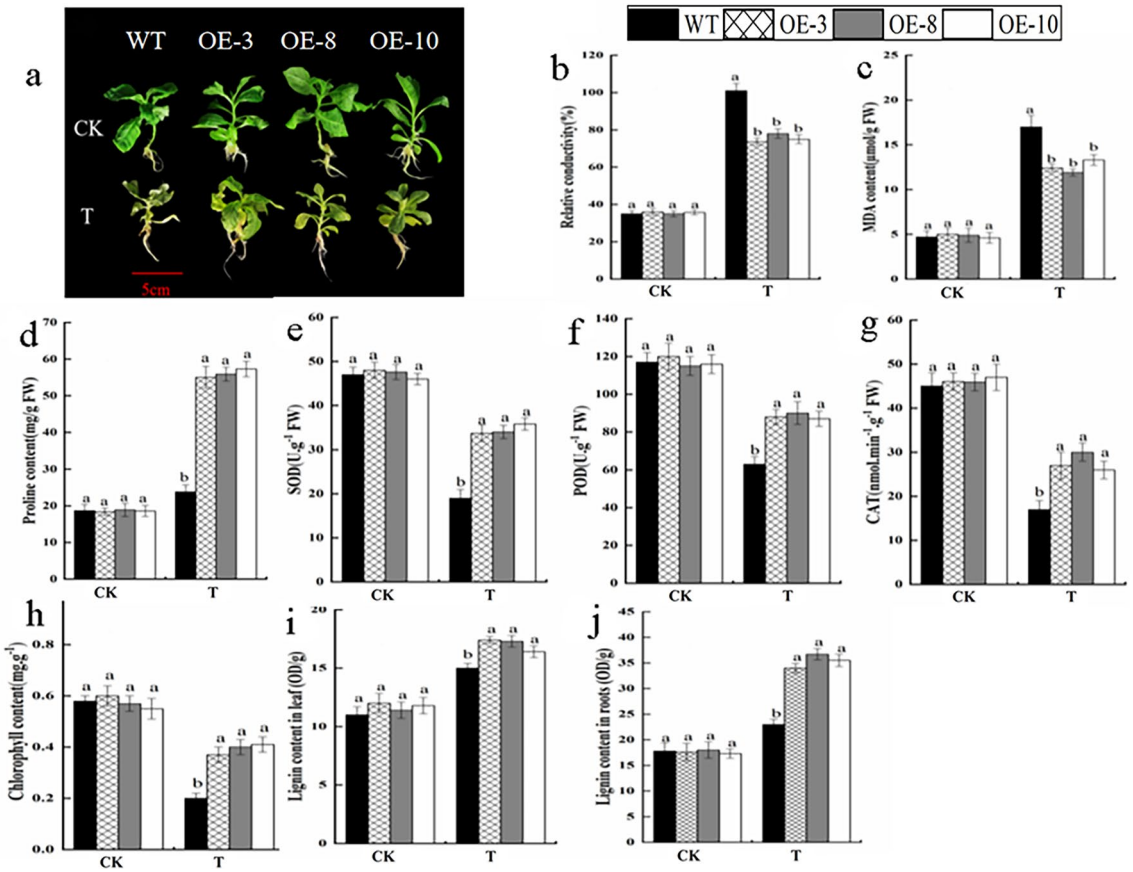


Fig. 5 Overexpression of *MhCCR1* improves saline-alkali resistance in tobacco. **a** The phenotypes of *MhCCR1*-OE and WT tobacco under normal conditions (CK) and saline-alkali stress (T). **b** Relative conductivity. **c** MDA content. **d** Pro content. **e** SOD activity. **f** POD activity. **g** CAT activity. **h** Chlorophyll content. **i** Lignin content in leaf. **j** Lignin content in roots

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 co-transformed into yeast receptor cells for reciprocal validation. The co-transformed 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 (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

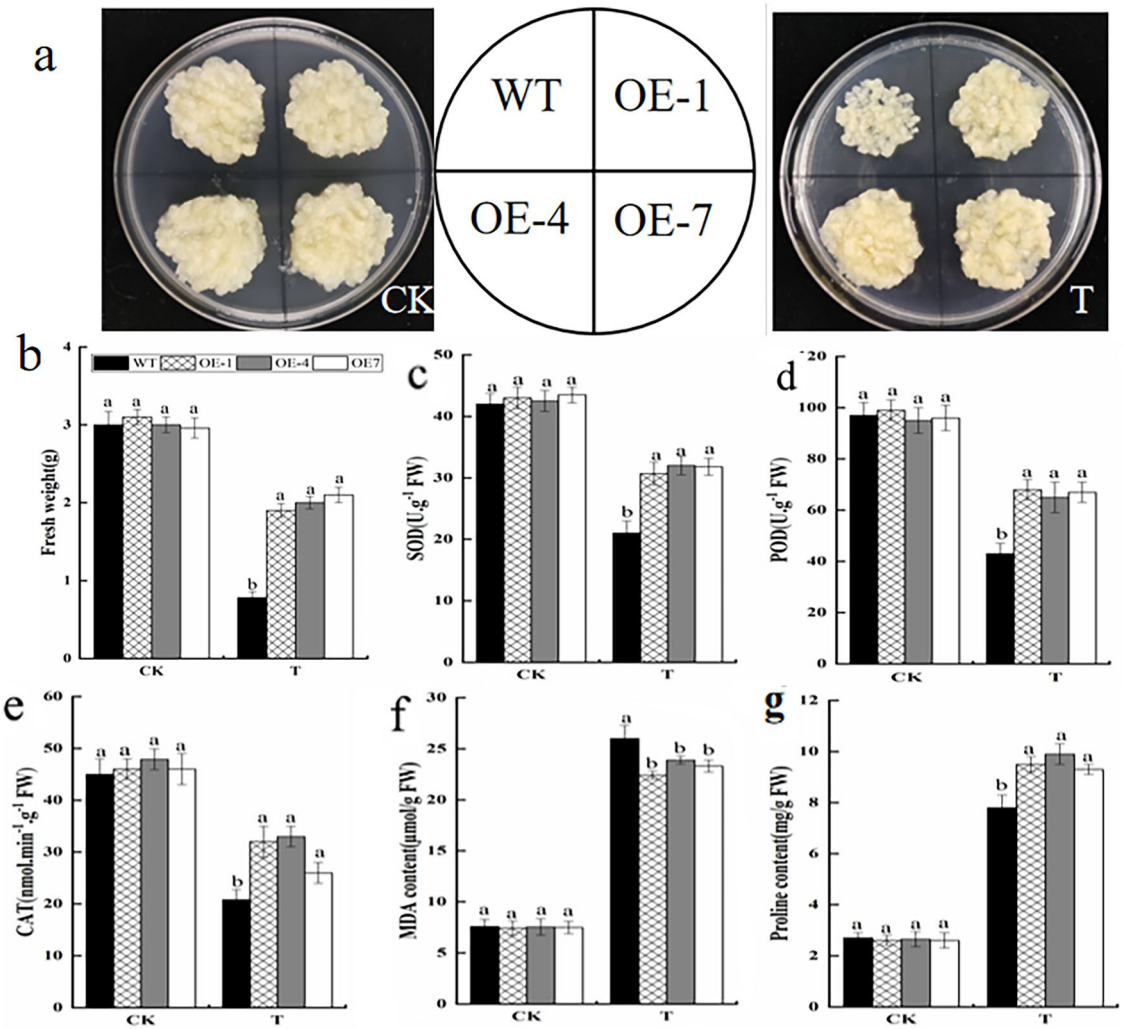


Fig. 6 Overexpression of *MhCCR1* improves saline-alkali resistance in apple calli. **a** The phenotypes of *MhCCR1*-OE and WT apple calli under normal conditions (CK) and saline-alkali stress (T). **b** Fresh weight. **c** SOD activity. **d** POD activity. **e** CAT activity. **f** MDA content. **g** Pro content

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 (*Trifolium repens* 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

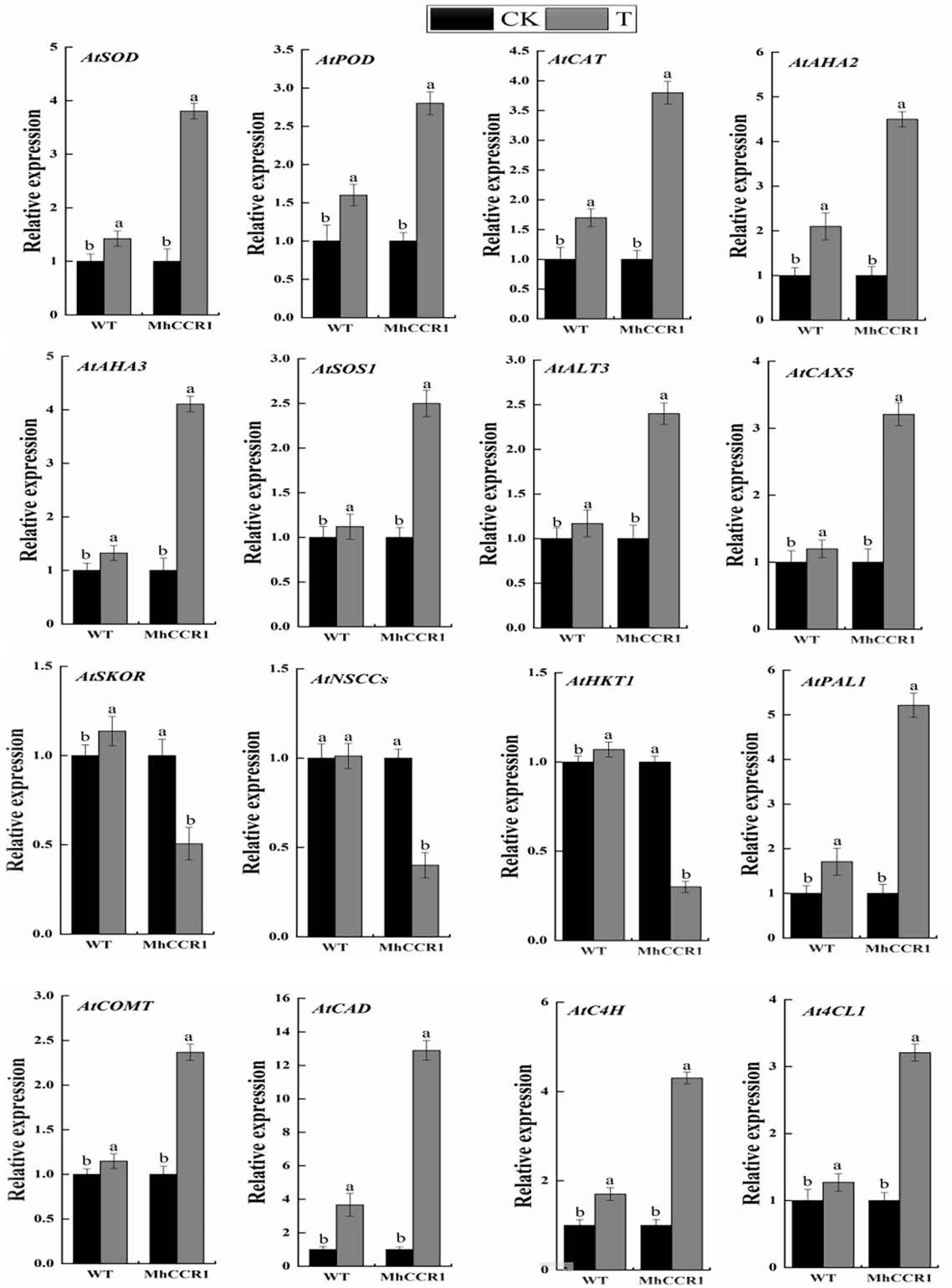


Fig. 7 Expression levels of WT and overexpressed *A. thaliana* (CCR1) lignin pathway and saline-alkali stress response genes under normal conditions (CK) and salinity stress (T). Significance using Student's test: values not followed by the same letter indicate significant differences between treatments

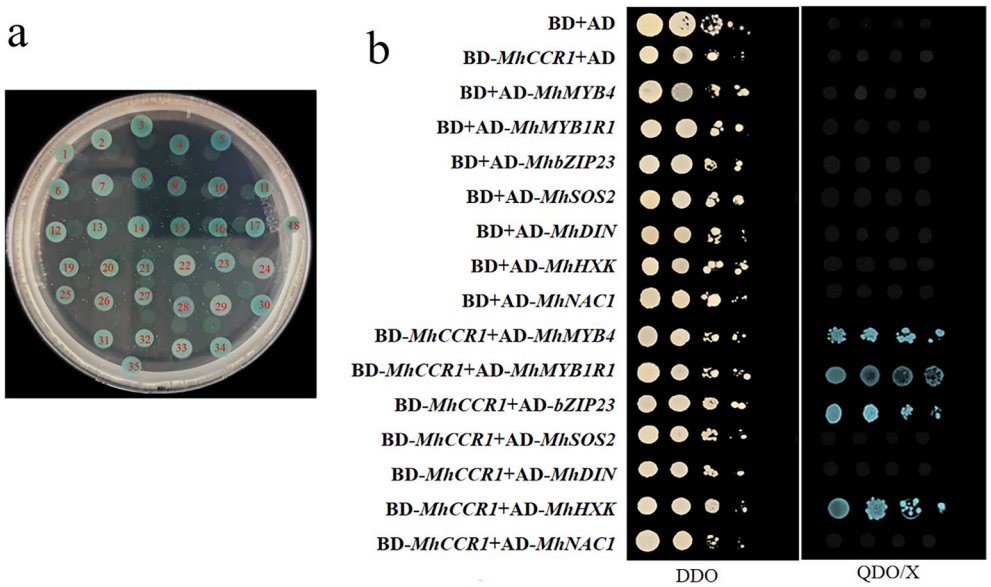


Fig. 8 Yeast two-hybrid screening for *MhCCR1*-interacting proteins. **a** Screening of positive clones on plates. **b** *MhCCR1* has no transcriptional activation activity and interacts with *MhMYB4*, *MhMYB1R1*, *MhHXK*, and *MhbZIP23*. DDO and QDO/X represent SD/-Leu/-Trp and SD/-Leu/-Trp/-His/-Ade/X-α-Gal medium, respectively

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.

We 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 [53, 54]. In this experiment, the expression levels of Na^+ transporters (*AtCAX5*, *AtSOS1*, and *AtALT3*) were significantly increased under saline-alkali stress, and the expression levels of Na^+ transporter genes were higher in overexpressed *A. thaliana* than in stressed WT *A. thaliana*, indicating that overexpressed *A. thaliana* had a higher capacity of Na^+ excretion than WT under saline-alkali stress. In addition, the expression levels of K^+ transporters (*AtSKOR*, *AtNSCCs*, and *AtHKT1*) were significantly reduced under saline-alkali stress, and the expression levels of K^+ transporter genes were lower in overexpressed *A. thaliana* than in stressed WT *A. thaliana*. 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

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40538-024-00565-1.

Additional file 1: Fig. S1. The outline of the lignin biosynthetic pathway. *PAL* phenylalaine ammonia-lyase, *C4H* cinnamate 4-hydroxylase, *4CL* 4-coumarate: CoA ligase, *CCR* cinnamoyl-CoA reductase, *HCT* p-hydroxycinnamoyl-CoA: quinate shikimate p-hydroxycinnamoyltransferase, *C3H* p-coumarate 3-hydroxylase, *CCoAOMT* caffeoyl-CoA o-methyltransferase, *CAD* cinnamyl alcohol dehydrogenase. **Fig. S2.** Heat map analysis of DEGs related to calcium signaling, plant hormone signal transduction and key metabolite synthesis pathways in leaves of *M. halliana* under saline-alkali stress. **Fig. S3.** Information on the *MhCCR1* protein sequence. **a**

Electrophoresis of PCR products for cloning of *MhCCR1*. **b** Electrophoresis images of pRI101-*MhCCR1* PCR amplification. **Fig. S4.** Protein sequence analysis of *MhCCR1* gene in *M. halliana* and the protein of other species. **Fig. S5.** Phylogenetic analysis of this protein of *MhCCR1* from *M. halliana* and other species. **Table S1.** List of primers for RT-PCR. **Table S2.** List of CCR1 gene and information on Its encoded proteins. **Table S3.** Some important *cis*-acting regulatory elements in the upstream regulatory sequences of *MhCCR1*.

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Author contributions
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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Availability of data and materials
The data that support the finding of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
This manuscript is an original paper and has not been published in other journals. The authors agreed to keep the copyright rule.

Competing interests
The authors declare that they have no competing interests.

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