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ORIGINAL ARTICLE

Molecular cloning and functional characterization of the CEP RECEPTOR 1 gene *MdCEPR1* of Apple (*Malus* × *domestica*)

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Abstract

Leucine-rich repeat-receptor-like kinases (LRR-RLKs) are major gene families that play an important role in in many aspects of plant growth and development particularly in the process of signal transmission. RLK XYLEM INTERMIXED WITH PHLOEM 1 (XIP1)/C-TERMINALLY ENCODED PEPTIDE (CEP) RECEPTOR 1 (CEPR1) has been identified as a leucine-rich repeat (LRR) receptor kinase. In this study, the *MdCEPR1* gene (GenBank ID: DQ221207) from apple (*Malus* × *domestica*), was isolated and characterized. *MdCEPR1* transcripts were highly accumulated in roots and leaves, and *MdCEPR1* was significantly induced under low nitrate conditions. In addition, suppressing the *MdCEPR1* gene in apple calli increased anthocyanin content. Overexpression of *MdCEPR1* promoted growth of apple calli and *Arabidopsis thaliana* under low nitrate condition by increasing nitrate assimilation and up regulating the expression of genes involved in nitrate assimilation. Ectopic expression of *MdCEPR1* also promoted lateral root development in transgenic *Arabidopsis*. Taken together, our results indicated that *MdCEPR1* acts as a positive regulator of plant nitrate utilization and lateral root development.

Key message

In this study, the *MdCEPR1* gene from apple, was isolated and characterized, and our results indicated that *MdCEPR1* acts as a positive regulator of plant nitrate utilization and lateral root development. *MdCEPR1* may be a useful target for marker-assisted breeding to improve crop yield and reduce the use of chemical fertilizer.

Keywords Apple · LRR-RLKs · *MdCEPR1* · Anthocyanin biosynthesis · Nitrate response

Introduction

Nitrogen is the key nutrient that determines fruit yield and quality (Crawford and Glass 1998; Williams and Miller 2001), and is one of the essential mineral elements for fruit trees. It is an important component of cell protoplasts, nucleic acids, hormones, alkaloids, and enzymes, so a supply of nitrogen is necessary for normal plant growth (Pallardy 2008). The supply of nitrogen is directly related to organ differentiation and formation of plant structures. Nitrogen is mainly taken up as ammonium nitrogen and nitrate nitrogen for utilization by plants (Lee et al. 1992). Nitrate is the main source of nitrogen for most plants and acts as a signaling molecule to regulate gene expression associated with metabolism and development (Gutiérrez et al. 2008).

Plant receptor-like protein kinases (RLKs) belong to a major gene family, with more than 610 and 1,132 members in *Arabidopsis* and rice, respectively (Shiu et al. 2004). Typical RLKs of plants contain three domains: an

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extracellular ligand-binding domain, a transmembrane domain, and a C-terminal intracellular kinase domain (Christiaan et al. 2012). Leucine-rich repeat-receptor-like kinases (LRR-RLKs) are the largest branch of the plant RLK family, including over 200 members that contain many LRRs in the extracellular domain (Shiu and Bleecker 2003). In plants, a number of LRR-RLKs have been functionally identified and play important roles in hormone receptors and disease resistance. For example, BRASSINOSTEROID-INSENSITIVE 1 (BRI1) is a component of the brassinosteroid receptor, which interacts with ligands to participate in the stress response in plants (Li et al. 2002). FLAGELLIN-SENSITIVE 2 (FLS2) is a component of the flagellin receptor in *Arabidopsis*, which is involved in defense responses to pathogens (Gómez-Gómez and Boller 2000). Some LRR-RLKs are also involved in plant developmental processes. CLAVATA1 (CLV1) receptor kinase controls meristem size and stem cell number (Nimchuk et al. 2011). ERECTA participated in the regulation of organ growth, stomatal differentiation, and inflorescence structure (Shpak et al. 2004). *AtSERK1* responds to biotic and abiotic stress and enhances embryogenic competence in culture (Salaj et al. 2008). RLK XYLEM INTERMIXED WITH PHLOEM 1 (XIP1)/C-TERMINALLY ENCODED PEPTIDE (CEP) RECEPTOR 1 (CEPR1) has been identified as a LRR receptor kinase that is necessary for development of the vascular system (Bryan et al. 2012). More recently, CEPR1 and CEPR2 have been shown to be the receptor for CEP1 and other members of the CEP family. CEPR1 contains a short secretory signal peptide sequence, an N-terminal extracellular LRR receptor domain, a C-terminal cytoplasmic serine/threonine kinase domain, and a single helical transmembrane region (Roberts et al. 2016). *CEPR1* belongs to the LRR XI family and loss-of-function mutants despite their general morphology and normal fertility show ectopic lignification of the phloem and accumulation of leaf anthocyanins (Bryan et al. 2012). Interestingly, the *cepr1 cepr2* double mutant has a variety of phenotypes, including smaller light green leaves and reduced expression of the nitrate transporter. Other studies have shown that *CEPR1* regulate the nitrogen-dependent responses in the long-distance systemic signaling pathway (Tabata et al. 2014). *CEP5* and *CEPR1* are involved in the regulation of lateral root initiation in *Arabidopsis* and cepr1 has a phenotype with reduced lateral root density compared to the wild-type (WT) (Roberts et al. 2016).

In this study, a *CEPR1* gene (*MdCEPR1*) was cloned from apple (*Malus* × *domestica*), followed by an expression analysis and functional characterization. Our results suggest that *MdCEPR1* regulate the nitrogen response and construction of root morphology. *MdCEPR1* plays a critical role in optimal root system deployment of apple for more effective access to nutrition.

Materials and methods

Plant materials and growth conditions

The apple calli used in this study were obtained from the young embryos of 'Orin' apple, and subcultured on modified 'Orin' calli medium (MS medium supplemented with 1.3 mg L^{-1} 2,4-dichlorophenoxy (2,4-D) and 0.6 mg L^{-1} 6-benzylaminopurine (6-BA) at 25 ℃ in the dark (An et al. 2017). The in vitro shoot cultures of the apple variety 'Gala' were grown on MS medium containing 0.5 mg L^{-1} 6-benzylaminopurine (6-BA), 0.1 mg L^{-1} gibberellins (GA_3), and 0.2 mg L^{-1} 1-naphthaleneacetic acid (NAA) and rooted in MS medium containing 0.15 mg L^{-1} NAA at 16 h light/8 h darkness (You et al. 2014). One-month-old uniform 'Gala' seedlings were selected and transplanted to PVC pots (25 cm long, 20 cm wide and 15 cm high) containing 6L nutrient solution. Floating plates were used to support seedling growing in the solution. The composition of modified Hoagland's nutrient solution (Hoagland and Arnon 1950) was as follows: 10 mM KNO_3, 2 mM $MgSO_4$, 1 mM KH_2PO_4, 0.045 mM H_3BO_3, 0.01 mM $MnCl_2$, 0.8 μM $ZnSO_4$, 0.3 μM $CuSO_4$, 0.4 μM Na_2MoO_4, and 0.02 μM Fe-EDTA. The solution for the low nitrate treatment contained 0.1 mM KNO_3, and K was compensated by KCl. *Arabidopsis thaliana* (Col-0) was used in this study for genetic transformation. The seeds were surface-sterilized with 4% NaClO. The sterilized seeds were stored at 4 °C for 2 days and sown onto plates containing MS medium with 0.6% agar at 22 °C.

Sequence alignment and phylogenetic analysis

A BLASTP search (http://www.ncbi.nlm.nih.gov/BLAST/) was performed to obtain the MdCEPR1 homologs. Multiple sequence alignments of the CEPR1 sequences were performed using ClustalX (version 2.1). Phylogenetic analysis of CEPR1 was performed according to the neighbor-joining method in the MEGA5 program (http://www.megasoftware.net/) (Hall 2013). The MdCEPR1 secondary protein structure was predicted using Simple Modular Architecture Research Tool (SMART) software (http://smart.embl-heidelberg.de/).

Generation of the *MdCEPR1* transgenic calli and transgenic *Arabidopsis*

The open reading frame (ORF) and the reverse complement DNA fragment of *MdCEPR1* were obtained by real time-polymerase chain reaction (RT-PCR). The ORF region of

the *MdCEPR1* cDNA was used for sense overexpression (MdCEPR1-OX), and the DNA fragment of *MdCEPR1* reverse complement were used for antisense suppression (MdCEPR1-anti). The PCR products were inserted into the transformed vector pRI101, and then the plasmids were introduced into *Agrobacterium* strain LBA4404 for obtaining transgenic apple calli according to the method as described by An et al. (2015). To create *MdCEPR1* transgenic *Arabidopsis*, the plant transformation vectors described above (pRI101- MdCEPR1) were transformed into *Arabidopsis* plants (Columbia) by the floral dip method (Clough and Bent 1998). Transcription levels of *MdCEPR1* in related lines were monitored by qRT-PCR. The T1 generations of transgenic plants were used for phenotypic analysis, and the phenotypes were confirmed in the following 2 to 3 generations.

Gene expression analysis

All samples were stored at − 80 °C. RNA was extracted from triplicate biological replicates of the above samples using the Trizol kit. Two micrograms of total RNA were used to synthesise first-strand cDNA by reverse transcription. RT-qPCR analysis was performed using the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) on a Bio-Rad iQ5 instrument (USA). The reactions were performed using the following cycling parameters: 5 min initial denaturation at 94 °C, 30 cycles of denaturation at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. *MdActin* was used as the control and the $2^{-\Delta\Delta Ct}$ calculation method was used for data analysis. All of the primers used are listed in Supplementary Table S1. All of the samples were repeated at least three times. The results were based on the average of three times biological replicates.

Determination of anthocyanin content

The 'Orin' apple calli were cultured on medium and were place in a phytotron (temperature: 22 °C, photon flux density: 70 μmol $m^{-2}s^{-1}$) for 1 week. Then, total anthocyanin content was extracted from both transgenic and WT calli using a methanol-HCl method as described by An et al. (2015).

Nitrogen deficiency treatment and determination of nitrate content and nitrate reductase activity

To investigate different growth rates under nitrogen deficiency conditions, the 15-day-old individual genotype apple calli were treated with different concentrations of nitrate medium (0.1 and 10 mM). Subsequently, these apple calli were grown for 2 weeks under continuous dark conditions. The 10-day-old *Arabidopsis* seedlings were grown on vermiculite for 3 weeks, and watered with a nutrient solution containing 10 mM NO^{3-} and 0.1 mM NO^{3-} respectively. Then, the plant material was collected for determination of nitrate content and nitrate reductase activity.

Nitrate content was measured using the salicylic acid method (Cataldo et al. 1975; Li et al. 2017). First, samples (1 g) were frozen in liquid N_2 and milled into powder. Then, samples were boiled at 100 °C for 20 min with 10 mL of deionized water, centrifuged at 15,000×*g* for 10 min. Next, 0.1 mL of the supernatant and 0.4 mL of 5% salicylic acid-sulfuric acid solution were added to a new tube for reaction. Twenty minutes later, 9.5 mL of 8% NaOH solution was added slowly to the tubes and cooled to room temperature. The optical density of the reaction solution at 410 nm was measured and the deionized water served as the control. The nitrate content were normalized according to the following formula: N = C·V/W (N, nitrate content; C, nitrate concentration calculated using OD_{410} in the regression equation; V, total volume of extracted sample; W, weight of sample). The standard curve was made using 10–120 mg L^{-1} KNO_3, and the regression equation was calculated according to the standard curve.

Nitrate reductase activity (NRA) was measured as described by Freschi et al. (2010) and Li et al. (2017). First, samples (0.5 g) were washed with distilled water and placed in tubes. Next, 9 ml of 0.1 M phosphate buffer (pH7.5) with 3% propanol and 0.1 M KNO_3 were added to the tubes for reaction at 30 °C in the dark. Thirty minutes later, 1 mL of trichloroacetic acid was added to stop the reaction. Then, the 2 mL of supernatant was transferred into a new tube, and 4 mL of 0.2% *N*-1-naphthyl-ethylene-diamine and 4 mL of sulfanilamide mixed with 3 M HCl were added. The optical density of the reaction solution at 540 nm was measured after 30 min. The standard curve was made using 0–2 g $NaNO_2$, and the regression equation was calculated according to the standard curve. The nitrate reductase activity (NRA) was normalized according to the of nitrite content produced per hour per gram of fresh weight (nmol $nitrite^{-1}$ g^{-1} FW).

Nitrate uptake assay using $^{15}NO_3^-$

The $^{15}NO_3^-$ was used to analyze nitrate uptake activity as described by Han et al. (2016). Ten-day-old *A. thaliana* seedlings were transferred to 0.1 mM $CaSO_4$ for 1 min, then to their respective hydroponic nutrient solutions with $^{15}NO_3^-$ (99% atom) replacing unlabeled NO_3^- for 30 min. Finally, the *A. thaliana* seedlings were transferred to 0.1 mM $CaSO_4$ for 1 min, after which the roots were washed with deionized water. Then, the seedlings were dried at 70 ℃ to constant weight and ground. ^{15}N content was analyzed using a continuous-flow isotope ratio mass spectrometer coupled with an elemental analyzer.

Statistical analysis

All experiments were performed at least three times and the results were based on the average of three replicates. The data were analyzed with appropriate methods using DPS (7.05) software (http://www.dpsw.cn/dps_eng/). A p-value < 0.05 was considered significant.

Results

MdCEPR1 protein structure and phylogenetic tree analysis

To investigate the functions of CEPR1 in apple, the AtCEPR1 (AT5G49660) sequence was used as a query to search similar sequences in apple genome database (https://www.rosaceae.org/blast) with the BLASTP program. As shown in Supplementary Fig. S1, we identified the apple *AtCEPR1* homologous gene and named it *MdCEPR1* (MDP0000122376). A sequence analysis revealed that the full-length *MdCEPR1* cDNA sequence consisted of 3392 nucleotides with an ORF of 2892 nucleotides, which encoded a 963 amino acid peptide. Alignment with the CEPR1 protein sequences from *Arabidopsis* showed that MdCEPR1 had 63% identity to AtCEPR1. Then the MdCEPR1 amino acid sequence was analyzed, and five LRR regions were predicted, with two conserved regions, including a transmembrane region and a serine/threonine protein kinase catalytic domain (Fig. 1a). To explore the evolutionary relationships among the CEPR1 proteins in plants, 22 CEPR1 family members were aligned and a

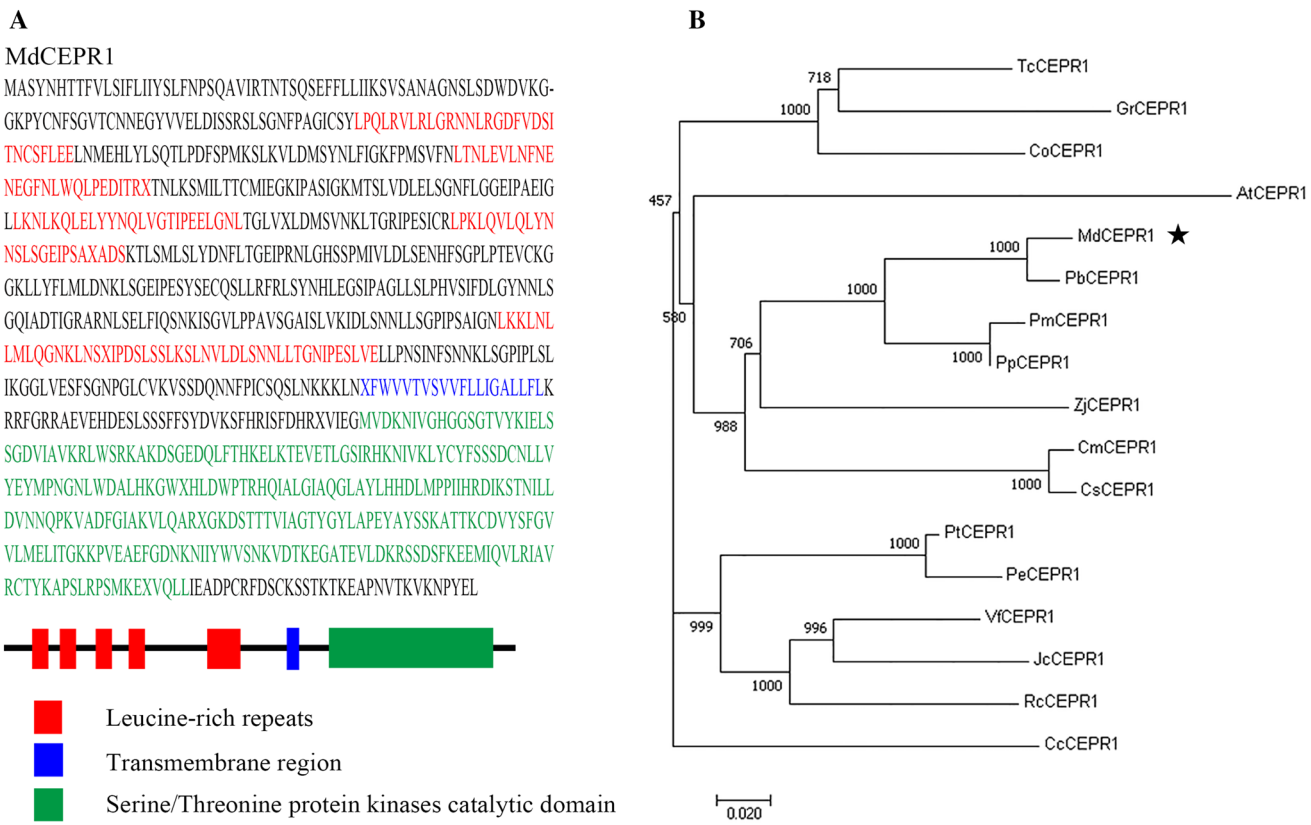

Fig. 1 Protein structure and phylogenetic tree analysis of MdCEPR1. Functional domains analysis of MdCEPR1. **a** The amino acid sequence of MdCEPR1 was analyzed using SMART (http://smart.embl-heidelberg.de/). Red represents the five LRR repeat domains (100–128 aa, 171–197 aa, 245–269 aa, 293–317 aa, and 485–533 aa). Blue represents the transmembrane region (593–612 aa). Green represents the serine/threonine protein kinase catalytic domain (654–931 aa). **b** Phylogenetic analysis of MdCEPR1 and CEPR1 from 16 other plants using the neighbor joining method. MdCEPR1 is denoted by an asterisk, and the distance indicated by "0.2" refers to the percent sequence divergence.TcCEPR1: *Theobroma cacao*, XP_007018366.1; GrCEPR1: *Gossypium raimondii*, XP_012446740.1; CoCEPR1: *Corchorus olitorius*, ACI42311.1; AtCEPR1: *Arabidopsis thaliana*, AT5G49660.1; PbCEPR1: *Pyrus bretschneideri*, XP_009357235.1; PmCEPR1: *Prunus mume*, XP_008219158.1; PpCEPR1: *Prunus persica*, XP_007227013.1; ZjCEPR1: *Ziziphus jujube*, XP_015887774.1; CmCEPR1: *Cucumis melo*, XP_008466101.1; CsCEPR1: *Cucumis sativus*, XP_004136411.1; PtCEPR1: *Populus trichocarpa*, XP_002301126.1; PeCEPR1: *Populus euphratica*, XP_011017021.1; VfCEPR1: *Vernicia fordii*, AMM42884.1; JcCEPR1: *Jatropha curcas*, XP_012068114.1; RcCEPR1: *Ricinus communis*, XP_002510008.1; CcCEPR1: *Citrus clementine*, XP_006442751.1. (color figure online)

phylogenetic tree was constructed using the neighbor-joining method. The results clearly indicate that MdCEPR1 has a close genetic relationship with PbCEPR1 and shares clades with PmCEPR1 and PpCEPR1 (Fig. 1b).

MdCEPR1 expression patterns

The spatial expression pattern of *MdCEPR1* was investigated by qRT-PCR to elucidate the function of *MdCEPR1* in plants. The results show that the *MdCEPR1* transcript accumulated in five apple tissues (roots, stems, leaves, flowers, and fruit). The amount of the transcript was significantly higher in roots and leaves than in stems, flowers, or fruit (Fig. 2a). To investigate whether *MdCEPR1* expression was induced by nitrogen deficiency, the expression of *MdCEPR1* was examined under a 0.1 mM L^{-1} nitrate condition. Transcript analysis revealed that the transcript levels of *MdCEPR1* were significantly increased in response to nitrogen deficiency treatments in roots and shoots compared with the control treatment (Fig. 2b). The *MdCEPR1* expression level in the root reached the maximum 8 h after treatment, which was earlier than that in the shoot. This finding indicates that the root system first senses nitrogen deficiency stress and then signals the shoot, causing the *MdCEPR1* response in the shoot. These results suggest that *MdCEPR1* is probably involved in the response to a nitrogen deficiency.

MdCEPR1 negatively regulates anthocyanin accumulation and inhibits expression levels of flavonoid structural genes in apple calli

AtCEPR1 loss-of-function mutants regulate leaf anthocyanin accumulation (Bryan et al. 2012). To investigate whether *MdCEPR1* also regulates the accumulation of anthocyanins in apple, *MdCEPR1* overexpression and antisense expression vectors were constructed, and were transformed into 'Orin' apple calli. Then, total DNA of the transgenic materials were extracted, and PCR were used to determine gene transformation (Supplementary Fig. S2A) and qRT-PCR were used to detect the expression level of *MdCEPR1* (Supplementary Fig. S2B). As a result, overexpressing calli (MdCEPR1-OX) and antisense suppressing calli (MdCEPR1-anti) were obtained (Fig. 3a). The accumulation of anthocyanins in MdCEPR1-anti was significantly higher than in WT, and MdCEPR1-OX accumulated less anthocyanin compared to the WT under light conditions (Fig. 3b–d). The expression levels of flavonoid structural genes were analyzed by qRT-PCR in the WT and transgenic calli (MdCEPR1-OX and MdCEPR1-anti). The results show that the expression of major genes related to the anthocyanin biosynthetic pathway were slightly suppressed in MdCEPR1-OX compared with WT, including *MdMYB1*, *MdPAL*, *MdCHI*, *MdF3H*, *MdDFR*, *MdANS1*, *MdANR1*, and *MdUFGT* (Fig. 4). In contrast, expression of these genes was significantly upregulated in MdCEPR1-anti compared with WT and MdCEPR1-OX.

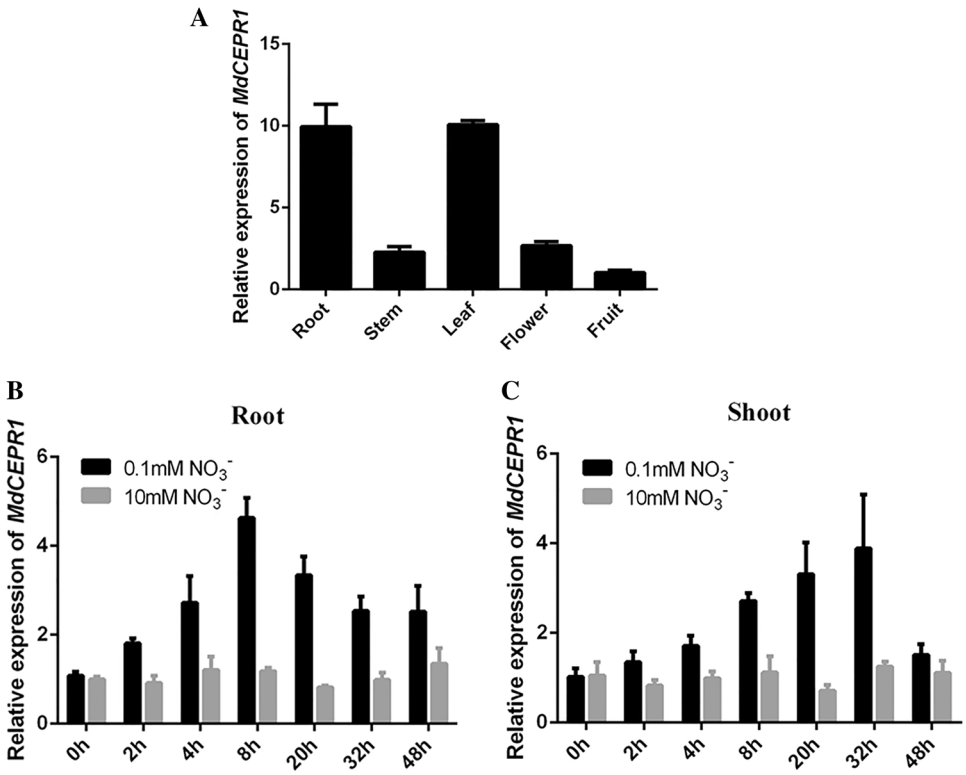

Fig. 2 MdCEPR1expression patterns. **a** Transcript levels of *MdCEPR1* in different organs. The value for fruit was set to 1. **b** Time-course expression levels of *MdCEPR1* at different periods in the roots and shoots of 'Gala' seedlings under 10 mM NO_3^- and 0.1 mM NO_3^- conditions. These data were analyzed by the $2^{-\Delta\Delta CT}$ method

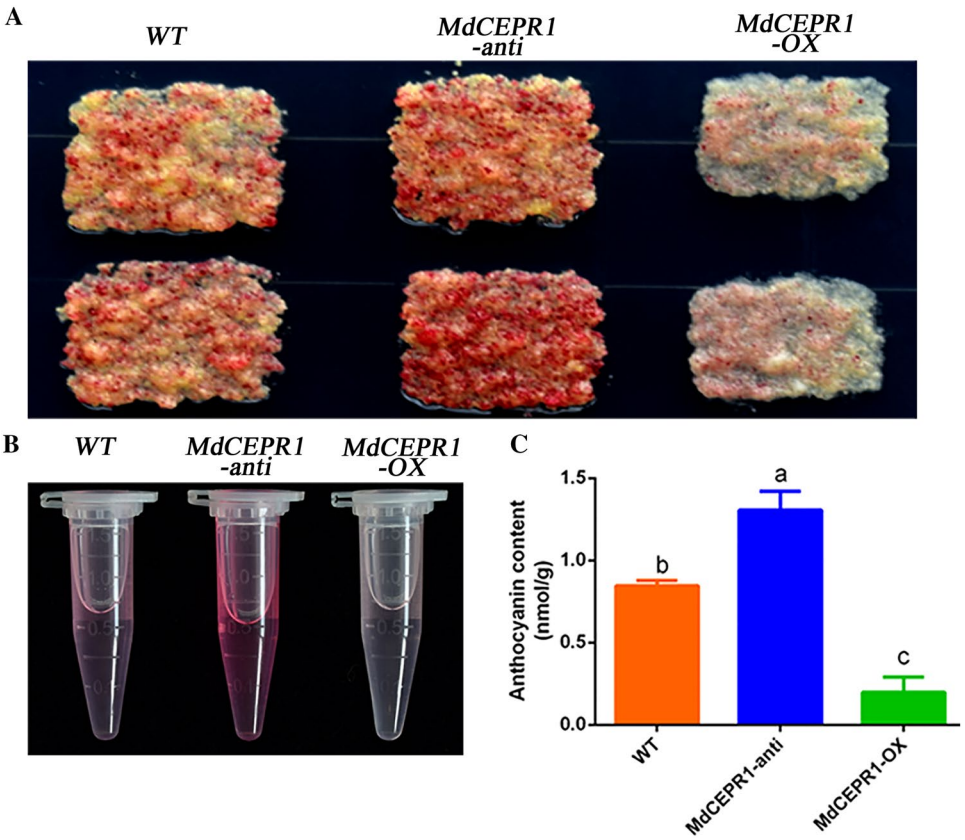

Fig. 3 MdCEPR1 negatively regulates anthocyanin accumulation. **a** and **b** Anthocyanin accumulation in wild type (WT) and transgenic calli (MdCEPR1-OX and MdCEPR1-anti). **c** Anthocyanin contents in transgenic calli (MdCEPR1-OX and MdCEPR1-anti) and WT control

These results indicate that *MdCEPR1* and *AtCEPR1* share similar functions in regulating anthocyanin accumulation.

Overexpression of *MdCEPR1* enhances tolerance to nitrogen deficiency by increasing nitrate assimilation in apple calli

The 15-day-old individual genotype calli were transferred to different concentrations of nitrate medium (0.1 and 10 mM) for 2 week to assess the role of *MdCEPR1* in nitrate assimilation in apple. As shown in Fig. 5a, the fresh weight of MdCEPR1-anti was slightly lower than that of WT and MdCEPR1-OX when grown on nitrogen-rich medium (10 mM NO_3^-). However, growth of MdCEPR1-OX was significantly superior to the WT and MdCEPR1-anti, and MdCEPR1-anti did not grow well on nitrogen-deficient medium (0.1 mM NO_3^-) compared with WT and MdCEPR1-OX (Fig. 5b). This result demonstrates that *MdCEPR1* rescued the phenotypic defects of the apple calli under nitrogen-deficient conditions. To further analyze the actual role of *MdCEPR1* in nitrate absorption, nitrate content and NRA of WT and transgenic calli were determined under N-rich and N-limited conditions. Statistical analyses indicated that overexpressing *MdCEPR1* increased nitrate content and NRA (Fig. 4c, d). However, the nitrate contents and NRA were significantly lower compared with the WT in MdCEPR1-anti (Fig. 5c, d). Then, the transcript levels of genes related to nitrate uptake, transport, and assimilation were analyzed by qRT-PCR in transgenic calli. The expression levels of *MdNRT1.1*, *MdNRT2.1*, and *MdNIA1* in WT and transgenic calli were assayed. As shown in Fig. 5, all tested nitrate-related genes had significantly higher expression levels in MdCEPR1-OX compared with the WT control, and the expression levels were significantly downregulated in MdCEPR1-anti (Fig. 5e–g). These results demonstrated that *MdCEPR1* plays a vital role in nitrate assimilation and signaling in apple.

Ectopic expression of MdCEPR1 enhances tolerance to nitrogen deficiency in *Arabidopsis thaliana*

To investigate whether *MdCEPR1* modulates nitrate assimilation in different plant species, *MdCEPR1* transgenic *Arabidopsis* plants were generated after repeated selection through kanamycin resistance assays and PCR detection. Then, the expression levels of *MdCEPR1* were measured by qRT-PCR in the transgenic lines, and three independent ectopic transgenic lines (MdCEPR1-L1, L2, and L3) were selected for the subsequent analysis (Supplementary Fig. S3). As shown in Fig. 6a, 10-day-old wild-type (Col) and transgenic *Arabidopsis* seedlings were grown on vermiculite, and watered with a nutrient solution containing 10 mM

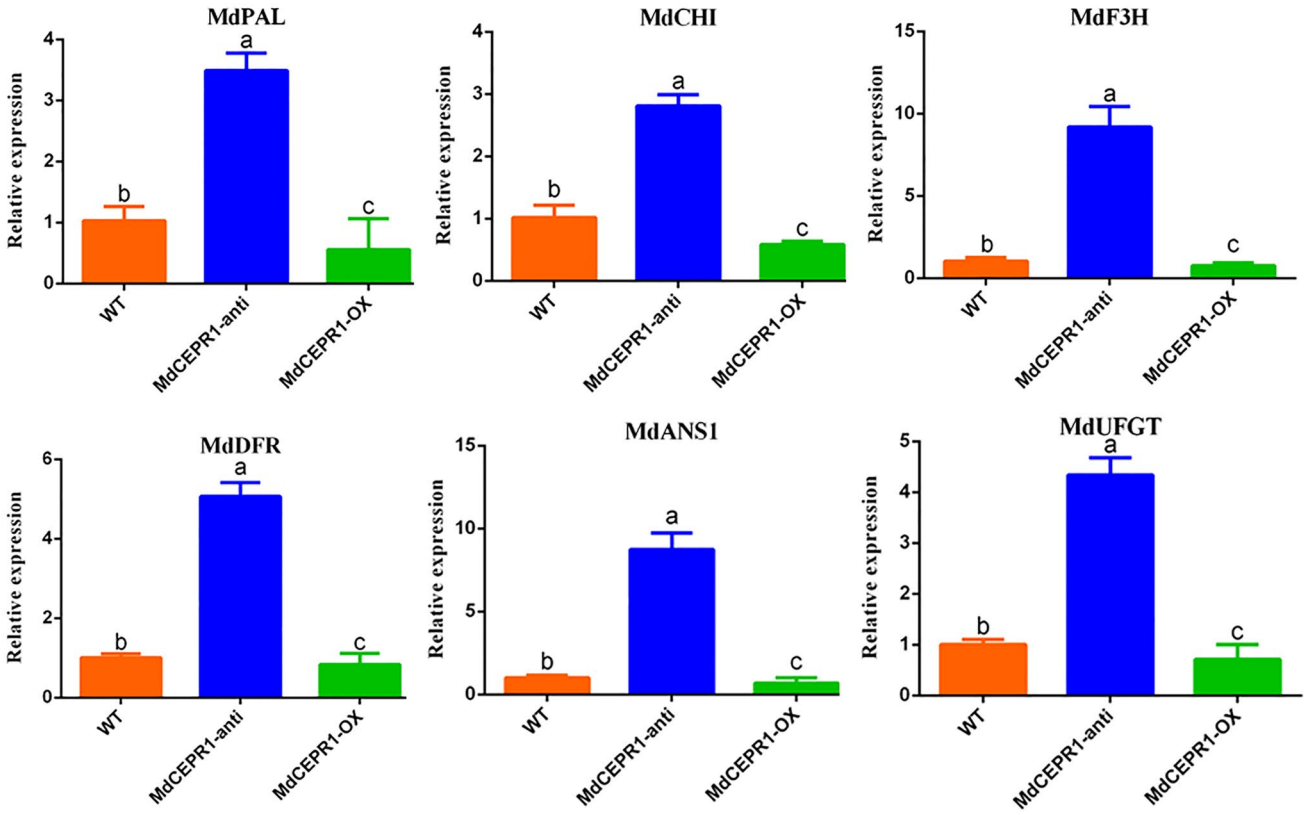

Fig. 4 MdCEPR1 inhibits expression levels of flavonoid structural genes. Expression levels of MdMYB1, MdPAL, MdCHI, MdF3H, MdDFR, MdANS, MdUFGT, and MdANR1 in transgenic calli (MdCEPR1-OX and MdCEPR1-anti) and the wild type (WT) control

NO_3^- and 0.1 mM NO_3^- for 3 weeks, respectively. Phenotypic observations showed that the *MdCEPR1* overexpressing plants developed dark blue leaves compared with WT under the 10 mM NO_3^- condition, and had a higher fresh weight (Fig. 6b) and chlorophyll content than the WT under both nitrogen concentrations (Fig. 6c). In addition, the root phenotype of the *MdCEPR1* transgenic lines was analyzed under the 0.1 mM NO_3^- condition. As shown in Figs. S4 and S5, the *MdCEPR1* transgenic lines had more root biomass than the WT.

Ectopic expression of MdCEPR1 increases nitrate uptake and utilization in *Arabidopsis thaliana*

Nitrate content and NRA were examined to further analyze the function of *MdCEPR1* in nitrate absorption by *Arabidopsis*. The results show that the *MdCEPR1* transgenic plants exhibited a much higher nitrate content and NRA under the two nitrogen concentrations (Fig. 7a, b). Then, 10-day-old transgenic *Arabidopsis* seedlings were treated with different concentrations of $^{15}NO_3^-$ (0.1 mM and 10 mM), and $^{15}NO_3^-$ content was determined. The results show that *MdCEPR1*-overexpressing plants exhibited much higher nitrate uptake activity at both the 0.1 mM and 10 mM $^{15}NO_3^-$ external concentrations, and the effect was more pronounced at the lower concentration (Fig. 7c). The expression levels of the genes involved in nitrate uptake and assimilation in WT and transgenic *Arabidopsis* plants were analyzed by qRT-PCR. The results show that the transcript levels of *AtNRT1.1*, *AtNRT2.1*, and *AtNIA1* increased remarkably in the transgenic lines (Fig. 7d–f). These results further confirm the function of *MdCEPR1* in promoting nitrate uptake and assimilation.

Ectopic expression of MdCEPR1 increases lateral root density under different nitrate concentrations

Previous studies have demonstrated that root development is regulated by nitrate availability (Zhang and Forde 2000), and that *AtCEPR1* plays an important role in lateral root initiation (Roberts et al. 2016). Therefore, we analyzed the root phenotype of *MdCEPR1* transgenic *Arabidopsis* seedlings under different concentrations of nitrate medium (0.1 and 10 mM) to examine the function of *MdCEPR1* in root development (Fig. 8a). A detailed analysis of the root showed that overexpressing *MdCEPR1* significantly increased the number of

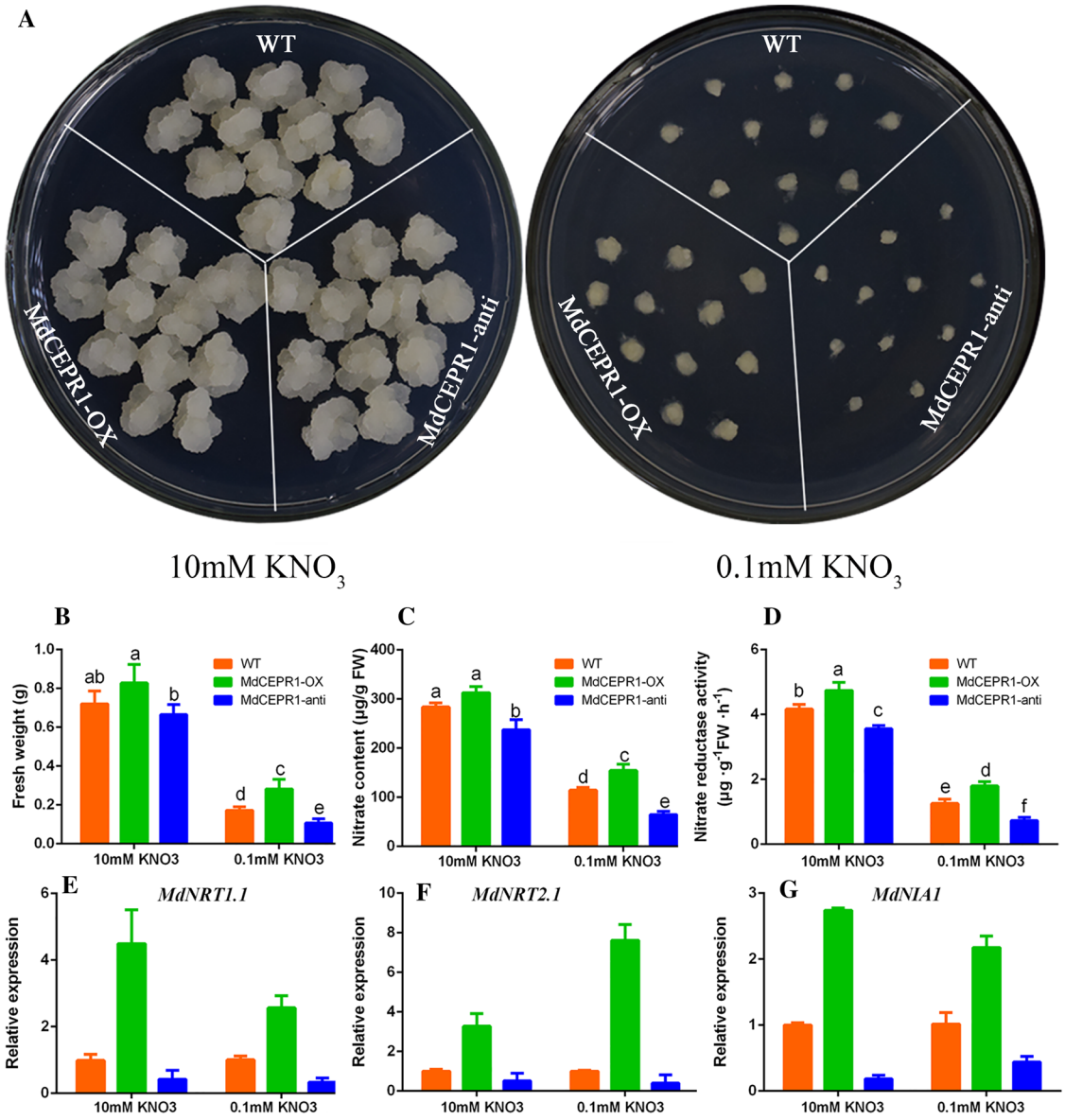

Fig. 5 MdCEPR1promotes growth of apple calli under a nitrogen-deficient condition by increasing nitrate assimilation and upregulating the expression of genes involved in nitrate assimilation in apple (**a**) Wild type (WT) and transgenic calli (MdCEPR1-OX and MdCEPR1-anti) were grown in a medium containing different concentrations of nitrate (10 and 0.1 mM) for 10 days. **b** Fresh weight, **c** nitrate content, **d** nitrate reductase activity, and relative expression levels of (**e**) MdNRT1.1, **f** MdNRT2.1 and **g** MdNIA1 of WT and transgenic calli (MdCEPR1-OX and MdCEPR1-anti). Values are mean ± SD of three replicate experiments.

lateral roots on *Arabidopsis* seedlings, but no obvious difference in the length of the primary root was detected when the seedlings were grown in 10 mM nitrate medium. In addition, the *MdCEPR1* transgenic lines had a significantly longer primary root than the WT on 0.1 mM nitrate medium, verifying the function of *MdCEPR1* to enhance tolerance to a nitrogen deficiency.

Discussion

MdCEPR1 may play an important role in the adaptation to nitrogen-limiting conditions

Nitrogen is one of the most basic elements of organisms and has an irreplaceable role in fruit metabolism, morphogenesis, biochemical processes, stage development, and

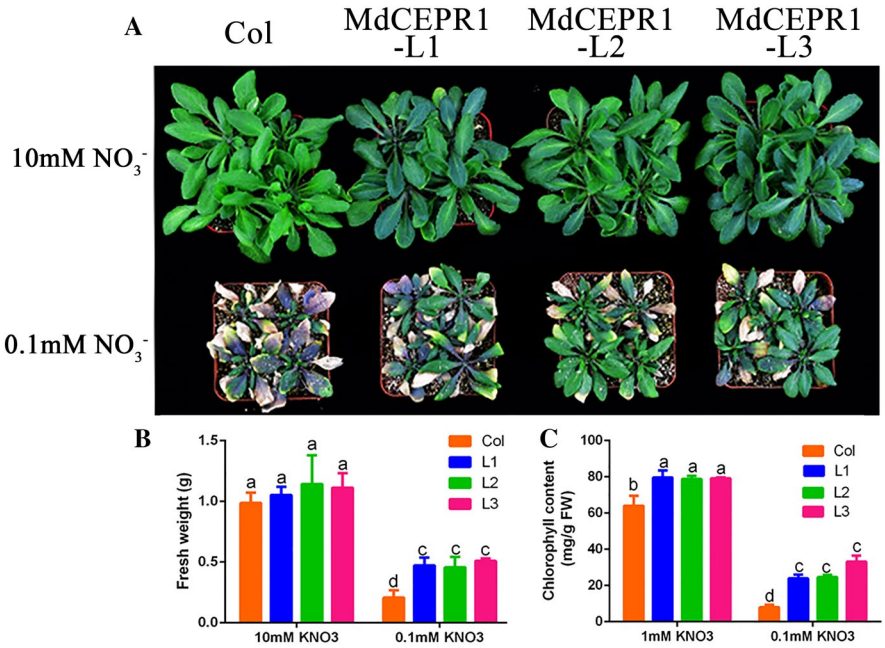

Fig. 6 Ectopically expressedMdCEPR1enhances tolerance to nitrogen deficiency in *Arabidopsis thaliana*. **a** Phenotypes of 4 weeks old MdCEPR1 transgenic *Arabidopsis* seedlings (L1, L2, and L3) and the wild-type (Col-0) growing in vermiculite and watered with a nutrient solution containing 10 mM NO^{3-} and 0.1 mM NO^{3-}, respectively. **b** Fresh weight and **c** chlorophyll contents of the wild type (Col) and transgenic *Arabidopsis* (L1, L2 and L3). Values are mean ± SD of three replicate experiments.

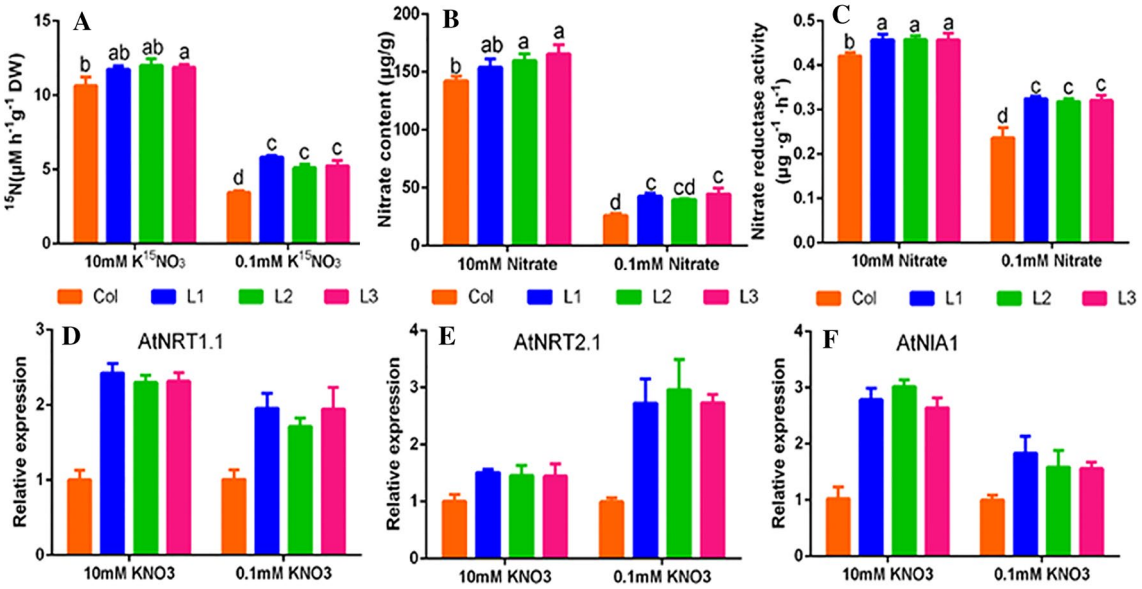

Fig. 7 Ectopically expressedMdCEPR1increases nitrate uptake and utilization in *Arabidopsis thaliana*. **a** The 15N concentration, **b** nitrate content, **c** nitrate reductase activity, and relative expression levels of **d** AtNRT1.1, **e** AtNRT2.1, and **f** AtNIA1 of wild type (Col) and MdCEPR1 transgenic *Arabidopsis* (L1, L2, and L3). Values are mean ± SD of three replicate experiments.

fruit yield and quality (Miner et al. 1997; Sánchez et al. 2002). However, plants are constantly challenged by different nutrient conditions, and the uneven distribution of nitrogen in the soil always affects plant growth (Tabatabaie et al. 2004). Therefore, plants need to carry out long-distance transduction of nitrogen-sensing signals and promote nitrogen absorption in nitrogen-rich areas. Plant RLKs play an important role sensing environmental changes at the cell surface. Environmental signals are transduced by RLKs via activated signaling pathways to trigger adaptive responses (Kim et al. 2009). Recent studies have provided evidence that HYPERNODULATION ABERRANT ROOT FORMATION1 (HAR1) is involved in the autoregulation and nitrate inhibition of nodulation (Okamoto and Kawaguchi 2015). The CLE-CLAVATA signaling pathway plays key roles in the regulation of systemic auto-regulation of nodulation integrated with nitrogen signaling mechanisms and lateral root development (Araya et al. 2014). CEPR1 is another LRR-RK

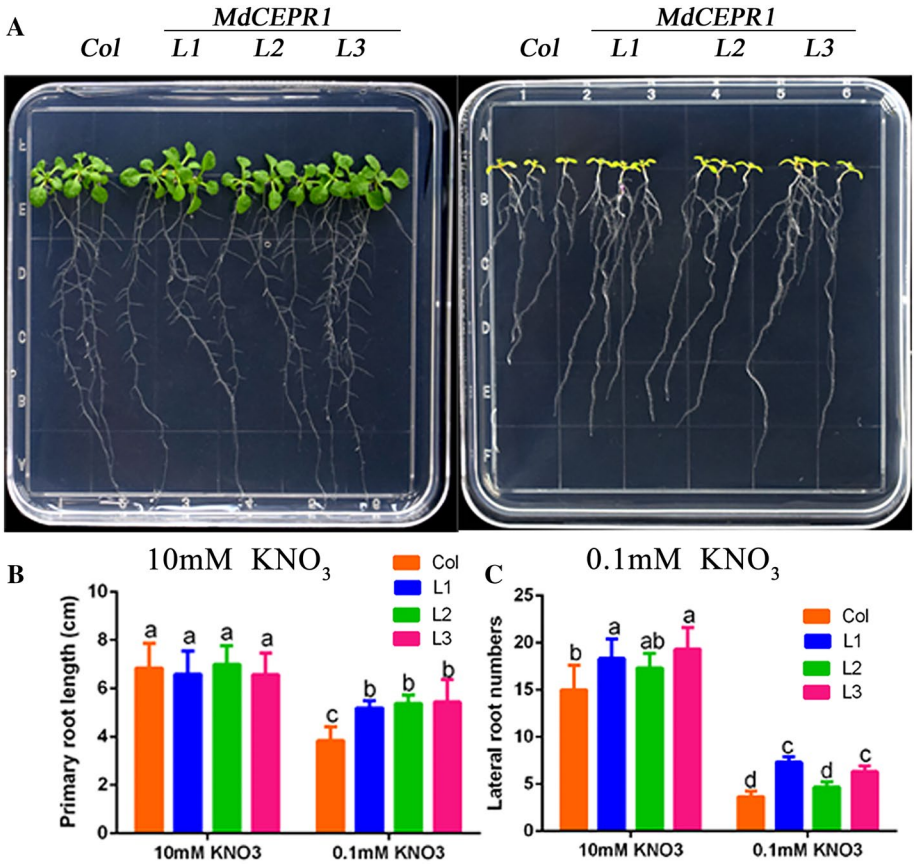

Fig. 8 Ectopically expressed-MdCEPR1increases lateral root density under different nitrate concentrations (10 and 0.1 mM). **a** The root-growth phenotypes, **b** primary root (PR) length, and **c** lateral root (LR) number of wild type (Col) and MdCEPR1 transgenic *Arabidopsis* seedlings (L1, L2, and L3) growing in a medium with different concentrations of nitrate (10 and 0.1 mM). Values are mean ± SD of three replicate experiments.

that affects N uptake and assimilation in *Arabidopsis* via cell-to-cell communication (Tabata et al. 2014).

Here, we cloned *MdCEPR1*, the apple homolog of *AtCEPR1*. The protein sequence alignment analysis revealed that MdCEPR1 had five LRR regions and two conserved regions, including a transmembrane region and a serine/threonine protein kinase catalytic domain. The hydrophobic LRR domain forms a helix that easily interacts with the ligand (Bell et al. 2005). After interacting with the ligand, the signal is transferred from the extracellular to the intracellular space via the intracellular kinase domain, so the MdCEPR1 protein is used as a signaling receptor to transfer the signal from the extracellular to the intracellular space. In this study, *MdCEPR1* was expressed in all tissues, including the roots, stems, leaves, flowers, and fruit. A particularly high *MdCEPR1* transcript level was observed in the roots and leaves (Fig. 2a). This result, as explained by (Bryan et al. 2012), indicates the *MdCEPR1* may play a role in the vasculature of all organs. Moreover, the MdCEPR1-anti calli exhibited much less growth than the control and MdCEPR1-OX under nitrogen-deficient conditions, and had lower nitrate content and NRA compared with the WT. The qRT-PCR results show that the genes related to nitrate uptake, transport and assimilation in *MdCEPR1*-overexpressing (MdCEPR1-OX) calli were significantly upregulated compared with the WT. These results suggest that *MdCEPR1* may play an important role in adaptation to nitrogen-limiting conditions.

Nitrate content was not the only factor affecting anthocyanin accumulation in MdCEPR1

Anthocyanin is an important plant pigment that accumulates in the vacuoles of various cells and tissues. Evidence suggests that low temperature and UV wavelengths induce anthocyanin synthesis (Bryan et al. 2012). Deficiencies in phosphorus or nitrogen can cause anthocyanin to increase (Henry et al. 2012; Ji et al. 2015). In this study, the MdCEPR1-anti calli showed the same phenotype of anthocyanin accumulation, and the anthocyanin-related genes in MdCEPR1-anti were significantly upregulated. Therefore, the accumulation of anthocyanin from MdCEPR1-anti calli may be caused by a nitrogen deficiency. Interestingly, some LRR-RLK mutants also affect anthocyanin accumulation. For example, the sucrose stress-induced anthocyanin accumulation phenotype is inhibited in *A. thaliana flg22* or *elf18* (Saijo 2010). *PXY* mutants exhibit a red cotyledon (Fiume and Fletcher 2012). It can be speculated that the effect of *MdCEPR1* on the accumulation of anthocyanin may be present in other pathways.

MdCEPR1 affects plant root development as a CEP receptor

Plants absorb soil moisture and nutrients through continuous production of lateral roots. Previous research has shown that lateral root development and nutrient uptake are controlled by local and systemic responses in plants (Michael et al. 2016; Mounier et al. 2013; Ruffel and Coruzzi 2011). The CEP family is a class of long-distance signaling molecules that participates in signal transmission triggered by N-starvation from roots to shoots in *Arabidopsis* (Tabata et al. 2014). *AtCEP1* has been reported to negatively regulate plant root growth (Ohyama et al. 2008). Recent research has shown that *CEP* genes are induced by limited nitrogen, and CEP family peptides act on the downstream receptor kinase CEP Receptor 1 (CEPR1), which induces upregulation of nitrate transporter genes to compensate for local nitrogen starvation (Tabata et al. 2014). Roberts et al. 2016 showed that CEP5 participates in lateral root initiation locally through XIP1/CEPR1. The present study showed that *MdCEPR1* may also affect root development. However, the *MdCEP* gene in apple has not been reported. Further experiments are needed to demonstrate that *MdCEPR1* is also affected by *MdCEP* during root development.

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Author contributions Y-JH, X-FW and RL designed the research. RL, J-PA, and C-XY performed the experiments and analyzed the data. Y-JH, RL and X-FW wrote the manuscript text.

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