A peptide encoding gene *MdCLE8* regulates lateral root development in apple

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**Abstract**

Nitrogen is not only an essential nutrient for plant, but also an important signal molecule to integrate and regulate gene expression, metabolism and growth. Plant peptides are considered as a new type of hormone, and play an important regulatory role in plant growth and development. However, little is known about the co-regulation network between nitrogen and peptide hormones in plant. Here we identified an apple *MdCLE8* gene, which encodes a putative peptide, induced by nitrogen deficiency in apple. Ectopic expression of *MdCLE8* inhibited lateral root formation in *Arabidopsis* under nitrogen deficiency. Similarly, overexpression of *MdCLE8* inhibited lateral root development in apple adventitious roots, and this inhibition was amplified under nitrogen deficiency treatment. Further studies showed that *MdCLE8* may inhibit the expression of several key genes during lateral root emergence stage in *Arabidopsis*, thereby inhibiting the emergence of lateral root from root cortex cells. Collectively, our study not only broadened the gene regulatory network under the influence of nitrogen in apple, but also expanded the function of CLE peptide hormones in apple.

**Key message**

The apple peptide encoding gene *MdCLE8* is induced by nitrogen deficiency signaling and negatively regulates lateral root formation in plants.

**Keywords** Apple · CLE peptide · Nitrogen · Lateral root development

**Introduction**

Nitrogen (N) is not only one of the most important nutrients for plants, but also a signal substance that affects plant growth and development (Vidal et al. 2010, 2013). Root system architecture (RSA) plays an important role in nutrient and water absorption, and exhibits considerable developmental plasticity to environmental changes (Malamy 2005; Osmont et al. 2007). Lateral root (LR) accounts for most of the total root system and displays higher plasticity to external nutrient utilization than primary root (Giehl et al. 2014). In *Arabidopsis*, nitrogen is an important environmental factor affecting lateral root development: local nitrate treatment can not only stimulate the initiation of lateral root (Vidal et al. 2013), but also significantly promote lateral root elongation (Zhang and Forde 1998; Remans et al. 2006a). Mild nitrogen deficiency can promote the growth of lateral root, while prolonged nitrogen deficiency or excessive nitrogen supply is not conducive to lateral root growth (Forde and Lorenzo 2001; Remans et al. 2006b; Ruffel et al. 2011; Gruuber et al. 2013).

The postembryonic and multistage nature of lateral root development makes it subject to more complex gene network regulation: in *Arabidopsis*, lateral roots originate from founder cells formed from xylem pole pericycle cells, and then undergo cell division and expansion to develop lateral root primordium (LRP) (Malamy and Benfey 1997; Casimiro et al. 2001; Dubrovsky et al. 2001). Lateral root primordium...
must pass through three cell layers: endodermis (EN), cortical layer (CO), and epidermal layer (EP) successively before it can appear on the surface of primary root (Péret et al. 2009a, b; Vilches-Barro and Maizel 2015). In this process, the cell layer provides the greatest resistance, which may affect the number of lateral roots. To reduce this resistance, cells covering the lateral root primordium undergo cell wall remodeling (CWR) to promote cell separation and thus expand the growth pathway of lateral root primordium, a process that requires proper spatiotemporal expression of cell wall remodeling genes (Lewis et al. 2013). CWR genes are expressed not only in the floral abscission zones, but also in regions where cell separation occurs, including dehiscence zones of the siliques and the cells overlaying emerging lateral root primordium (González-Carranza et al. 2007; Cai and Lashbrook 2008; Ogawa et al. 2009; Lewis et al. 2013). During lateral root emergence, auxin induces the expression of LAX3 in cortical cells directly covering lateral root primordium, which is also where some CWR genes are specifically expressed. LAX3 encodes a high affinity auxin influx carrier and functions in lateral root emergence by targeting the auxin-inducible expression of CWR genes (Fig S1) (Swarup et al. 2008; Kumpf et al. 2013). In addition, peptide signaling also plays a key role in lateral root emergence by regulating the expression of CWR genes. INFLORESCENCE DEFICIENT IN ABScession (IDA) encodes a small protein ligand that co-regulates floral organ abscission with its receptor HAE/HSL2 (Sung et al. 2008; Santiago et al. 2016). Moreover, IDA also has an overlapping expression region with CWR genes in the root (Kumpf et al. 2013). IDA-HAE/HLS2 module and its downstream MPK4/MKK5–MPK3/MPK6 module jointly regulate the expression of CWR genes and thus play a key role in the lateral root emergence process (Fig S1) (Kumpf et al. 2013; Zhu et al. 2019).

Peptides are typically processed from a small protein precursor, of which hundreds are expected in Arabidopsis (Hanada et al. 2007, 2013; Murphy et al. 2012). Recent studies have shown that peptides play an important role in plant growth and development (Czyzewicz et al. 2013; Fletcher 2020). The CLAVATA3/EMBRYO SURROUNDING REGION-related (CLE) gene encodes a large peptide family (Goed et al. 2017). Among 32 CLE genes reported in Arabidopsis (Cock and McCormick 2001), CLV3 and CLE40 are the two best characterized members, which play key roles in stem cell population maintenance in shoot apical meristem and root apical meristem, respectively (Fletcher 1999; Hobe et al. 2003). The CLE gene is also widely present in other species (Goed et al. 2017). We previously identified 25 MdCLE genes in the apple genome, and they have very similar structural characteristics to CLE genes reported in other species (Zhang et al. 2021), but their functions in apple are unknown.

CLE peptide hormones play an important role in regulating plant growth and development (Fletcher 2020), but the regulation of apple root architecture has not been reported. Here, we identified and analyzed the function of the MdCLE8 gene, which is adjacent to CLE1/3/4/7 of Arabidopsis in the evolutionary tree and encodes highly similar CLE motif (Fig. S2 and S3). Our results suggest that MdCLE8 regulates the nitrogen response and affects apple root system architecture. The N-responsive MdCLE8 peptide is an important signal element required for translating an N-deficient nutritional status into effect lateral root formation in apple.

Materials and methods

Plant materials, growth conditions and treatments

Tissue cultured ‘M26’ apple plants were subcultured on Murashige and Skoog (MS) medium (MS + 3% sucrose + 0.5 mg/L 6-BA + 0.2 mg/L NAA + 0.2 mg/L GA, pH5.8) under a long-day conditions (16 h light/8 h dark) at 24 °C for 30 d.

The Columbia ecotype (Col-0) Arabidopsis seeds were cultured on 1/2 MS medium for 4 days, then transferred to new 1/2 MS medium or N-deficient 1/2 MS medium (Nitrogen concentration was 0.1 mM, KNO3 was replaced by KCl and nitrogen was supplemented by NH4NO3).

To determine the expression levels of MdCLE genes in response to different nitrogen concentrations, 1-month-old ‘Pingyitiancha’ (Malus×hupehensis) apple seedlings were pretreated with 1/2 medium for 3 days, then treated with 0 mM, 0.1 mM, 1 mM and 10 mM nitrogen medium (KNO3 was replaced by KCl and nitrogen was supplemented by NH4NO3) for the indicated times, respectively. The seedlings were grown at 24 °C with a 16 h light/8 h dark photoperiod (The light intensity is 100–150 µmol m−2 s−1).

Vector construction and plant transformation

The open reading frame (ORF) of MdCLE8 was fused to pRI-GFP to generate 35S::MdCLE8-GFP. Then 35S::MdCLE8-GFP and empty expression vector were introduced into Agrobacterium rhizogenes strain K599 and transformed into ‘M26’ apple stem segments to induce hairy roots (Zhou et al. 2019), and the empty expression vector (CK) was used as a control. GFP fluorescence of transgenic adventitious roots was identified by fluorescence microscopy.

35S::MdCLE8-GFP vector was transformed into Arabidopsis by the Agrobacterium-mediated floral dip method (Clough and Bent 1998).
Gene expression analysis

Total RNA was extracted from apple seedlings, ‘M26’ transgenic adventitious roots, and Arabidopsis using RNA Plant Plus reagent (Tiangen, Beijing, China) following the instructions, and reverse transcription assay was performed by using the PrimeScript cDNA Synthesis Kit (Takara, Liaoning, China). RT-qPCR was performed with the UltraSYBR mixture (Takara, Liaoning, China) by an ABI7500 RT-PCR system (An et al. 2020). Primers used for RT-qPCR are listed in supplementary Table S1, 18S rRNA and MdAc-tin (GenBank accession number CN938024) or AtActin were used as internal control genes.

GUS staining

After germinating and growing for 4 days on 1/2 MS medium, DR5-GUS and DR5-GUS/MdCLE8-OE transgenic Arabidopsis seeds were transferred to N-deficient 1/2 MS medium for 3 days. 7-day-old transgenic Arabidopsis seedlings were stained using a GUS solution, then the total number of lateral root primordium was counted under the microscope.

Determination of pectinase and pectinase activities

Polygalacturonase and pectinase activity detection kits were purchased from Comin Biotechnology (Suzhou, China).

Root measurement

For Arabidopsis, the primary root length and the lateral root number were analyzed by Digimizer software. For apple seedlings, the phenotypes of apple transgenic adventitious roots were scanned with a root scanner (Perfection V850 Pro Photo, Epson), and the lateral root length and number were measured with Digimizer software.

Statistical analysis

SPSS v17.0 software was used for statistical analysis. Statistical analysis was performed using a Student’s t test, where ns P > 0.05, *P < 0.05 and **P < 0.01. Different letters indicate significant difference (P < 0.01) as obtained by one-way ANOVA test.

Accession numbers

MdCLE1 (MDP0000312687), MdCLE3 (MDP0000223926), MdCLE4 (MDP0000825799), MdCLE5 (MDP0000226320), MdCLE6 (MDP0000262449), MdCLE8 (MDP0000240955), MdCLE10 (MDP0000149789), MdCLE23 (MDP0000119279), MdLAX-like1 (MDP0000885425), MdLAX-like2 (MDP000020317), MdLAX-like3 (MDP0000155113), MdLAX-like4 (MDP000089124), MdLAX-like5 (MDP0000080407), LAX3 (AT1G77690), PGLR (AT5G14650), PGAZAT (AT2G41850), XTR6/XTH23 (AT4G25810), EXP17 (AT4G01630). The MdXTH gene family information as described by Atkinson et al. (Atkinson et al. 2009). All primers used are listed in Table S1.

Result

MdCLE8 is a nitrogen-responsive gene that encodes a putative peptide in apple

Nitrogen is one of the essential elements for plant growth and development. In order to investigate the relationship between nitrogen and MdCLE peptide hormones in apple, we quantitatively detected the expression levels of MdCLE genes in subfamily III under different nitrogen concentrations (Fig. S2). The results showed that compared with the other MdCLE family members, the response of MdCLE8 gene expression was most significantly under low nitrogen treatment (Fig. 1), suggesting that MdCLE8 might be a functional gene under the influence of nitrogen in apple.

To investigate further, we cloned the MdCLE8 gene. By sequence alignment, we found that MdCLE8 protein had a conserved CLE motif at the C terminal similar to that of AtCLE1/3/4/7 (Fig S3). Our previous study showed that the external application of MdCLE8 synthetic peptide (MdCLE8p) had no significant effect on the normal growth of Arabidopsis, showing similar activity to AtCLE3p (Zhang et al. 2021).

Ectopic expression of MdCLE8 inhibits lateral root formation in Arabidopsis under nitrogen deficiency

To investigate the function of MdCLE8 gene, we constructed MdCLE8 overexpression vector, and transformed it into wild type Arabidopsis (MdCLE8-OE1, MdCLE8-OE2, and MdCLE8-OE3). Subsequently, we treated transgenic Arabidopsis lines with 1/2 MS and N-deficiency 1/2 MS medium, and used the wild type (WT) as a control. The results showed that there was no difference between WT and MdCLE8-OE transgenic Arabidopsis growing on 1/2 MS medium, the primary root length, lateral root number and density (Fig. 2A, C and D). In contrast, the growth of MdCLE8-OE transgenic Arabidopsis was significantly inhibited in N-deficient 1/2 MS medium. Compared with the WT, although the primary root length of MdCLE8-OE transgenic Arabidopsis was only slightly reduced, the lateral root number and density were significantly reduced (Fig. 2B,
Fig. 1 Expression patterns analysis of MdCLE genes under different nitrogen concentrations (0 mM, 0.1 mM, 1 mM, 10 mM). The significance difference was analyzed by SPSS v17.0 software. Different letters indicate significant differences (P < 0.01) as obtained by one-way ANOVA test. Data are shown as means ± SE.

Fig. 2 Ectopic expression of MdCLE8 inhibits Arabidopsis lateral root development. A, B The phenotypes of wild type (WT) and MdCLE8 overexpressed Arabidopsis under 1/2 MS and N-deficiency 1/2 MS conditions, respectively. Bar = 10 mm. C–E Primary root length, lateral root number and density of Arabidopsis in (A) and (B). Results shown are means ± SE, based on three independent biological replicates. Statistical significance was determined using Student’s t-test: **P < 0.01
C and D). The above results revealed that overexpression of *MdCLE8* inhibited lateral root growth and development under nitrogen deficiency condition.

**MdCLE8 inhibits lateral root formation in apple adventitious roots under nitrogen deficiency**

In order to explore the effect of *MdCLE8* on apple root growth, 35S:: *MdCLE8*-GFP vector was constructed, and compound apple plants composed of *MdCLE8* transgenic roots and wild-type shoots were obtained by *Agrobacterium rhizogenes*-mediated transgenic technology (Fig S4).

Our results showed that prolonged nitrogen deficiency increased the root length of the empty expression vector transgenic adventitious, but significantly decreased the lateral root tips number and density (Fig. 3A, C, E–G). This finding indicated that prolonged nitrogen deficiency would seriously affect the growth and development of apple lateral root. Under the 1/2 MS condition, the total lateral root tips number and density of 35S::*MdCLE8*-GFP transgenic adventitious roots were significantly decreased (Fig. 3A, B, E–G). Moreover, nitrogen deficiency intensified the effect of 35S::*MdCLE8*-GFP on the growth of apple adventitious roots (Fig. 3C–G). The root length of *MdCLE8*-OE transgenic adventitious roots decreased significantly, the lateral root number decreased from 45.64 to 79.58%, and the lateral root density decreased from 44.37 to 68.71%. Taken together, our results indicate that *MdCLE8* inhibited lateral root formation in apple, and this inhibition was enhanced by nitrogen deficiency treatment.

**MdCLE8 is involved in the spatiotemporal regulation of key genes during lateral root emergence**

The above experimental results revealed that the overexpression of *MdCLE8* gene inhibited lateral root formation in *Arabidopsis* under nitrogen deficiency. An unsolved question is what is the underlying mechanism? To answer this question, we obtained a hybrid *Arabidopsis* material of DR5-GUS and *MdCLE8*-OE (DR5-GUS/MdCLE8-OE). The development of lateral root primordia of DR5-GUS/MdCLE8-OE was observed by GUS staining (Fig. S5A), and DR5-GUS was used as control. The results showed that the total lateral root primordia number and density of DR5-GUS/MdCLE8-OE was not significantly different from control (Fig. SSB-D), suggesting that *MdCLE8* does not affect lateral root primordium formation in *Arabidopsis*.

We hypothesized that the inhibition of *MdCLE8*-OE on lateral root development was caused by affecting lateral root emergence stage. Therefore, we examined the expression levels of key genes in *Arabidopsis* that affect the lateral root emergence, including auxin influx vector gene *LAX3* (Swarup et al. 2008), cell wall remodeling gene *PGAZAT, PGLR, XTR6 and EXP17* (Laskowski et al. 2006; González-Carranza et al. 2007; Swarup et al. 2008; Kumpf et al. 2013). As hypothesized, the results showed that overexpression of *MdCLE8* inhibited the expression of *LAX3, PGLAZAT, PGLR* and *XTR6* genes in *Arabidopsis* under nitrogen deficiency conditions (Fig. 4A). At the same time, we detected the expression levels of *MdLAX3*-like and *MdXTH* genes, as well as polygalacturonase (PG) and pectinase activity in apple. The results showed that overexpression of *MdCLE8* inhibited the expression of some *MdLAX*-like and *MdXTH* genes (Fig. 4B, C), and decreased the activities of PG and pectinase (Fig. 4D, E), and this inhibition was particularly obvious under nitrogen deficiency conditions. In conclusion, *MdCLE8* may inhibit the formation of lateral root by affecting lateral root emergence stage.

**Discussion**

Root system is an important organ for plants to obtain water and nutrients from the soil. The growth and development of the aboveground parts of plants as well as the yield and quality of fruits are highly dependent on the underground parts (Takatsuka and Umeda 2014). Studies in a variety of plants have shown that the root system architecture of plants is highly plastic under the influence of the environment (Malamy 2005; Osmont et al. 2007). However, the genetic transformation of apple and many plants is difficult and takes a long time, which greatly hindrance the research on their root growth and development. In recent years, the technique of obtaining transgenic adventitious roots by *Agrobacterium rhizogenes* has been established and applied to many plants (Shajahan et al. 2017; Xue et al. 2017; Meng et al. 2019; Zhou et al. 2019). It provides a rapid and simple molecular biological tool for studying plants root development.

Plant peptides are considered as a new type of plant hormones, which play critical regulatory roles in many aspects of plant growth and development (Motomitsu et al. 2015; Gancheva et al. 2019). In our previous study, we identified 25 *MdCLE* genes in apple, and by applying their presumed synthetic peptides (MdCLEp) to *Arabidopsis*, we found that most of the MdCLEp showed strong effects on root growth and development (Zhang et al. 2021). In fact, CLE genes and their synthetic peptides found in other plant species also showed inhibitory effects on plant root growth (Kinoshiba et al. 2007; Whitford et al. 2008; Tian et al. 2019), suggesting that CLE peptide plays an important role in the regulation of root system architecture. Here, we found a low-nitrogen induced expression of *MdCLE8* gene in apple, which encodes a putative plant peptide and plays an important regulatory role in the growth and development of apple lateral roots.
Our previous research has shown that 1 μM MdCLE8p had no significant effect on the root system of Arabidopsis cultured in 1/2 MS medium (Zhang et al. 2021). Similarly, in this study, we found that the ectopic overexpression of MdCLE8 did not affect the growth of Arabidopsis in 1/2 MS medium, but significantly inhibited lateral root formation in N-deficiency 1/2 MS medium (Fig. 2). Furthermore, we also overexpressed the MdCLE8 gene in apple adventitious roots.

We found that MdCLE8 gene also inhibited apple lateral roots formation, and this inhibition was further enhanced by nitrogen deficiency treatment (Fig. 3), suggesting that MdCLE8 gene and nitrogen deficiency signal co-regulate the development of apple lateral roots.

Lateral root formation in plants is a complex physiological process. To put it simply, lateral root primordia are first produced from xylem pole pericycle cells, and then further...
developed and grow until they successfully drill out of the parent root epidermis to produce lateral roots (Malamy and Benfey 1997; Casimiro et al. 2001; Dubrovsky et al. 2001). The growth of the lateral root primordium needs to pass through three cell layers, and the substances in the cell layer such as pectin provide resistance to this process (Lewis et al. 2013). Our results further showed that the overexpression of MdCLE8 strongly inhibited the expression of cell wall remodeling genes (Fig. 4), thus impeding the normal separation of the cell layer. Therefore, we suggest that the influence of MdCLE8 on lateral root formation may be due to the growth and emergence

Fig. 4 MdCLE8 affected the expression of related genes during lateral root emergence. A Expression of key genes during lateral root emergence of DR5-GUS and DR5-GUS/MdCLE8-OE transgenic Arabidopsis. B, C Quantitative analysis of MdLAX-like and MdXTH in transgenic adventitious roots of empty vector and 35S::MdCLE8-GFP. D, E Detection of polygalacturonase and pectinase activities in transgenic adventitious roots of empty vector and 35S::MdCLE8-GFP.
of lateral root primordium rather than the origin of lateral root primordium.

Previous studies have shown that the CLE-CLV1 signaling pathway is a core module that regulates the expansion of the lateral root system of Arabidopsis under low nitrogen conditions (Araya et al., 2014a, b). The MdCLE8 protein in apple has a highly similar CLE motif to that of AtCLE1/3/4/7 in Arabidopsis (Fig S3), and MdCLE8 and AtCLE3 have similar regulatory functions for plant lateral root formation (Fig. 2), suggesting that CLE genes with similar CLE motif in different species are functionally conserved. However, more detailed anatomical analysis of apple root system, and the receptors that CLE peptides act on remain to be studied. Therefore, the specific mechanism by which MdCLE8 regulates lateral root development in apple still needs to be further analyzed.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11240-021-02182-4.

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Authors contribution C-XY, QZ, and T-EZ conceived and designed the experiments. C-XY and QZ supervised the experiments. T-EZ, YS and X-ML performed the experiments. All the authors read and approved the final manuscript.

Data availability All data generated or analysed during this study are included in this published article [and its supplementary information files].

Declarations Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors have no conflict of interest to declare.

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