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Molecular cloning and characterization of the chloride channel gene family in trifoliate orange

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Abstract

Chloride channels (CLCs) play pivotal roles in plant development and anion transport. However, little research has been conducted about the CLC in fruit-bearing plants. Here we provide an insight into the evolution and expression patterns of *CLC* gene family members in various tissues of trifoliate orange [*Poncirus trifoliata* (L.) Raf.] and their responses to several treatments. Genome-wide analysis identified six *PtrCLC* genes. The predicted proteins had similar numbers of amino acids, but shared a low sequence identity. Phylogenetic analysis revealed that PtrCLC were classified into two separate subgroups, and PtrCLC4 and PtrCLC6 in subgroup II were more closely related to bacterial CLCs. Sequence comparison with EcCLCA from *Escherichia coli* reveals that PtrCLC showed amino acid divergence in anion selectivity of CLC proteins. Real time qPCR analysis shows that *PtrCLC* genes, particularly *PtrCLC6*, preferentially expressed in leaves. Nitrogen deficiency irreversibly inhibited expression of *PtrCLC* genes except for *PtrCLC1*. In contrast, NaCl stress profoundly induced expression of *PtrCLC* genes, particularly *PtrCLC2* and *PtrCLC4*, both of which were also upregulated by ABA treatment. The results presented here provide a solid foundation for a future functional research on citrus *CLC* genes.

Additional key words: abscisic acid, gene expression, nitrogen supply, *Poncirus trifoliata*, salt stress, sequence analysis.

Introduction

Chloride channels (CLCs) are ubiquitously expressed from bacteria to animals and make significant contributions to various biological processes. Studies on prokaryotic CLCs provided a substantial progress in the understanding of the CLC family (Matulef and Maduke 2007). In higher plants, CLC proteins also appear as key players in regulation of cell pressure potential, stomatal movement, generation of action potentials, nutrient transport, and metal tolerance (Zifarelli and Pusch 2010). The first plant CLC genes have been cloned independently in tobacco and *Arabidopsis* (Hechenberger *et al*. 1996, Lurin *et al*. 1996). Since then, many plant CLCs have been isolated from rice, soybean, and other plant species (Diedhiou and Golldack 2005, Li *et al*. 2006, Zhou and Qiu 2010). Among these plant CLCs, *Arabidopsis* CLCs are the most thoroughly investigated proteins. Seven *CLC* genes exist in *Arabidopsis* genome, named *AtCLCa-g*. The AtCLC proteins are targeted to various membranes including the vacuolar membrane (AtCLCa, AtCLCb, AtCLCc, and AtCLCg), Golgi vesicles (AtCLCd and AtCLCf), or chloroplast membrane (AtCLCe). Physiological characterization of *Arabidopsis* mutants suggests the involvement of AtCLCa and AtCLCb in controlling NO_3^- exchange across the tonoplast (Geelen *et al*. 2000, Von der Fecht-Bartenbach *et al*. 2010). Application of patch-clamp technique to isolated vacuoles has revealed that AtCLCa is able to accumulate nitrate in the vacuole and functions as a $2NO_3^-/1H^+$ antiporter (De Angeli *et al*. 2006). This provides a direct evidence that AtCLCa plays a major role in NO_3^- homeostasis, probably partly in cooperation with AtCLCe which is postulated to be an NO_3^- channel/ transporter on thylakoid membranes (Monachello *et al*. 2009). The AtCLCc is also suggested as another major

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Abbreviations: CBS - cystathionine β-synthase; CLC - chloride channel; PtrCLC - CLC of *Poncirus trifoliata*; RT-qPCR - real time quantitative PCR; TM - trans-membrane regions.

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component for NO_3^- accumulation. Harada *et al*. (2004) has reported that *clcc-1* mutant plants show a reduced NO_3^- content in shoots as compared with wild-type shoots. However, in contrast with AtCLCa mutants, the plants have also an altered Cl^- content indicating a broader anion specificity of AtCLCc compared with AtCLCa. Indeed, AtCLCc were recently reported to be related to the movement of Cl^- across the tonoplast and hence contributes to opening stomata or salt resistance of plants (Wege *et al*. 2010). The localization of AtCLCd is coincident with that of V-type ATPase VHA1a in agreement with a functional link between these two proteins (Von der Fecht-Bartenbach *et al*. 2007). The expression of the plant AtCLCd and AtCLCf proteins, both residing in Golgi vesicles, complements the *gef1* yeast mutant phenotype (Hechenberger *et al*. 1996, Gaxiola *et al*. 1998, Marmagne *et al*. 2007) suggesting a functional similarity with *gef1*. The AtCLCg has been suggested to have vacuole localization, but its functional properties are still unknown.

Although *CLC* genes in *Arabidopsis* have been studied extensively, a comprehensive analysis of the CLC gene family in other plant species has not been conducted. Citrus is a major world horticultural commodity and it is sensitive to NaCl stress, particularly to Cl^- toxicity. We previously reported that a citrus *CLC* homologue (*CsCLCc*) can alleviate NaCl stress in *CsCLCc*-over-expressing *Arabidopsis* by controlling Cl^- accumulation (Wei *et al*. 2013). This impels us to gain more knowledge about the CLC genes in citrus. Recently, the genome sequence of citrus has been published (Xu *et al*. 2013) and provided an excellent opportunity for systematic study of citrus *CLCs*. In the present study, we identified *CLC* genes in the genome of trifoliate orange which is widely used as rootstock in citrus-producing regions. Then we report on comprehensive sequence analysis and phylogenetic construction. Organ expression profiles and gene expression patterns under abscisic acid or stress treatments were also examined using real-time quantitative PCR (RT-qPCR) method.

Materials and methods

Plant growth and treatments: The seeds of *Poncirus trifoliate* (L.) Raf were surface-sterilized using 5 % (m/v) NaClO and germinated at a temperature of 30 ºC and subsequently transferred to black barrels filled with *Vermiculite* moistened with a half-strength Hoagland′s solution. Two-month-old seedlings with fully expanded leaves were grown in a chamber at day/night temperatures of 28/24 ºC, a 14-h photoperiod, an irradiance of 300 μmol m^{-2} s^{-1}, and a relative humidity of 75 %. These seedlings were used for cloning *CLC* genes and analysis of their expression. To investigate gene expression at tissue-specific levels, the plants were grown under above mentioned conditions and five parts, including the taproot (TR), lateral root (LR), stem (S), mature leaf (ML), and young leaf (YL) of these plants, were separately harvested. To investigate *CLC* gene expression in response to different treatments, mature leaves were collected from the seedlings which were treated as follows. For nitrogen treatment, the plants were first grown in a nutrient solution without nitrogen for the nitrogen starvation, and the samples were collected 7 d after the treatment. Then, the plants were transferred to a nutrient solution with a low nitrogen concentration (2 mM NO_3^-), and the samples were collected 1 and 2 d after the treatment. For salt or ABA treatments, the plants were submerged in a nutrient solution containing 150 mM NaCl or 100 μM ABA. The samples were collected 1, 3, and 7 d after the salt treatment and 6 h, and 1 and 2 d after the ABA treatment. Different tissues were collected from three biological samples. After collection, the samples were immediately frozen in liquid nitrogen and stored at -80 ºC until use.

Identification of *PtrCLC* genes (*PtrCLCs*): Total RNA was extracted from leaves of trifoliate orange using a *TRIzol* reagent according to the manufacturer's instruction. After being treated by RNase-free *DNase I* (*Takara*, Dalian, China) to remove genomic DNA contamination, the RNA (2 μg) was reversely transcribed into cDNA using a *First Strand* cDNA synthesis kit (*Toyobo*, Osaka, Japan). The cDNA was then used for gene amplification. To identify putative members of the *CLC* gene family in citrus, the sequences of the citrus *CLC* gene (GenBank ID: GU942490) were subjected to *BLASTN* searches in the database of the *Citrus sinensis* annotation project (http://citrus.hzau.edu.cn/orange/index.php). Homology searches were also performed using CLC as keyword in the *Phytozome* database (http://www.phytozome.net/). Redundant sequences were removed after similarity comparison. These sequences were used as templates to design primers for PCR amplification of full-length cDNA of *CLC* genes in trifoliate orange (primers in Table 1 Suppl.). All PCR products were cloned into the pMD18-T vector (*Takara*). Two positive clones for each fragment were sequenced.

Sequence analysis: Homology searches were performed using the *BLASTN* algorithm at *NCBI*. The sequence alignment of the deduced proteins was generated using the *Clustal X* (*v. 2.1*). Phylogenetic analysis was based on the neighbor-joining method using *MEGA* (*v. 5.0*), and 1 000 replicates were used for bootstrap test. The transmembrane regions (TMs) and cystathionine β-synthase (CBS) domains were predicted by *TMHMM* (http://www.cbs.dtu.dk/services) and *SMART* (http://smart.embl-heidelberg.de/), respectively. The

molecular mass (M_r) and theoretical isoelectric point (pI) for the predicted proteins were identified by an internet server *ExPASy* (http://www.expasy.org/tools). The amino acid identity of PtrCLC proteins (PtrCLCs) was calculated by the *DNAMAN* (*v. 6.0*) software.

Real-time quantitative PCR analysis: The RNA isolation and cDNA synthesis of the collected samples were performed as mentioned above. Quantitative PCR was performed in an optical 96-well plate using *LightCycler 480* (*Roche Diagnostics*, Basel, Switzerland) and gene-specific primers listed in Table 1 Suppl. Reaction mixtures were prepared in a total volume of 20 mm^3; they contained 100 ng of cDNA, 0.4 mm^3 of each 10 μM primer, 10 mm^3 of a *SYBR Green I* qPCR mix (*Takara*); and double distilled H_2O. The procedure was as follows: 50 ºC for 2 min and 95 ºC for 10 min, followed by 45 cycles of 95 ºC for 15 s and 60 ºC for 30 s. Following the PCR, melting curve analysis was performed to confirm the specificity of the amplified fragments. To determine relative quantity of each sample, the C_T (cycle threshold) value of target genes was normalized to the C_T value of the citrus *β-actin* gene and calculated relative to a calibrator using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008). Each cDNA sample was amplified in four independent replicates.

Results

A total of six putative CLC coding sequences were identified. All the six sequences were verified in trifoliate orange by sequencing after sub-cloning them into the T-vector. Here we designate these sequences as *PtrCLC1* to *PtrCLC6*, respectively, corresponding to the sequences of Cs2g25150, Cs3g03450, Cs4g05340, Cs4g07570, Cs9g06580, and orange1.1t03372 in the database. The properties of deduced PtrCLCs are shown in Table 1. Molecular analysis of the deduced polypeptides reveals that PtrCLCs contained 748 - 789 amino acids with M_r between 80.57 and 87.67 kDa. The pI of PtrCLC4 (6.50) and PtrCLC6 (5.80) were far lower than those of other members which ranged from 8.06 (PtrCLC1) to 8.87 (PtrCLC3). Sequence comparison found that the *PtrCLC* genes shared 23.55–61.24 % of sequence identity at the amino acid level (Table 1). Further, PtrCLC4 and PtrCLC6 showed a lower amino acid identity than other CLC members, only approximately 25 %. But, the amino acid sequences of PtrCLC4 and PtrCLC6 were more similar to each other with a 41.07 % identity. In addition, PtrCLCs were predicted to contain 9–12 TMs (Fig. 1). These proteins were all predicted to contain two CBS domains at the carboxyl end.

Table 1. The description of trifoliate orange PtrCLCs and the chromosomal location of respective genes.

Protein	Location	M_r [kDa]	pI	Identity [%] PtrCLC2	PtrCLC3	PtrCLC4	PtrCLC5	PtrCLC6
PtrCLC1	Chr 2	87.67	8.06	55.37	55.47	24.18	46.56	24.86
PtrCLC2	Chr 3	86.09	8.52		61.24	24.53	50.81	25.32
PtrCLC3	Chr 4	86.62	8.87			25.84	48.87	25.28
PtrCLC4	Chr 4	80.57	6.50				23.55	41.07
PtrCLC5	Chr 9	84.73	8.26					24.93
PtrCLC6	unknown	83.65	5.80					

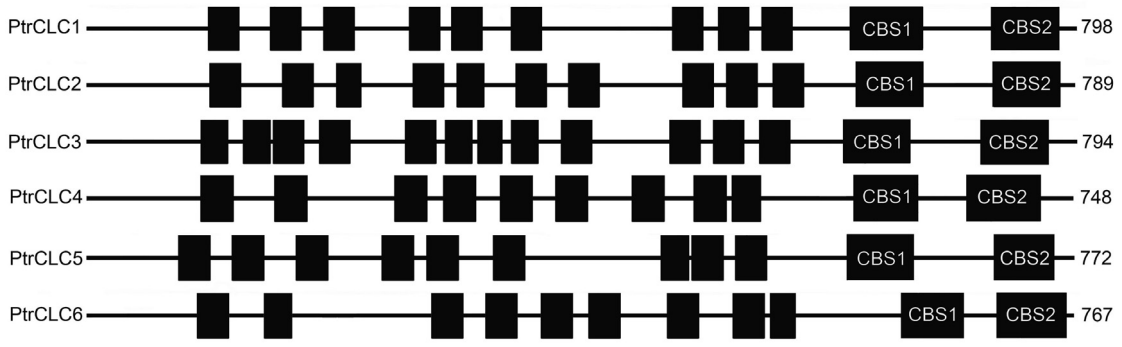

Fig. 1. The scaled graphic representation of different domains of *PtrCLCs*. The *black bars* stand for TM regions and the *black boxes* correspond to CBS domains.

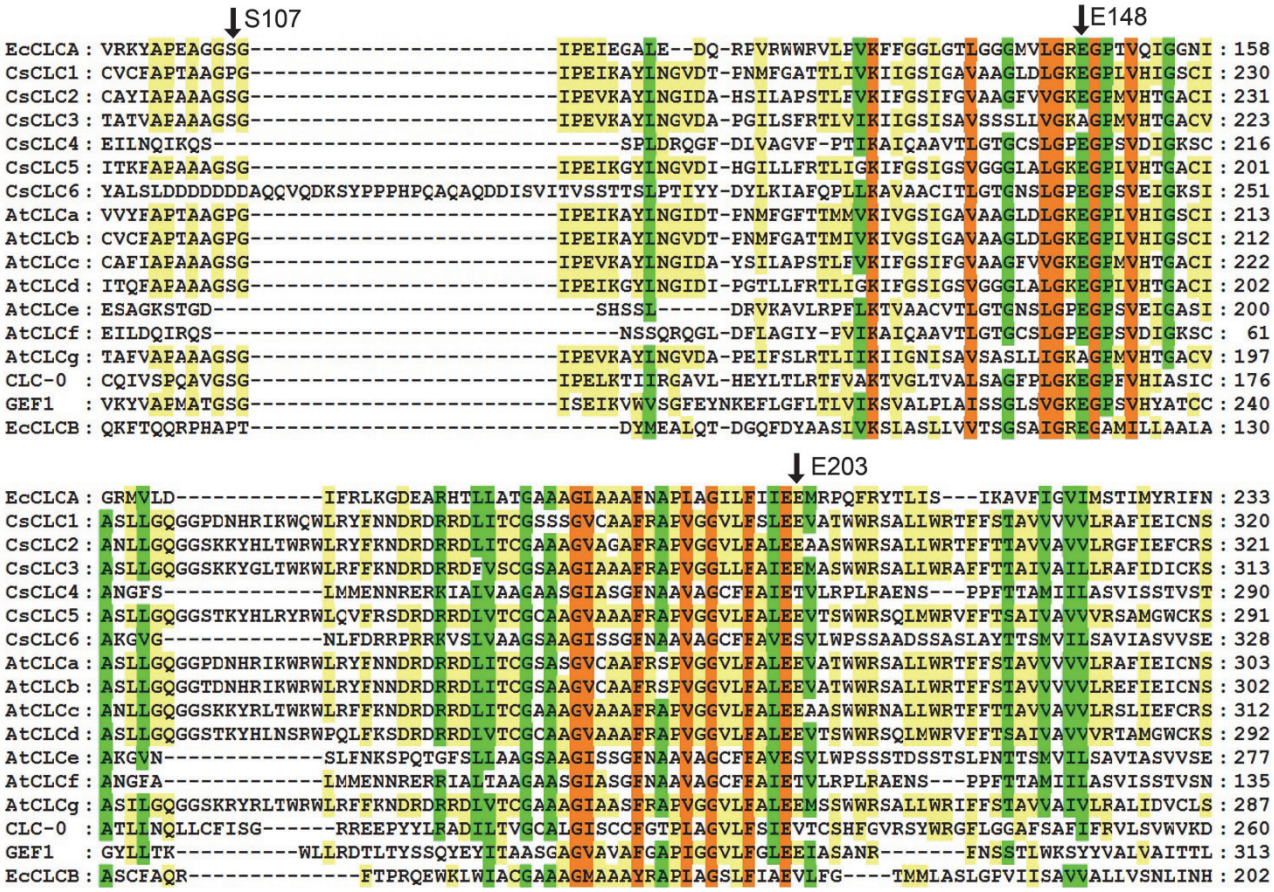

Fig. 2. The multiple sequence alignments of PtrCLCs with other CLC proteins. The sequence of EcCLCA (P37019) added as reference, and critical positions of amino acids involved in anion selectivity are indicated by the *arrows* above the sequence. The accession numbers of the corresponding proteins are as follows: AtCLCa (NP_198905), AtCLCb (NP_189353.1), AtCLCc (NP_199800), AtCLCd (NP_197996.1), AtCLCe (NP_567985.1), AtCLCf (NP_564698.1), AtCLCg (NP_198313.2), CLC-0 (P35522), GFE1 (P37020), and EcCLCB (P76175).

Multiple sequence alignment reveals that PtrCLCs shared a low similarity, but they were relatively conserved in three motifs: GxGIPE, GKxGPxxH, and PxxGxLF (Fig. 1 Suppl.). It has been reported that amino acid divergence in these motifs is related to an isoform-specific anion selectivity of CLC proteins in bacteria and animals (Basilio *et al*. 2014). To explore the alterations of PtrCLCs, we compared these sequences with other CLC sequences (Fig. 2). In EcCLCA, the amino acids of the selectivity filter are S107, E148, and E203 and are involved in interaction with Cl^- (Dutzler *et al*. 2002). Sequence comparison reveals that PtrCLC2, PtrCLC3, and PtrCLC5 possessed serine residues in equivalent positions of S107 in EcCLCA, whereas PtrCLC1 displayed a proline instead of a serine. As for glutamate residues (E148), it was highly conserved in PtrCLCs except for PtrCLC3, which displayed an alanine (A213) instead of a glutamate. Furthermore, PtrCLC1-3 and PtrCLC5 also possessed glutamate residues in equivalent positions of E203, whereas PtrCLC4 and PtrCLC6, similar to AtCLCf and AtCLCe, respectively, displayed a threonine (T260) and serine (S295) at this position (Fig. 2).

To characterize the relatedness of PtrCLCs, *MEGA* was employed and a neighbor-joining phylogenetic tree was created (Fig. 3). All collected CLCs were classified into three clusters, and plant CLCs were divided into two individual subgroups, plant CLC subgroup I and plant CLC subgroup II. PtrCLC1-3 and PtrCLC5 that affiliated to larger plant CLC subgroup I were phylogenically distant from PtrCLC4 and PtrCLC6 that were affiliated to plant CLC subgroup II. It is worth noting that when compared with CLC from *Arabidopsis*, PtrCLCs established a one-to-one correspondence with AtCLCs. For example, PtrCLC1 and AtCLCa/AtCLCb closely belonged to the same branch of the phylogenetic tree.

Expression of all the six *PtrCLC* genes in various citrus tissues were examined using RT-qPCR (Fig. 4), and *PtrCLCs* exhibited a ubiquitous expression in all detected tissues with a predominant expression in leaves. However, individual differences in the expression pattern existed among the *PtrCLC* genes. The expression of

PtrCLC1 was lower in LR and S and higher in YL and ML. The expression of *PtrCLC2* was also more abundant in ML than in the other tested tissues. The expressions of both *PtrCLC3* and *PtrCLC5* were highest in ML, followed by YL, TR, LR, and S. Remarkably, the *PtrCLC6* expressions in YL and ML were almost 20- and 25-fold higher than in TR (Fig. 4).

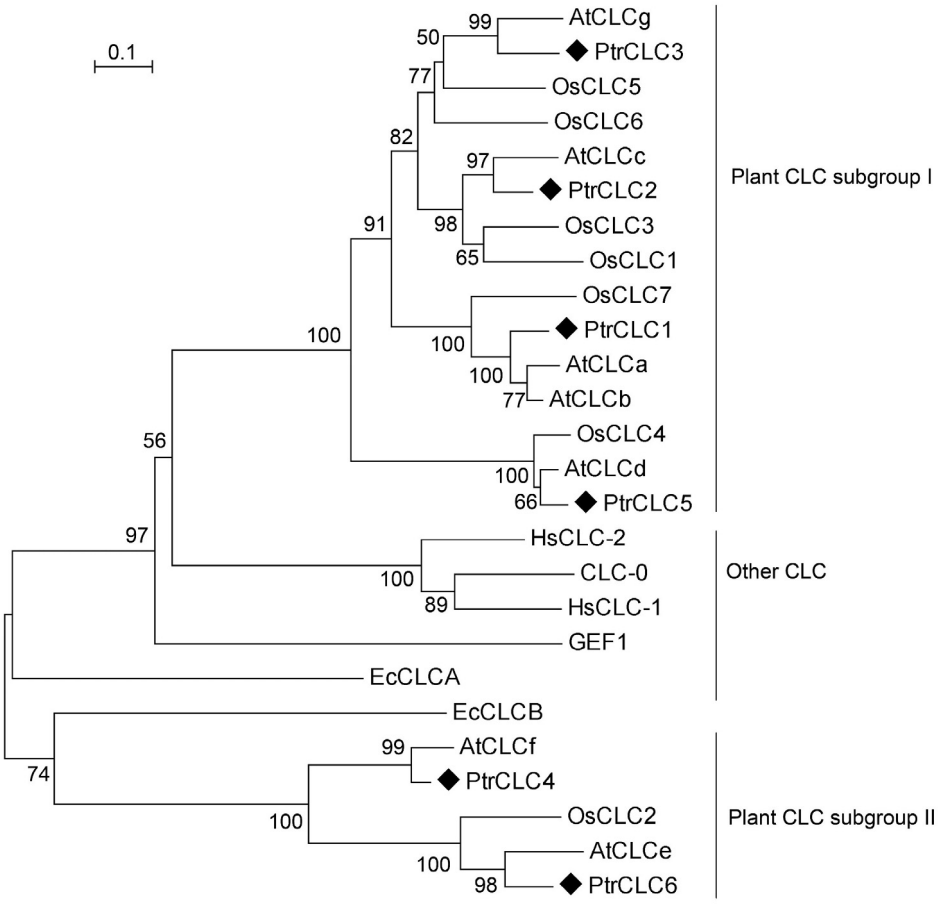

Fig. 3. A phylogenetic tree constructed based on amino acid sequences of PtrCLCs and other CLCs, including AtCLCa-g from *Arabidopsis thaliana*; OsCLC1 (Os01g65500), OsCLC2 (Os01g50860), OsCLC3 (Os02g35190), OsCLC4 (Os03g48940), OsCLC5 (Os04g55210), OsCLC6 (Os08g20570), and OsCLC7 (Os12g25200) from *Oryza sativa*; HsCLC-1 (P35523) and HsCLC-2 (P51788) from *Homo sapiens*; CLC-0 from *Torpedo californica*; GFE1 from *Saccharomyces cerevisiae*; EcCLCA and EcCLCB from *Escherichia coli.* The neighbor-joining method was used, and the *numbers* beside the branches represent bootstrap values based on 1 000 replications. The branch lengths are proportional to divergence, with the scale of 0.2, representing a 20 % change.

We further examined the expression profiles of *PtrCLCs* in response to abiotic stresses using RT-qPCR method (Fig. 5). After nitrogen starvation for 7 d, *PtrCLC1-3* and *PtrCLC6* were downregulated, whereas *PtrCLC4* and *PtrCLC5* were slightly upregulated. When 2 mM NO_3^- was supplemented, the *PtrCLC1* expression rebounded and was higher than that of the control 2 d after the treatment. In contrast, the expressions of *PtrCLC4* and *PtrCLC5* were persistently inhibited. All *PtrCLC* genes were induced by the NaCl treatments. Among them, the *PtrCLC2*, *PtrCLC3*, and *PtrCLC4* expressions sharply increased until the end of the treatment up to 31-, 12-, and 22-times of the initial levels, respectively. The transcript abundances of *PtrCLC1* and *PtrCLC5* gradually rose with the prolonging treatment and reached the highest values 7 d after the treatment. Likewise, the transcript abundance of *PtrCLC6* increased, but it seems not to be sensitive to the high salt treatment as the increase was limited when compared with the other *PtrCLC* members. Furthermore, we found that both the *PtrCLC2* and *PtrCLC4* expression was induced by the ABA treatment with increases ranging from 5- to 9-times of the control (Fig. 5). The expressions of *PtrCLC3* and *PtrCLC5* were also induced steadily by about 2 times of the control during the experimental period. The expression of *PtrCLC1* showed no obvious changes, and *PtrCLC6* showed a slight increase 6 h after the ABA treatment and subsequently reverted to the basal level.

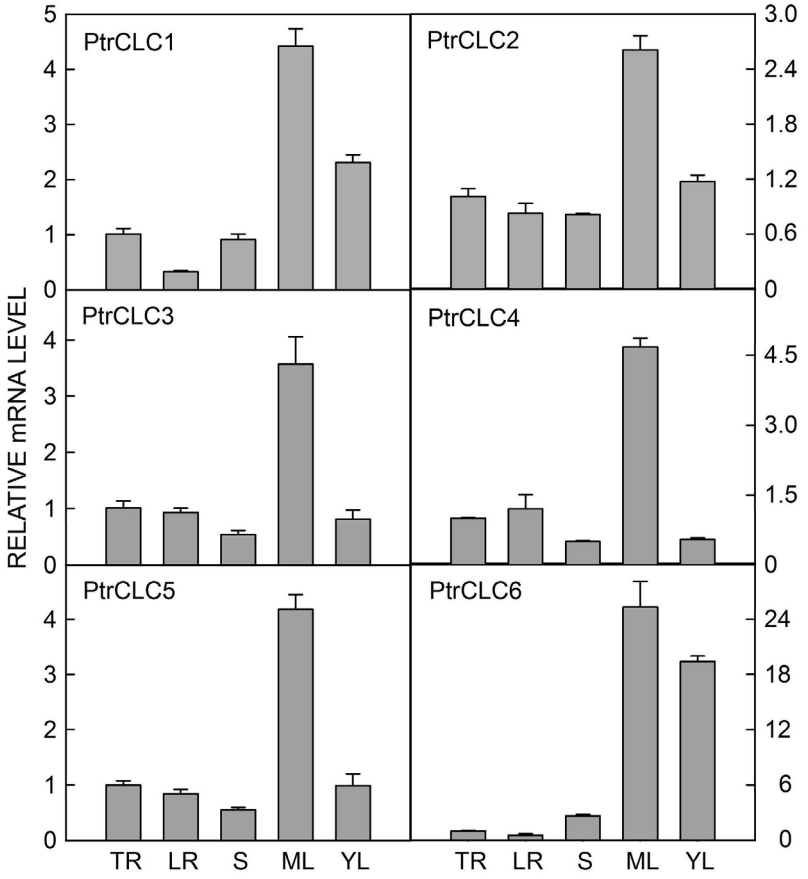

Fig. 4. The transcription of *PtrCLCs* in the taproot (TR), lateral root (LR), stem (S), mature leaf (ML), and young leaf (YL) of trifoliate orange. The relative mRNA level of PtrCLC in TR (= 1) was used as calibrator to determine the fold change of mRNA abundance among tissues. Values represent means ± SE (n = 3).

Discussion

CLC genes belong to a small gene family in higher plants, with seven homologues both in *Arabidopsis* and rice genomes, but their subcellular localizations and biological roles were reported to be diverse among plants (Jentsch *et al*. 2008). Thus, a comprehensive analysis of the *CLC* gene family in different plant species is essential. In the present study, we identified six *CLC* genes in the citrus genome database and then isolated these genes (*PtrCLC1-6*) from trifoliate orange. As other plant *CLCs*, *PtrCLCs* contained 9–11 TMs and two CBS domains. The TMs confer the properties of membrane localization of CLC proteins, and CBS is able to bind ATP, which led to a hypothesis that the CBS domain functions as sensors of intracellular metabolites and exerts a regulatory role on transport activity of the protein (De Angeli *et al*. 2009). Sequence analysis reveals that *PtrCLCs* shared a relatively low similarity among individual members and were distributed on different chromosomes (Fig. 2). This indicates that functional diversities have evolved among PtrCLC members, particularly across the two ancient subgroups. Our finding is supported by a previous study on CLC in *Arabidopsis* (Lv *et al*. 2009).

Crystallography analysis revealed that some conserved residues in the CLC proteins are closely related to anion selectivity and transport. The EcCLCA has three Cl^- binding sites, namely S107, E148, and E203 (Dutzler 2006). Among these sites, the S107 is reported to be critical for a higher selectivity for Cl^- over NO_3^-. The mutations of E148 severely affect or abolish gating in CLC proteins and lead to the transporters not sensitive to pH (Pusch *et al*. 2004). This suggests that this residue is an essential determinant of the CLC protein, and thus it has been termed as the gating glutamate (Bergsdorf *et al*. 2008). Another glutamate residue E203 plays a key role in anion/H^+ coupling (Dutzler 2006, Zifarelli and Pusch 2010). At the equivalent position of S107, AtCLCa and AtCLCb display a proline residue and function as NO_3^-/H^+ antiporter indicating that the replacement of proline at this position can turn the ionic selective property of the CLC transporter (De Angeli *et al*. 2006, Von der Fecht-Bartenbach *et al*. 2010). Here, sequence

analysis reveals that PtrCLC1 also displayed a proline (P177) residue at the S107 position but had glutamate residues conserved. Together with the phylogenetic analysis, we speculate that *PtrCLC1* is orthologous with *AtCLCa*/*AtCLCb*, and the protein may function in NO_3^- transport like AtCLCa/AtCLCb does. The E203 is considered to be conserved in all transporters (Dutzler 2006). Threonine and serine are phosphorylation sites, and mutations of E203 to serine influence human CLC-3 protein activity (Robinson *et al*. 2004). Hence, the mutations of E203 in PtrCLC4 (T260) and PtrCLC6 (S295) imply that both CLC proteins function as channels but not transporters (Jentsch *et al*. 2005).

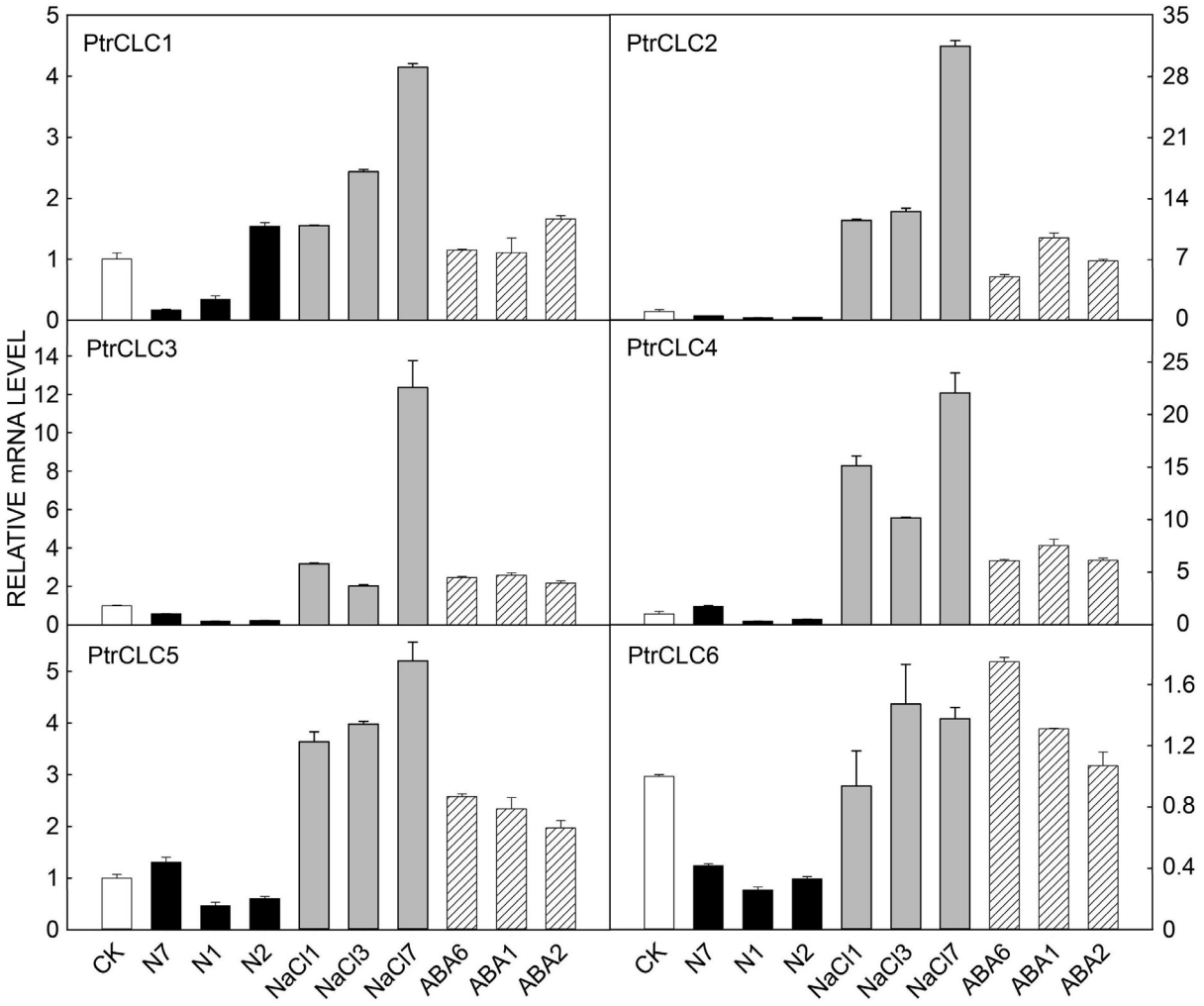

Fig. 5. The transcription of *PtrCLCs* in ML of trifoliate orange under nitrogen, NaCl, and ABA treatments. N7, N1, and N2 indicate samples collected 7 d after the nitrogen deficiency treatment and 1 and 2 d after the limiting nitrogen (2 mM NO_3^-) supply, respectively; NaCl1, NaCl3, and NaCl7 indicate samples collected 1, 3, and 7 d after the 150 mM NaCl treatment. ABA6, ABA1, and ABA3 indicate samples collected 6 h, and 1 and 2 d after the 100 μM ABA treatment. The transcription in leaves from untreated plants (CK) was set to 1, and values represent means ± SE (n = 3).

In *Arabidopsis*, *AtCLC* members of subgroup I exhibit a higher expression than those of subgroup II in various tissues at different development stages (Lv *et al*. 2009). However, no such expression tendency was observed between *PtrCLC* members of subgroup I and subgroup II. In addition, Lv *et al*. (2009) has reported that *AtCLCb* in *Arabidopsis* is strongly expressed in roots rather than in leaves or inflorescences. This is inconsistent with our observations that all *PtrCLCs* including *PtrCLC1* showed a relatively low expression in roots (Fig. 4). Therefore, the tissue expression patterns of CLC members may depend on plant species. It is worth noting that *PtrCLC6* displayed a leaf-specific expression pattern indicating that it had a distinct function. The high expression in leaves is consistent with earlier findings that AtCLCe functions in chloroplasts, and mutations of *AtCLCe* lead to a low photosynthetic activity in plants (Marmagne *et al*. 2007).

Patch-clamp technique revealed that one CLC protein can transport various anions, but the permeability for

anions is different. For example, the selectivity sequence of AtCLCa is $NO_3^- \approx I^- > Br^- > Cl^- > SO_4^{2-}$ > glutamate (De Angeli *et al*. 2006). Among the different anions, NO_3^- plays a prominent role in plant physiology. Nitrate compartmentalization in intracellular organelles has been long recognized as critical for plant physiology (Wang *et al*. 2012). AtCLCa and AtCLCb have been shown to function as NO_3^-/H^+ antiporter that play roles in the translocation of NO_3^- into the vacuole (Wege *et al*. 2010). However, our result shows that all the *PtrCLC* genes seemed to be involved in NO_3^- translocation as their expression varied after the nitrogen deficiency (Fig. 5), and the opposite expression changes among certain *PtrCLCs* were probably due to their diversified intercellular roles. The expression of *PtrCLC1* rapidly increased when NO_3^- was supplemented (Fig. 5), confirming the above conclusion that PtrCLC1 has similar roles with AtCLCa/AtCLCb.

The CLCs were also suggested to contribute to plant salt-tolerance as their roles in mediating Cl^- transport across the tonoplast (Nakamura *et al*. 2006, Wong *et al*. 2013, Wang *et al*. 2015). Control of Cl^- transport and Cl^- exclusion from shoots contributes to salt tolerance in many species, such as wheat, barley, avocado, grapevine, and citrus (Teakle and Tyerman 2010, Tregeagle *et al*. 2010). In the present study, the expressions of *PtrCLC2* and *PtrCLC4* were rapidly upregulated by the NaCl stress (Fig. 5) indicating that both genes responded to the salt stress and might function in Cl^- sequestering in the vacuole to detoxify the cytoplasm. The *PtrCLC2* and *PtrCLC4* were also induced by application of ABA. We conclude that these genes are ABA dependent and may be involved in the regulation of stomatal movement as *AtCLCc* does (Cutler *et al*. 2010, Jossier *et al*. 2010). Comprehensively, PtrCLC2/PtrCLC4 and PtrCLC3/PtrCLC5 play interconnecting but not redundant roles in Cl^- sequestration. As for *PtrCLC6*, it showed a great difference in the sequence characteristic and expression pattern compared with other *PtrCLC* genes. Considering *AtCLCe*, the *PtrCLC6* homologue in *Arabidopsis*, localized at the thylakoid membrane in chloroplasts and implicated in photosynthesis (Marmagne *et al*. 2007), we speculate that *PrtCLC6* may be essential for improving photosynthetic activity of citrus plants under NaCl stress. However, further studies are needed to explore the biological roles of PtrCLC6 in the photosynthetic system.

In conclusion, the trifoliate orange *CLC* family contained six members, *PtrCLC1-6*, that can be classified into two distinct subgroups. The *PtrCLC4* and *PtrCLC6* in subgroup II showed a great difference in sequence identity when compared with other *CLC* members. Expression of *CLC* genes was influenced by nitrogen deficiency and NaCl stress indicating that they play a role in control of anion homeostasis in plant resistance regulation.

This study presents a comprehensive overview of the *PtrCLC* gene family and provides a theoretical basis for a further functional analysis of *CLC* genes in citrus.

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