International Journal of Current Research in
Biosciences and Plant Biology
Volume 4 • Number 1 (January-2017) • ISSN: 2349-8080 (Online)
Journal homepage: www.ijcrbp.com

Original Research Article **doi:** http://dx.doi.org/10.20546/ijcrbp.2017.401.002

Molecular Cloning and Characterization Analysis of a Gene Encoding Cup-shaped Cotyledon (*CUC1*) from *Phoebe neurantha* (Hemsl.) Gamble

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Abstract

Cup-shaped cotyledon (*CUC*) gene is a kind of border-specific gene, which is leaf edge cracking and complex leaflet development. *CUC* plays an important role in shoot apical meristem formation and leaf margin development in *Phoebe neurantha* (Hemsl.) Gamble. In this paper, a *CUC* gene designated as *PnCUC1* was successfully cloned from *P. neurantha* (Hemsl.) Gamble. The full-length of *PnCUC1* was 1317-bp and contained a 1008-bp open reading frame. It encoded a 335-amino-acid protein with a calculated molecular weight of about 37.8 kDa and isoelectric point of 7.04. Sequence comparison revealed PnCUC1 showed extensive homology with CUCs from other plant species. Phylogenetic tree analysis revealed that PnCUC1 were high similar to previously described CUC1 proteins from other plant species and shared the same ancestor in evolution with other CUCs, indicating that PnCUC1 belonged to a multi-gene family. The cloning and characterization of *PnCUC1* gene will be helpful to further study the role of *PnCUC1* gene in the regulation of leaf margin and braches development in *P. neurantha* (Hemsl.) Gamble.

Article Info

Accepted: 18 December 2016
Available Online: 06 January 2017

Keywords

Leaf development
Phoebe neurantha (Hemsl.) Gamble
PnCUC1

Introduction

Phoebe neurantha (Hemsl.) Gamble is a evergreen tree species of *Phoebe* Nees, Lauraceae. Due to its straight trunk and beautiful tree shape, *P. neurantha* (Hemsl.) Gamble is a good landscape tree and widely used in the garden, which is usually considered as plant landscaping tree and landscape background tree. *P. neurantha* (Hemsl.) Gamble is also a kind of precious timber trees resource of Chinese traditional timber trees, because of its unique fragrance and fine texture (Hai-Shan et al., 2014).

Leaf development and mechanism of leaf morphogenesis are hot topics of plant developmental biology. With the aid of the model plant mutants research, many key genes of leaf development has been cloned and identified (Nicotra et al., 2011). However, the regulatory mechanism of leaf development in other plant remains to be illuminated. During pattern formation in plants and animals, groups of cells are divided into domains that acquire different developmental fates. This process requires the establishment of precise gene expression patterns that are maintained despite continuous growth and cell division. A recently discovered class of small RNAs, the microRNAs (miRNAs) involved in gene expression regulation may contribute to this (Laufs

et al., 2004). MiRNAs are small, single-stranded RNAs molecules of about 21bp in length, that post-transcriptionally regulate gene expression (Kidner, 2010; Pulido and Laufs, 2010). The biological function of miRNAs has investigated in many plants, including *Arabidopsis thaliana* (Sunkar and Zhu, 2004), *Oryza sativa* (Sunkar et al., 2005), *Zea mays* (Mica et al., 2006), *Triticum aestivum* (Yao et al., 2007), *Nicotiana tabacum* (Billoud et al., 2005), *Populus tomentosa* (Lu et al., 2005) and Pteridophyta (Axtell and Bartel, 2005).

The cup-shaped cotyledon (*CUC*) genes *CUC1*, *CUC2*, and *CUC3* are required to demarcate primordium boundaries as well as promote meristem formation (Takada et al., 1991). Recent studies have shown that miR164 plays the key role in the development of the leaf margin. The target gene of miR164 is the transcription factor of *CUC1*, *CUC2*, NAC1 of NAM / CUCc family (Mallory et al., 2004). *CUC1* and *CUC2*, a kind of border-specific gene, plays an important role in leaf edge cracking and complex leaflet development. The balance between the miR164 and *CUC2* determines the depth of cleft lobes, the stronger *CUC2* activity, the deeper the lobes, the miR164 mutant and the phenotype of *CUC* overexpression, Mutation occurs so that miR164 cannot mediate cleavage of the CUC mRNA, resulting in the accumulation of CUC mRNA, Resulting in Arabidopsis leaf margin to produce deep cleavage, while overexpression of miR164 Arabidopsis plant leaf margin cleft engraved disappearance (Nikovics et al., 2006). Expression of mutated miRl64 causes uncontrolled post-transcriptional regulation of the *CUC* gene while driving *CUC1* expression with the *CUC2* promoter, and the *Arabidopsis* single leaf becomes a compound leaf (Hasson et al., 2011). However, the enzymes involved in the leaf margin development in *P. neurantha* (Hemsl.) Gamble are not well understand. The identification of the *CUC* genes and the genetic sequences are important for further studies of leaf development in *P. neurantha* (Hemsl.) Gamble. To date, little is known about the gene encoding *CUC* in *P. neurantha* (Hemsl.) Gamble. Therefore, we cloned a new *CUC1* gene from *P. neurantha* (Hemsl.) Gamble and analysis the sequence of *PnCUC1*.

Materials and methods

Phoebe neurantha (Hemsl.) Gamble leaves were collected from botanical garden at Yangtze University, and immediately placed in a -80°C freezer. Both the primers synthesis and DNA sequencing were performed by Shanghai Sangon Biotechnology Company, in China. Agarose Gel DNA purification Kit Ver.4.0, pMD18-T vector kit, AMV Reverse Transcriptase, dNTPs, RNase in and Taq DNA polymerase were purchased from Takara Company (Dalian, China).

Cloning the full-length cDNA of PnCUC1

Total RNA of *P. neurantha* (Hemsl.) Gamble seedlings was isolated using the TaKaRa MiniBEST Plant RNA Extraction kit (Dalian, China) according to the manufacturer's instructions. The concentration and quality of the RNA were measured *via* spectrophotometry and agarose gel electrophoresis. The primers PnCUC1-u (5′-ATGGCCCCAGCAGCTGTAGT-3′) and PnCUC1-d (5′-TTTCGTTGTGGAACTTCTG-3′) were designed and synthesized according to the gene annotation of *P. neurantha* (Hemsl.) Gamble in the transcriptome database. The full-length cDNA sequence of *PnCUC1* was amplified with the one-step RT-PCR kit (Dalian TaKaRa, Dalian, China) under the following condition: 94°C for 3 min; 33 cycles of amplification at 94°C for 20 s, 56°C for 40 s, and 72°C for 60 s; and a final extension at 72°C for 7 min. The PCR products were purified, ligated into pMD18-T vector, and introduced into *E. coli* strain DH5a followed by sequencing.

Bioinformatics and molecular evolution analysis

Sequence assembly was performed with programs of DNAstar (http://www.dnastar.com). The nucleotide sequence, deduced amino acid sequence and open reading frame (ORF) were analyzed, and the sequence comparison was conducted through database search (http://www.ncbi. nlm.nih.gov).

The calculated isoelectric point (pI) and molecular weight of the PnCUC1 protein were computed with the software of Compute pI/Mw Tool at http://web.expasy. org/compute_pi/. Multiple sequence alignment was performed with the software Vector NTI 11.5 program. Phylogenetic analysis of PnCUC1 and other CUC from other plants were aligned with CLUSTALX 2, and subsequently, a phylogenetic tree was constructed by the neighbor-joining (NJ) method with MEGA 6 software (Kumar et al., 2004).

Results

Cloning and characterization of the full-length cDNA of *PnCUC1* gene

A total of 49,561 unigenes have been identified in *P. neurantha* (Hemsl.) Gamble RNA-Seq database through annotation against public protein databases (data not published). Among these unigenes, one CUC member was identified based on the annotation information. Using a pair of specific primers based on the CUC unigene (Gene ID: c41381) of transcriptome data, we performed PCR using cDNA as the template, and a 1317-bp fragment was amplified. It contained a 1008-bp open reading frame (ORF) encoding a 335-amino-acid protein (Fig. 1). The nucleotide sequence of *PnCUC1* was 82, 80, 78, 76 and 74% identical to those of the *CUC1* genes from *Nelumbo nucifera*, *Theobroma cacao*, *Beta vulgaris*, *Eucalyptus grandis*, and *Glycine max*, respectively (Table 1). The results indicate that the gene we cloned is a member of the *CUC* gene family.

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1       CCTTGTTTTCTAGAGACAAAGGAGGTATCCTGCCAAGACCCAATAATACTCCTCTTCATT
61      CCTTTTTATCTTTTTTGTACTCTTGAAATGGAGGAGAATCTGCCACCAGGGTTTAGATTT
21                                  M  E  E  N  L  P  P  G  F  R  F
121     CACCCCACAGATGAAGAACTCATCACATACTATCTGACTCACAAGGTCTCTGATTTCAAT
41       H  P  T  D  E  E  L  I  T  Y  Y  L  T  H  K  V  S  D  F  N
181     TTCGTTACCAGGGCGATCACTGACGTCGATCTCAATAAGTGCGAGCCTTGGGATCTTCCA
61       F  V  T  R  A  I  T  D  V  D  L  N  K  C  E  P  W  D  L  P
241     GGGAAAGCTTCTATGGGAGAAAAAGAGTGGTATTTCTTCAACTTAAGAGATCGAAAATAC
81       G  K  A  S  M  G  E  K  E  W  Y  F  F  N  L  R  D  R  K  Y
301     CCCACAGGCCTTCGAACTAATCGAGCCACAGAGGCAGGCTATTGGAAGACCACAGGAAAA
101      P  T  G  L  R  T  N  R  A  T  E  A  G  Y  W  K  T  T  G  K
361     GATAAGGAGATCTTTCATTCCGGAGTGATGGTTGGAATGAAGAAAACCCTAGTATTCTAC
121      D  K  E  I  F  H  S  G  V  M  V  G  M  K  K  T  L  V  F  Y
421     AAGGGTAGGGCTCCCAGAGGAGAGAAAAGCAACTGGGTGATGCATGAATATCGACTCCAA
141      K  G  R  A  P  R  G  E  K  S  N  W  V  M  H  E  Y  R  L  Q
481     AGCAAGTATGCCTACAGACCCGCCAAGGAGGAATGGGTGGTTTGTAGGGTTTTCCAGAAG
161      S  K  Y  A  Y  R  P  A  K  E  E  W  V  V  C  R  V  F  Q  K
541     AGCTCCTGTGCAAAGAAACCGCCATCAAGTCCAGCATCATTGCCCCATTTGGTTGAATCA
181      S  S  C  A  K  K  P  P  S  S  P  A  S  L  P  H  L  V  E  S
601     TCTTGCGATACTACCTTAATGAATGAAATAGAGGAGATCGATATGCCAATGCCAAACTTG
201      S  C  D  T  T  L  M  N  E  I  E  E  I  D  M  P  M  P  N  L
661     AATAGTCTAGTGAGTTTTTCAAGTGATTTTAGTTCAGTTTCTACTAACAACAACAACATC
221      N  S  L  V  S  F  S  S  D  F  S  S  V  S  T  N  N  N  N  I
721     ATCCCGAGTAACAACAATAACAACAACCACAACAACAATAGCAACATCAACAATGGCAGC
241      I  P  S  N  N  N  N  N  N  H  N  N  N  S  N  I  N  N  G  S
781     GGCAGCAACAACAATAACATGAACATGAACAATGAAATGAACTTGAATTGGGGTTTCGCG
261      G  S  N  N  N  N  M  N  M  N  N  E  M  N  L  N  W  G  F  A
841     AATGCAACAACAAACCAATCTTCTCTCAATTGGCCTTCAGGATTGTTGAACTCCAGTTTT
281      N  A  T  T  N  Q  S  S  L  N  W  P  S  G  L  L  N  S  S  F
901     TCAACAAATTCGTCAATTTTAAAGGCGTTGCAGTTTGCTTGTCAACCCCAAGAAGGAAGT
301      S  T  N  S  S  I  L  K  A  L  Q  F  A  C  Q  P  Q  E  G  S
961     AATCTAAGCTCTTTGATAGCGCAGGGAGAGTGTTTACAAACCAATATGAACTCCAATTTC
321      N  L  S  S  L  I  A  Q  G  E  C  L  Q  T  N  M  N  S  N  F
1021    GCATCTTCTTCATCTTCGAAGGGTACAAATTCTATGCAACAACAACCATTCCATATGGAT
341      A  S  S  S  S  S  K  G  T  N  S  M  Q  Q  Q  P  F  H  M  D
1081    TCGATTTGGCGTTGATATTCTCGACAATGAAAAACAACATGTAAATTTCCTTCTCCACTT
361      S  I  W  R  *
1141    ATTCATGGTAGGTAAATTGAACCCATGGTAAGATGGGATCGAACCCATGATTTCAATGTT
1201    GTGAACCACCTTTATCTCCTTACTCTGGCACACATGGTTCATACCTAGTACTTATGTAAG
1261    ATCATGGTTGCAAAGCTTCCTAGATATTAGCTAGAAGACAACTATATATTCCCCACG
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Fig. 1: Nucleotide sequence and deduced amino acid sequence of *PnCUC1*. The primer sequences are underlined.

Table 1. Nucleotide sequence of *PnCUC1* similarity to the *CUC* genes of other plant species.

Species	Accession no.	E-value	Homology (%)
Nelumbo nucifera	XM_010265607.1	8e-106	82
Theobroma cacao	XM_007036468.2	4e-104	80
Beta vulgaris	XM_010680148.1	5e-73	78
Eucalyptus grandis	XM_010038590.1	2e-56	76
Glycine max	XM_003541118.3	3e-26	74

Characterization of the deduced PnCUC1 protein

By using the software of Compute pI/Mw Tool, the calculated isoelectric point (pI) and molecular weight of the PnCUC1 were predicted to be 7.04 and 37.8 kDa, respectively. Sequence comparison by performing BlastP search showed that PnCUC1 exhibited high similarity to CUC proteins from other plants (Fig. 2). The deduced PnCUC1 protein sequence showed 77, 73, 73, 70, 69, 61 and 60% identities to the counterparts of *Manihot esculenta*, *Nicotiana tabacum*, *Nicotiana sylvestris*, *Solanum tuberosum*, *Nicotiana tomentosiformis*, *Arachis ipaensis* and *Elaeis guineensis*, thereby indicating that PnCUC1 belongs to plant CUC superfamily.

Molecular evolution analysis

To investigate the evolutionary relationships among PnCUC1 and CUC proteins from other plants, a phylogenetic tree was constructed by using software Clustal X2 and MEGA6 with the neighbor-joining (NJ) method. As showed in Fig. 3, the evolutionary tree was divided into five distinct categories. The results highlighted all plants derived from a common ancester in the evolution using CUC as outgroup, no matter whether they belonged to the Solanaceae, Leguminosae, Lauraceae, Rosaceae or Brassicaceae. PnCUC1 belonged to Lauraceae had a close relationship to other CUC proteins. These results suggest that PnCUC1 shares a common evolutionary with other plant CUC proteins based on conserved structure and sequence characteristics, such as amino acid homologies and conserved motifs, respectively.

Discussion

Leaf shape can significantly affect plant photosythesis, yield, and commercial characters and so on, its diversity is produced by alterations of the margin. Compared to complement leaf, a deeply labe leafed would more adapt

the adversity. Leaf-edge cleavage could reduce the distance of heat transfer and have more effective in combating to heat damage to facilitate plant adaptation to high temperatures (Vogel, 2009). In addition, leaf-edge cleavage imparts a plasticity that extends longitudinally to the leaves and can respond more quickly to compete for limited light sources (Semchenko and Zobel, 2007). Leaf-edge cleavage also could increase the hydraulic efficiency to better adapt the drought conditions (Siso et al., 2001; Jiang et al., 2000).

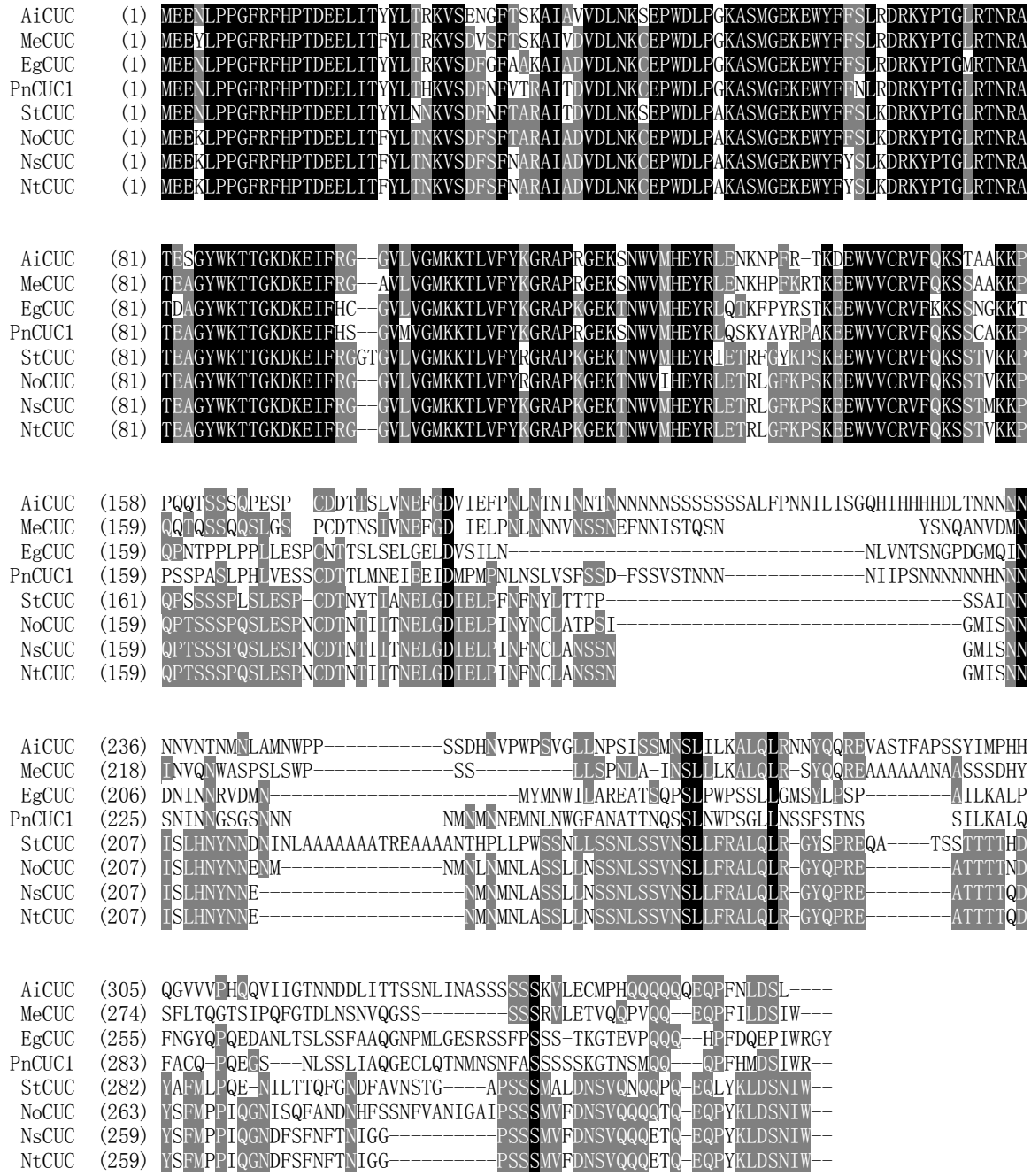

Fig. 2: The multiple alignments of PnCUC1 amino acid sequence with other CUC proteins. The completely identical amino acids are indicated with white foreground and black background. The conserved amino acids are indicated with white foreground and grey background. Non-similar amino acids are indicated with black foreground and white background. The species, protein names and GenBank accession numbers are as following: *Elaeis guineensis*: EgCUC (XP_010916375.1); *Nicotiana tabacum*: NtUC (XP_016433985.1); *Nicotiana tomentosiformis*: NoCUC (XP_009620409.1); *Nicotiana sylvestris*: NsCUC (XP_009779881.1); *Manihot esculenta*: MeCUC (ALC79053.1); *Solanum tuberosum*: StCUC (XP_006353806.2); *Arachis ipaensis*: AiCUC (XP_016203627.1).

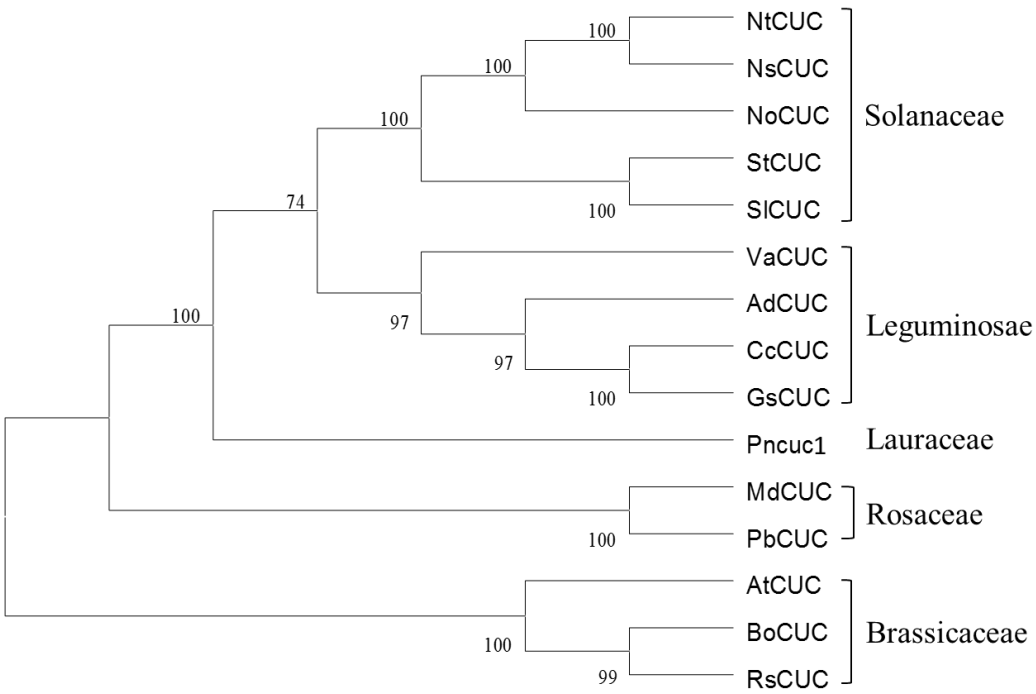

Fig. 3: Phylogenetic tree of CUC from different species using the Neighbor-joining method. The numbers of nodes represent the percentage of boot strap value obtained from 1000 sampling. Bar 0.01 shows the substitutions per nucleotide position. The species, protein names and GenBank accession number are as following: *Cajanus cajan*: CcCUC (KYP69700.1); *Glycine soja*: GsCUC (KHN20732.1); *Vigna angularis*: VaCUC (XP_017441934.1); *Arachis duranensis*: AdCUC (XP_015966676.1); *Nicotiana tabacum*: NtCUC (XP_016433985.1); *Nicotiana tomentosiformis*: NoCUC (XP_009620409.1); *Nicotiana sylvestris*: NsCUC (XP_009779881.1); *Solanum tuberosum*: StCUC (XP_006353806.2); *Solanum lycopersicum*: SlCUC (XP_004252235.1); *Malus domestica*: MdCUC (XP_008355378.1); *Pyrus x bretschneideri*: PbCUC (XP_009339299.1); *Arabidopsis thaliana*: AtCUC (AAP82630.1); *Brassica oleracea*: BoCUC (ADU76441.1); *Raphanus sativus*: RsCUC (ADU76440.1).

The microRNA miR164 family negatively regulates *CUC1* and *CUC2* expression levels: overexpression of miR164 in transgenic plants leads to a reduction in the mRNA levels of *CUC1* and *CUC2* (Kwon et al., 2006), whereas mutants that affect miR164 accumulation result in increased *CUC1* and *CUC2* expression, and in an enlarged boundary domain (Baker et al., 2005). Up until now, the genomic analysis of *CUC* gene has only been reported in some model-plants, such as *Arabidopsis thaliana* (Bellesboix et al., 2006; Aida et al., 1997) and rice (Hu et al., 2006).

Mapping and cloning of leaf lobe genes will provide an insight into mechanism of leaf shape development and improving cultivar in *P. neurantha* (Hemsl.) Gamble. In this study, a 1317 bp full-length cDNA of the *PnCUC1* gene was isolated from *P. neurantha* (Hemsl.) Gamble. The *PnCUC1* gene encodes a 335 amino acid protein. Our multiple alignments showed that the deduced PnCUC1 sequence exhibited high similarity to CUC proteins from other plants. The phylogenetic tree indicated that PnCUC1 has a distinct and ancient relationship with the CUCs from other plant species.

Conclusion

In conclusion, in this present investigation, we have successfully cloned and characterized the gene encoding cup-shaped cotyledon (CUC) involved in the regulation of leaf margin and braches development in *P. neurantha* (Hemsl.) Gamble. Multiple sequence alignment of the deduced PnCUC1 showed highest identity to other plants. Therefore, our study will be helpful to understand the mechanism of leaf shape development in *P. neurantha* (Hemsl.) Gamble. Further studies on the identification of genes related to leaf development of this plant would not only be useful for understanding the shoot apical meristem formation and leaf margin development but will also provide molecular wealth for cultivate new varieties of *P. neurantha* (Hemsl.) Gamble.

Conflict of interest statement

Authors declare that they have no conflict of interest.

Acknowledgement

This work was supported by the Natural Science Foundation of Hubei Province (2013CFA039), and the Science and Technology Support Plan of Hubei Province (2013BBB24).

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How to cite this article:
Yu, P., Tao, T., Wu, Y., Fei, Y., Xiao, B., 2017. Molecular cloning and characterization analysis of a gene encoding cup-shaped cotyledon (CUC1) from *Phoebe neurantha* (Hemsl.) Gamble. Int. J. Curr. Res. Biosci. Plant Biol. 4(1), 6-12. **doi:** http://dx.doi.org/10.20546/ijcrbp.2017.401.002