Scientific Research Publishing

American Journal of Molecular Biology, 2018, 8, 92-101
http://www.scirp.org/journal/ajmb
ISSN Online: 2161-6663
ISSN Print: 2161-6620

Isolation and Functional Analysis of the Regulation of Branching by Isopentenyl Transferase Gene *CmIPT*1 in *Chrysanthemum morifolium* cv. 'Jinba'

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How to cite this paper: Ishak, A., Dong, L., Rong, H., Zhang, S. and Zhao, L. (2018) Isolation and Functional Analysis of the Regulation of Branching by Isopentenyl Transferase Gene *CmIPT*1 in C*hrysanthemum morifolium* cv. 'Jinba'. *American Journal of Molecular Biology*, **8**, 92-101.
https://doi.org/10.4236/ajmb.2018.82008

Received: December 19, 2017
Accepted: April 10, 2018
Published: April 13, 2018

Abstract

Today's *chrysanthemums* are highly evolved flowering plants and they are considered as one of the most important ornamental cut flowers. In this research an isopentenyl transferase gene named *CmIPT*1 was isolated from *Chrysanthemum morifolium cv.* 'Jinba' using RACE and RT-PCR methods. The full cDNA sequence of *CmIPT*1 was 873 bp which encoded a deduced protein of 290 amino acids. It contained GxxGxGKS which is a conserved sequence of the typical domain of IPT family. The phylogenetic tree analysis of *CmIPT*1 in *Chrysanthemum morifolium cv.* 'Jinba' shows that it has the closest relationship with CcIPT1 in *Cynara cardunculus* var. *scolymus*. Expression of *CmIPT*1 was higher in stems and apex, whereas it was lower in leaves and roots. And the overexpression of *CmIPT*1 obviously increased the number of rosette branches in Arabidopsis. Here, in our study, we showed that *CmIPT*1 is a positive regulator of branch development in *Chrysanthemum* and may play a key role in regulating lateral branch formation of *Chrysanthemum* plants.

Keywords

Chrysanthemum, Cytokinin, *CmIPT*1, Shoot Branching

1. Introduction

The development of branching in plants involves the formation and subsequent growth of axillary buds [1]. Plant hormones, location on the plant, gene expression and environment all influence the growth of axillary buds. Hormones are

one of the primary factors regulating branching. At present, it has been considered that three types of hormones participate in regulating the growth of axillary buds. Cytokinins accelerate the growth of axillary buds [2], while auxin and strigolactones inhibit the growth of axillary buds [3] [4] [5] [6]. Cytokinin is considered to be the second messenger [7], and influences the growth of axillary buds under mutual antagonization with strigolactones [8]. Isopentenyl transferase (IPT) is the first reaction in the biosynthesis of cytokinin, and is also a limiting enzyme in cytokinin biosynthesis. There are two types of Isopentenyltransferase (IPT), namely tRNA-IPT which forms the cis-form zeatin, and adenylate isopentenyltransferase (A-IPT) which forms isopentenyladenine as well as anti-form zeatin [9]. The IPT family of genes has been identified in *Arabidopsis [10]*, rice [11], pea [12] and corn [13]. However, *IPT*1 is rarely studied. Research on *Physcomitrella patens* showed that *IPT*1 was the main enzyme which catalysed the prenylation of tRNA, and mutation of *IPT*1 increased the number of lateral branches [14]. Research showed that the level of expression of *PsIPT1* and *PsIPT*2 in peas increased significantly and axillary bud started germinating after removing the shoot top by pruning. This illustrated that cytokinin was synthesized promptly to accelerate the growth of axillary buds after removing the shoot top by pruning [15]. According to the research results, *IPT*1 plays an important role in regulating lateral branch formation and the development of plants.

Chrysanthemum is native to China, with a long history of cultivation and abundant cultural connotations, it is considered as one of the most important ornamental cut flowers. Plant architecture is one of the important factors which needs to be regulated when cultivating Chrysanthemum. In this study, we cloned an IPT1 homologous gene called CmIPT1 from *C. morifolium* cv. 'Jinba', and studied its expression pattern using fluorescent quantitative PCR. We also studied the role of the *CmIPT*1 gene in regulating branching by using allogenic transformation of *Arabidopsis* with the aim of breeding new varieties of cut-flower *Chrysanthemum* having less lateral branches or without any lateral branches by means of molecular biology techniques.

2. Materials and Methods

2.1. Materials

In this study, we used *Chrysanthemum morifolium* cv. 'Jinba' from the Germplasm Nursery of Anhui Agricultural University. *Chrysanthemum* cuttings and *Arabidopsis* wild-type (col-0) as well as transgenic *Arabidopsis* were all grown in a culture room at a temperature of (20 ± 2)˚C, photoperiod of 16 h/8 h (light/dark), illumination intensity of 100 - 120 μmol·m^{-2}·s^{-1}.

2.2. Method

2.2.1. Cloning of *CmIPT*1

Total RNA of *Chrysanthemum* was extracted from stems using Trizol reagent. Residual DNA was digested using RNase-free DNase. cDNA anti sense was syn-

thesized by using M-MLV reverse transcriptase. Degenerate primers were designed according to the conservative nucleotide sequence of *IPT*1 in *Cynara cardunculus* var. *scolymus*, *Medicago truncatula*, *Lotus japonicus* and *Arabidopsis thaliana.* An intermediate fragment was obtained by amplification with degenerate primers. Specific primers for 5' and 3' were designed according to the sequence of the intermediate fragment. The 5' and 3' terminal sequences of *CmIPT*1 were obtained using a SMART^{er}TM RACE cDNA Amplification Kit. DNAMAN 6.0 splices joints tripartite sequences, and finally the full length sequence of the *CmIPT*1 gene was obtained. *CmIPT*1 was amplified using TransStart Fast Pfu polymerase and was connected with the pEASY-Blunt Simple vector. DNA sequencing was conducted by Sangon Biotech (Shanghai). PCR amplification reaction procedure and conditions were: 95˚C initial denaturation 5 min; 95˚C denaturation 30 s, 55˚C annealing 30 s, 72˚C extension 2 min, overall 34 cycles; finally 72˚C extension 10 min, 12˚C preserved (Table 1).

Table 1. Primers used in this study.

Primers	Sequence 5'-3'	Usage
IPT1-PF	CTCCGACAAAATGCAAGTCTAC	Amplification of *CmIPT*1 middle fragment
IPT1-PR	TTCACAATCT TTACGCTTGG	Amplification of *CmIPT*1 middle fragment
IPT1-5'-R1	CGACCCGAGACTCCGAAAATCCGAG	Amplification of *CmIPT*1 5' end
IPT1-5'-R2	GGGTCGGGTCAATGGCTCCGAGAAG	Amplification of *CmIPT*1 5' end
IPT1-3'-F1	CACGTGTCAGTTGGCGAAAAGACAAATAGG	Amplification of *CmIPT*1 3' end
IPT1-3'-F2	GAGTGGATGCCACCGAGGCGTTTAA	Amplification of *CmIPT*1 3' end
IPT1-RTPF	CGCCAAGTTCTTTCCCGAGTATCC	qRT-PCR for *CmIPT*1
IPT1-RTPR	ATGCCAAGGTCTCTGAGCTTCTGC	qRT-PCR for *CmIPT*1
18S-F	CTCATGGGATGTGGCTTCTT	Reference gene in *Chrysanthemum*
18S-R	GCGTTCAAAAACTCGATGGT	Reference gene in *Chrysanthemum*
Tubulin-F	GAGCCTTACAACGCTACTCTGTCTGTC	Reference gene in *Arabidopsis*
Tubulin-R	ACACCAGACATAGTAGCAGAAATCAAG	Reference gene in *Arabidopsis*

2.2.2. *CmIPT*1 Sequence Analysis and Construction of a Polygenetic Tree

Multiple comparison analyses were conducted between the protein sequences of gene code and homologous sequences of other species in the NCBI data base, using clustalX 1.8 and MEGA 5.05 software to establish maximum likelihood phylogenetic tree, adopting the neighbor-joining method [16], and by setting up bootstrap analysis repeated 1000 times.

2.2.3. Expression Analysis of *CmIPT*1 from Different Tissues of *Chrysanthemum* and after Removing the Shoot Top by Pruning

RNA was extracted from root, stem, leaf and apex of the plant respectively, using 2 μg of each, and after DNase digestion, cDNA template was obtained by conducting reverse transcription using M-MLV reverse transcriptase. Real-time fluorescent quantitative PCR (qRT-PCR) was used, adopting the ABI 7500 Fast

real-time PCR system and SYBR®Premix Ex Taq™ II fluorochrome. The response procedure as follows: 95˚C denaturation 90 s, 95˚C 15 s, 60˚C 30 s, 40 cycles. Data analysis was conducted using 7500 software (version 2.0.4). *Tubulin* was used as the reference gene, and the $2^{-\Delta\Delta CT}$ method was used to compute relative expression [17].

2.2.4. Construction of a *CmIPT*1 Overexpression Vector

Sal Ⅰ and HindIII cutting sites were respectively added in the upstream and downstream primer, *CmIPT*1. The full length sequence was amplified using M-MLV and ligated into the PJL-Blue vector. Sequencing was conducted by Sangon Biotech (Shanghai). The correct plasmid from sequencing was ligated into the PFK205 expression vector through LR reaction.

2.2.5. Gene Transfer and Phenotypic Analysis

The vector plasmid was transformed into (*Agrobacterium tumefaciens*) ASE, and was then transformed into *Arabidopsis thaliana* wild type col-0 using the floral dip method [18]. Transformants were selected using 1/2 MS which contained 50 $mg \cdot L^{-1}$ kanamycin, and selected T3 generation transgenic plants were transplanted into pots. RNA was extracted from rosette leaves of the control and T3 generation transgenic plants respectively. cDNA was synthesized using reverse transcription and the level of expression of *CmIPT*1 was determined. The number of lateral branches and plant height were recorded after culturing the plant for 40 days in growth chamber.

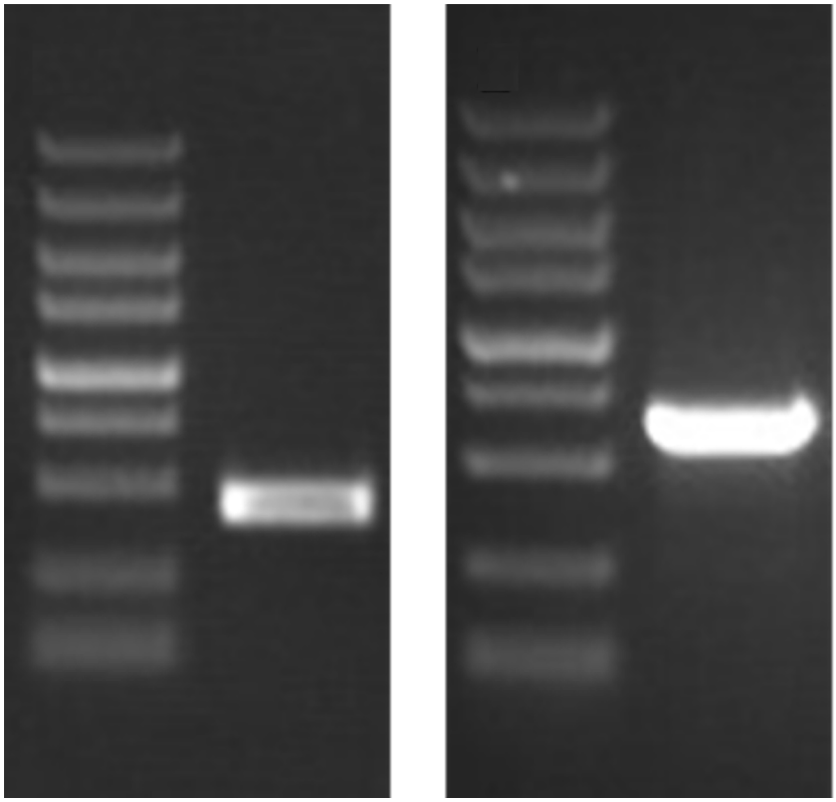
3. Results and Analysis

3.1. Cloning Full Length cDNA of the *CmIPT*1 Gene from *Chrysanthemum*

Based on the available *IPT*1 sequences from plant species such as *Brassica campestris*, *Arabidopsis thaliana*, *Raphanus sativus* and *Solanum tuberosum*, a 781 bp length DNA fragment was obtained from *Chrysanthemum morifolium* using PT1-PF/IPT1-PR. Amplification of the 5' end of the *CmIPT*1 gene was conducted using the gene-specific primer IPT1-5'F1/IPT1-5'R1, and a fragment with the length of 477 bp was obtained (**Figure 1(a)**). Amplifications of the 3' end sequence of the *CmIPT*1 gene were conducted using 3' Gene Racer outer and inner primers respectively with IPT1-3'F1 and IPT1-3'F2, and finally a 588 bp fragment was obtained with a poly A tail (**Figure 1(b)**). The 1615 bp *CmIPT*1 gene sequence was obtained by joining the intermediate fragment, the 3' end sequence and the 5' end sequences.

3.2. Sequence Analysis

The *CmIPT*1 gene with an overall length of 1155 bp was extracted from *Chrysanthemum* using the RACE method, and this gene encoded a protein with 384 amino acids. The predicted molecular weight was 43.41 kD and the theoretical isoelectric point was 8.68, using ExPaSy ProtParam online tool. The amino acid



(a) (b)

Figure 1. The amplification of *CmIPT*1. (a) Amplification results of 5'RACE; (b) Amplification results of 3'RACE.

sequence coded by the *CmIPT*1 gene (**Figure 2**) was compared with amino acid sequences of other plant species, and the result showed that *CmIPT*1 had the binding site GxxGxGKS sequence motif of ATP/ADP/AMP and DMAPP, which is the conserved domain of all *IPT* genes [10].

3.3. Phylogenetic Analyses of the IPT1 Homologous Protein

To confirm the genetic relationship between *CmIPT*1 and *IPT*1 from other plant species, multiple sequence alignment and phylogenetic analyses were conducted between *CmIPT*1 and *IPT*1 which had already been cloned from eight plant species such as *Cynara cardunculus* var. *scolymus*, *Lotus japonicus*, *Arabidopsis thaliana*, *Morus notabilis*, *Malus domestica*, *Medicago truncatula*, *Brassica napus* and *Solanum tuberosum* using clustal × 1.8 and MEGA 5.05 software (**Figure 3**). The results showed that the origin of *CmIPT*1 and *IPT*1 from other plant species was the same, and was separated together with the other plant species during periods of evolution. *CmIPT*1 was most similar to *CcIPT*1 of *Cynara cardunculus*, and was most distant from *StIPT*1 of potato.

3.4. Gene Expression Analysis of *CmIPT*1 in Different Tissues of *Chrysanthemum*

To reveal the expression features of *CmIPT*1 gene in Chrysanthemum, the

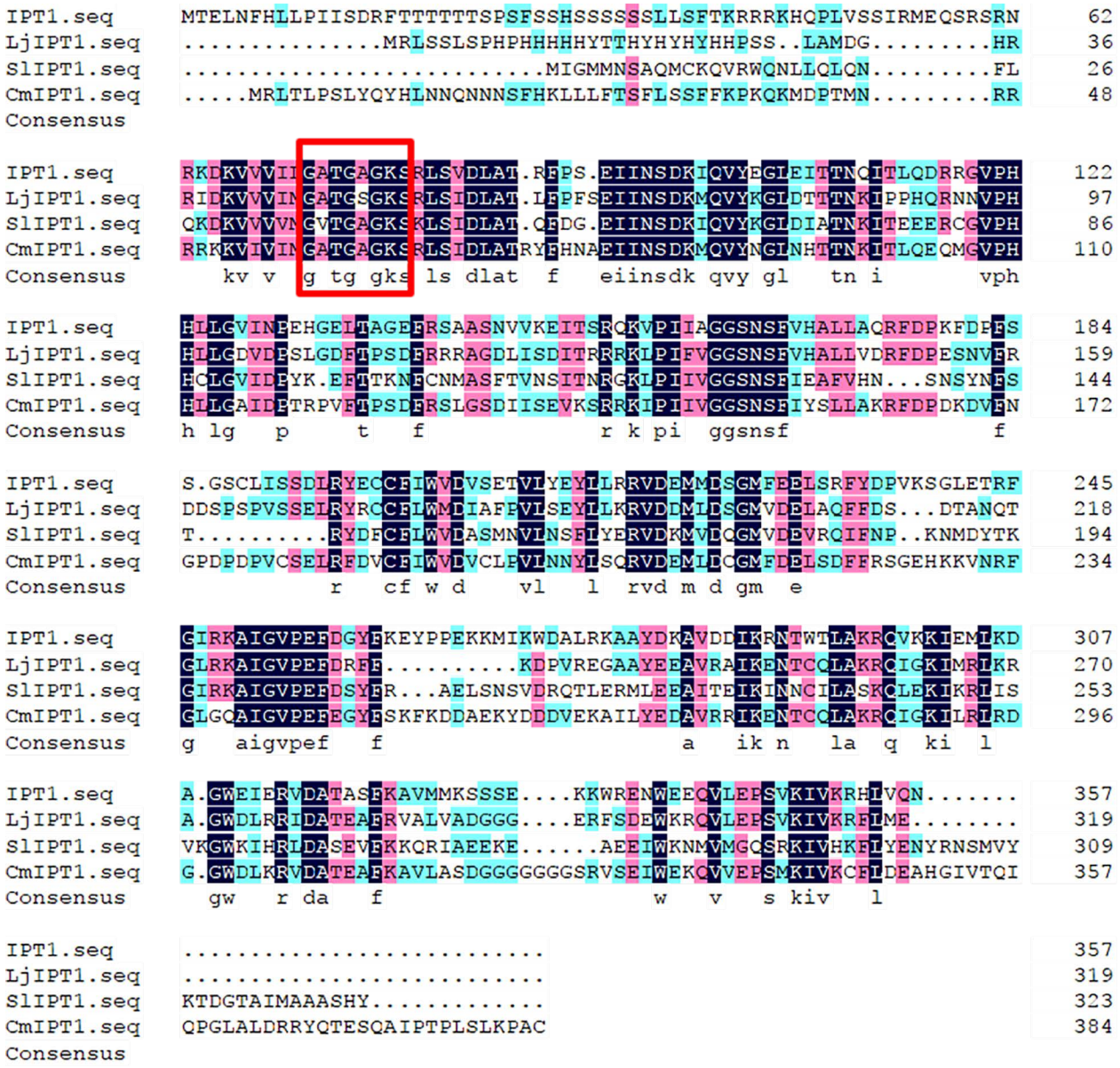


Figure 2. Alignment of the predicted amino acid sequences of *Chrysanthemum CmIPT*1 compared with *Arabidopsis thaliana* (*AtIPT*1), *Lotus japonicus* (*LjIPT*1) and *Solanum lycopersicum* (*SlIPT*1). Note: Black, red, and blue stands for completely conserved, partially conserved, and similar amino acids respectively. The conserved sequence is highlighted.

expression of *CmIPT*1 in different tissues of *C. morifolium* cv. 'Jinba' was detected. The results indicated that *CmIPT*1 gene was expressed in roots, stems, leaves and the apex of the plant. Expression of *CmIPT*1 in stem and apex was relatively high, while the expression in root and leaf tissues was relatively low (**Figure 4**).

3.5. Phenotypic Analysis of *CmIPT*1 Over-Expressed *Arabidopsis thaliana*

To construct a plant expression vector based on *CmIPT*1, the gene was transformed into wild type *Arabidopsis*, and six transgenic plants were obtained.

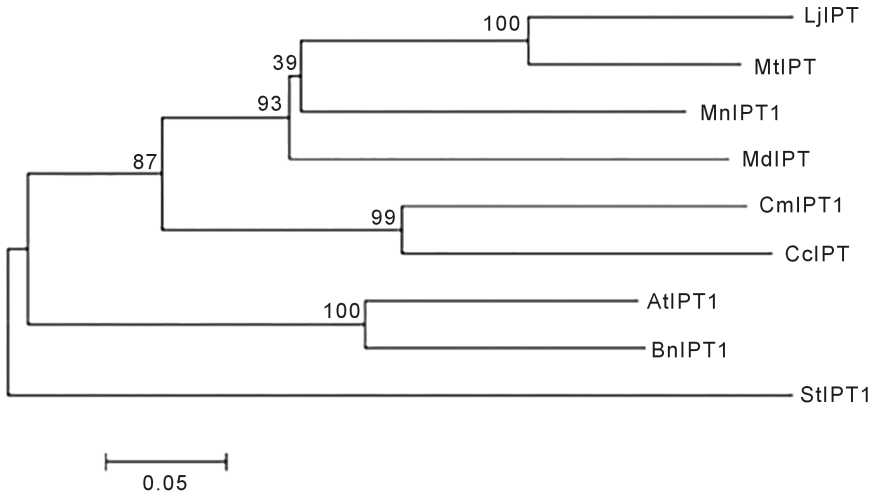


Figure 3. Phylogenetic analysis of IPT from different plant species. The sequence accession numbers of IPT from the different plant species are as follows: *Lotus japonicus LjIPT* (ABD93932), *Medicago truncatula MtIPT* (XP_00359265 3), *Morus notabilis MnIPT*1 (XP_010099238), *Malus domestica MdIPT* (ADY8 0561), *Cynara cardunculus* var. *scolymus CcIPT*1 (KVH96285), *Arabidopsis thaliana AtIPT*1 (B AB59040), *Brassica napus BnIPT*1 (XP_013691077), *Solanum tuberosum StIPT*1 (XP_006360459).

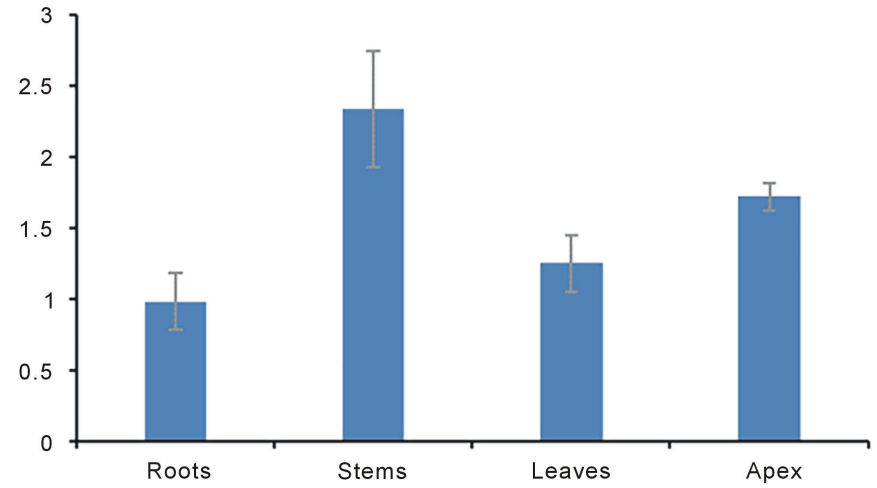


Figure 4. The relative expression of *CmIPT*1 in different tissues of *C. morifolium* cv. 'Jinba'.

*CmIPT*1 gene expression was detected in randomly selected transgenic plants. After 40 days growth, the number of lateral branches in wild type and transgenic plants was 3.5 and 6.3 respectively, with the number of lateral branches in transgenic plants being significantly greater. The mean heights of wild type and transgenic plants were 25.2 and 17.0 cm, respectively, with the transgenic plants being significantly shorter (Figure 5).

4. Discussion

Chrysanthemum is one of world's four major cut flowers. However, cut flower Chrysanthemums generally have many lateral branches. Controlling lateral

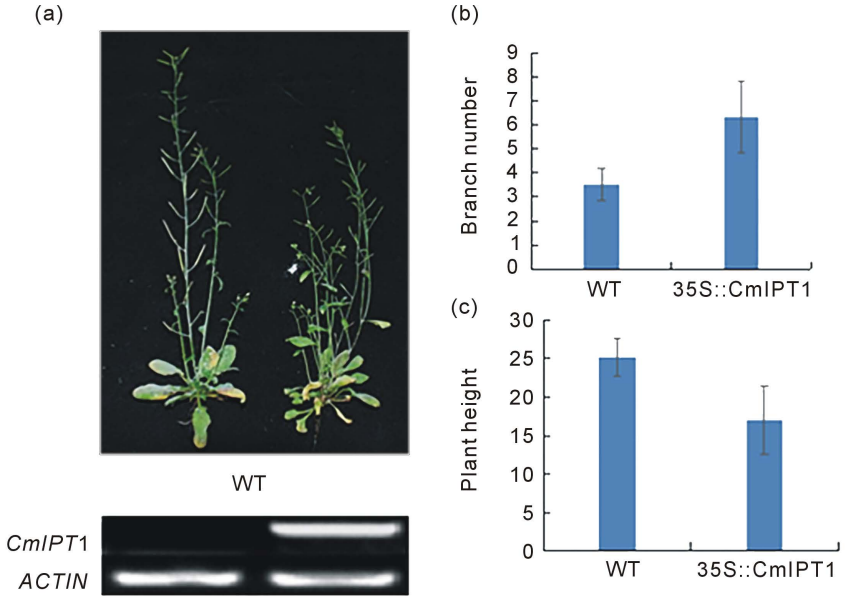


Figure 5. Comparison of number of branches and plant height of wild type *Arabidopsis* and transformants carrying 35*S*::*CmIPT*1. (a) Phenotype of wild type *Arabidopsis* and transformants carrying 35*S*::*CmIPT*1; (b) Branch number per plant of wild type Arabidopsis and transformants with 35*S*::*CmIPT*1; (c) Plant height of wild type Arabidopsis and transformants with 35*S*::*CmIPT*1.

branching using hormones has been very difficult, and doing it manually is a common practice, but is very time-consuming. For *C. morifolium* cv. 'Jinba', for instance, the cost of manual bud removal is 1/3 of the overall production cost. So, producing new varieties of *Chrysanthemum* with no lateral branches or with fewer lateral branches is the key to promoting the efficient production of this flower. In our study, a homologous gene of *IPT*1 from *C. morifolium* cv. 'Jinba' was cloned. The predicted amino acid sequence of the gene contains the conserved structural domain of *IPT* family genes, GATGAGKS, hence it was named *CmIPT*1. Phylogenetic tree analysis indicated that the origin of *CmIPT*1 and *IPT*1s from other plants was similar. The *Chrysanthemum IPT*1 gene *CMIPT*1 was most closely related to that of *Cynara cardunculus*, and was most distant from those of *Solanum tuberosum* and *Lotus japonicus*. *Asteraceae* family contains both *Chrysanthemum* and *Cynara*. So, some degree of closer relatedness is to be expected. After testing gene expression levels in root, stem, leaf, and apex of the Chrysanthemum plant, it was found that the *CmIPT*1 gene was expressed in all 4 different tissues, and the expression in stem and apex of the plant was relatively high while the expression was relatively low in root and leaf of the plant.

Direct application of cytokinin could accelerate the growth of axillary buds, and synthesis of cytokinin is controlled by auxin, so cytokinin is considered to be the second messenger of auxin [7]. The *CmIPT*1 gene was transformed into wild type Arabidopsis, and the number of lateral branches in transgenic plants was increased significantly, showing that that *CmIPT*1 could be the key gene

that controls lateral branch development in *Chrysanthemum* and t to produce single-stemmed plants a knockout mutant of *CmIPT*1 can be developed and tested.

5. Conclusion

In summary, after analyzing the expression features and functional analysis of *CmIPT*1 gene, it was found that there is a correlation between the expression of *CmIPT*1 and the number of lateral branches, and it therefore appears to be an important regulator of lateral branch development in *Chrysanthemum*. The *CmIPT*1 gene is worthy of further study, in order to provide insight into the possibility of breeding new varieties of cut-flower Chrysanthemum using molecular biology.

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