
Genetic Analysis of *AtPP16-1* and *AtPP16-2*

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Abstract

In plants, long-distance trafficking of macromolecules occurs through the phloem. CmPP16 is a 16-kD protein found in the pumpkin phloem. Several biochemical analyses showed that CmPP16 has the capacity to bind RNA and to move cell-to-cell depending on the interactions with NCAPP1 (Non-Cell Autonomous Pathway Protein 1); however, the in vivo function is still unclear. Since it is difficult to do genetic analyses on pumpkin plants, Arabidopsis was used due to its ease of genetic transformation, complete genomic information, and minimal resource requirement. To investigate the function of CmPP16 in Arabidopsis, AtPP16-1 and AtPP16-2, close homologues of CmPP16 in Arabidopsis were analyzed by using two genetic approaches. First, to overcome redundant function of AtPP16-1 and AtPP16-2, RNAi analyses were performed to knock-down the AtPP16 genes simultaneously. Because no phenotype was observed, T-DNA insertion lines were used. T-DNA insertion lines were obtained to observe the phenotypes of knock-out mutants. After the establishment of homozygous lines, the expression levels were measured by RT-PCR and no expression was detected. No single T-DNA insertion line showed any observable phenotype under normal growth conditions. Double KO plants for AtPP16-1 and AtPP16-2 were made by crossing single homozygous plants and screening by genomic PCR. One double KO candidate was found; however, no abnormal phenotype was observed. To further test for abnormal phenotype, double KO plants were grown under short-day and low temperature conditions. Under these conditions, the plants exhibited no abnormal phenotype. These results may be due to the AtPP16 genes' response to an environmental condition other than the ones tested, or to complementation of AtPP16 by other genes.

Introduction

In plants, long-distance transport of compounds occurs through the xylem and the phloem. The xylem is composed of dead cells and transports water and minerals from roots to leaf. In contrast, the phloem contains live cells, including enucleate sieve elements, that are sustained by companion cells through a pore known as the plasmodesmata. It has been widely accepted that the phloem functions to transport the products of photosynthesis and other nutrients via a pressure gradient. In recent studies, however, other compounds, including proteins and RNA, have been found to be selectively transported through the phloem. One such protein is 16-kD *Cucurbita maxima* Phloem Protein (CmPP16), which is found in the pumpkin phloem. It has been shown that CmPP16 is specifically expressed in the companion cell, binds RNA, and is transported cell-to-cell by interaction with NCAPP1 (Xoconostle-Cázares et al., 1999; Lee et al., 2003). A study of CmPP16 homologs in rice shows an expression pattern similar to that of CmPP16 (Asano et al., 2002). However, the biological function of CmPP16 or the homolog has not yet been elucidated. To investigate the *in vivo* function of CmPP16 using genetic methods, we used *Arabidopsis thaliana* Col-0 as the model plant in this study due to its short life cycle, ease of transformation and minimal resource requirement. The two closest homologs of CmPP16 were found in *Arabidopsis* and named *AtPP16-1* and *AtPP16-2*. Expression analysis showed that *AtPP16* genes are highly expressed in stem and flower. RNAi analysis was performed in order to observe the effect of knocking down both *AtPP16-1* and *AtPP16-2*. T-DNA insertion lines were used to observe the effects of individually knocking out either *AtPP16-1* or *AtPP16-2*. Single mutants did not show an observable phenotype. The T-DNA insertion lines were then crossed in order to observe the effects of knocking out both *AtPP16* genes simultaneously. Finally, all the plants were grown under several different conditions to observe the phenotype under different environmental conditions.

Materials and Methods

PLASMID CONSTRUCTION

For the RNAi construct, PCR Amplification of the ORF region of each gene was performed as follows: *AtPP16-1* was amplified using primers that were designed to contain a 5' restriction enzyme site and a 3' overlap with *AtPP16-2*, and *AtPP16-2* was amplified using primers that were designed to contain a 5' overlap with *AtPP16-1* and a 3' restriction enzyme site. PCR fragments were denatured, renatured and amplified again to ligate the PCR fragments. Amplified fragments were inserted into the *HindIII/XbaI* and *KpnI/EcoRI* sites of the pN6 vector (Wesley et al, 2001). The DNA sequence was confirmed by sequencing. The *NotI* fragment containing the 35S promoter, inverted repeat region and the OCS terminator was subcloned into pMLBART. The resulting plasmid was transformed into *Agrobacterium* C58C1.

PLANT TRANSFORMATION

Arabidopsis thaliana Col-0 was transformed by the dipping method of Logemann et al., 2006.

T-DNA INSERTION LINES

T-DNA insertion lines (SALK_003385, SALK_069424, SALK_059900) were obtained from the Salk Institute. T-DNA insertion was confirmed by genomic PCR using primers LBb1, 16-1-RS, 16-1-rt-F, 16-2-rt-F and 16-2-rt-R (Table 1). The T-DNA insertion point was confirmed by sequencing the PCR product.

Table 1. Primer sequences.

Primer	Sequence
16-1-RS	5'-GGTCGACCTAATCAACTTGGCTATGCTTCC-3'
16-1-rt-F	5'-TAGTCTGATCAGTGGCAAAGGTCTCAAGCGCTCT-3'
16-2-rt-F	5'-TGTTCTTGTTCAGCGCCAAAGGTCTCGAGGACGCA-3'
16-2-rt-R	5'-AGGACTCCTCGTCCATACCCCTGCTTCGGTTTTCC-3'
LBb1	5'-GCAAACCAGCGTGGACCGCTTGCTGCAACT-3'
MH-161-F	5'-TGGCTGTTGGAATCCTTGAGGTTAGTC-3'
MH-161-R	5'-CAACTTGGCTATGCTTCCATCCTCC-3'
MH-162-F	5'-CTCTTGAAGTTGTTCTTGTTCAGCGCCA-3'
MH-162-R	5'-CGCATCATCCTCTGTACCGACATCT-3'
At imp- α 5'	5'-ATGTCACTGAGACCCAACGCTAAGACGGAG-3'
At imp- α 3'	5'-GAATTAGTCGTTCAAGGGCGGGAAGAGCAG-3'

RT-PCR ANALYSIS

Total RNA was extracted from one month old *Arabidopsis thaliana* Col-0 plants with the Trizol reagent (Invitrogen) per the manufacturer's instructions. Total RNA was reverse transcribed to make cDNA using the Super Script III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. PCR was performed using the resulting cDNA and primers as listed in Table 2.

Table 2. RT-PCR Primer Sets.

mRNA	Primers	PCR product size
AtPP16-1	MH-161-F/MH-161-R	233 bp
AtPP16-2	MH-162-F/MH-162-R	465 bp
At imp- α	At imp- α -5'/At imp- α -3'	754 bp

PLANT GROWTH

Plants were grown under normal conditions (20°C, constant light), short-day conditions (20°C, 8h day, 16h night), or low temperature conditions (15°C, constant light).

Results

EXPRESSION ANALYSIS OF *AtPP16*

The two *Arabidopsis thaliana* genes that had highest similarity to CmPP16 were named *AtPP16-1* and *AtPP16-2* (Figure 1). To examine the tissue specific expression of *AtPP16*, total RNA was extracted from the flower, leaf and stem of one-month old wild-type *Arabidopsis thaliana* Col-0 and RT-PCR analysis was performed (Figure 2). Strong expression in stem and flower was observed for both *AtPP16-1* and *AtPP16-2*. In contrast, faint or no expression was observed in the leaf.

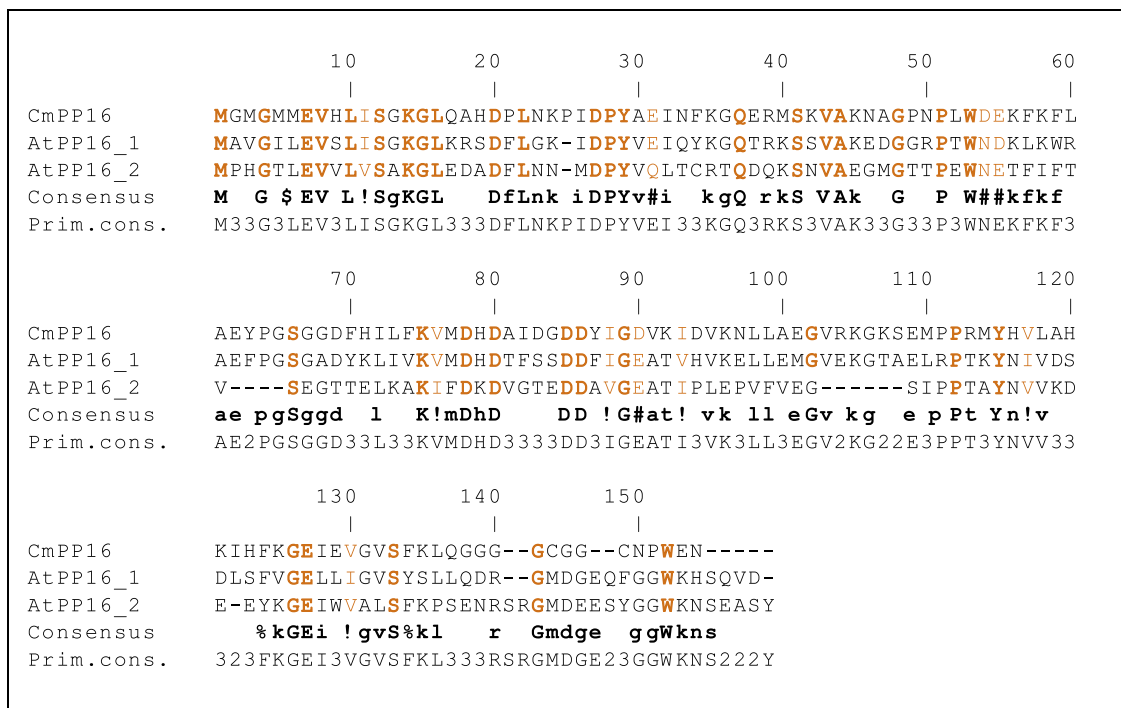


Figure 1. Protein Sequence alignment performed on CmPP16, *AtPP16-1* and *AtPP16-2*. Bolded Brown indicates conserved amino acids and brown indicates similar amino acids. Multalin (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_multalin.html) was used to perform alignment (Corpet, 1988).

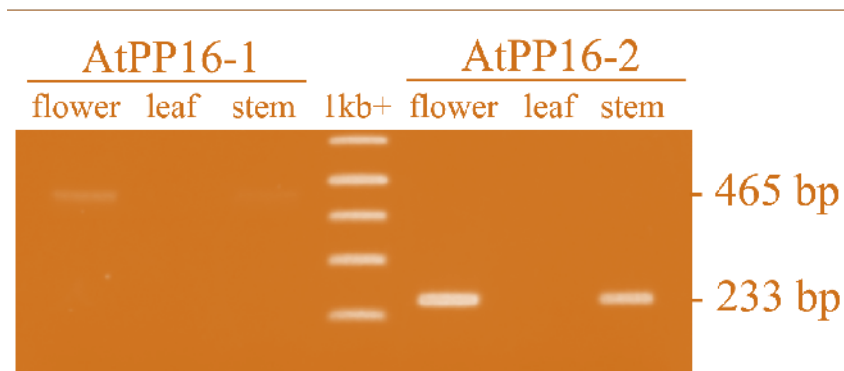


Figure 2. Tissue was collected from flower, leaf, and stem of wild-type *Arabidopsis* and RT-PCR was performed using RT-PCR primers. Strong expression was detected shown in the flower and stem tissues. 40 cycles of PCR were performed.

FUNCTIONAL ANALYSIS OF *AtPP16* BY RNAi APPROACH

Because of the high degree of similarity between *AtPP16-1* and *AtPP16-2*, the function could be redundant. To examine the *in vivo* function of the *AtPP16* gene, the RNAi approach was used to knock down *AtPP16-1* and *AtPP16-2* simultaneously. Wild-type plants were transformed with *Agrobacterium* containing the RNAi vector that was designed to express double-stranded RNA corresponding to *AtPP16-1* and *AtPP16-2*. After BASTA screening, five candidates of T1 plants were obtained. The T2 generation was used for observation.

When the plants were grown on soil under normal growth conditions, no abnormal phenotype was observed (data not shown). In order to determine whether environmental conditions would affect the phenotype, the plants were grown under short-day conditions or low temperature conditions; however, no abnormal phenotype was observed (data not shown).

FUNCTIONAL ANALYSIS OF *AtPP16* BY USING T-DNA INSERTION LINES

Two T-DNA insertion lines for *AtPP16-1* (Salk_003385 and Salk_069424) and one T-DNA insertion line for *AtPP16-2* (Salk_059900) were used for this analysis. To screen for homozygous insertion lines, genomic PCR was performed using primers as listed in Table 3. Plants with T-DNA insertion and no intact *AtPP16* gene were selected as homozygous T-DNA insertion lines. These plants were grown on soil under normal conditions and no abnormal phenotype was observed.

Table 3. Genomic PCR Primer sets.

	T-DNA	Primers	PCR Product Size
<i>AtPP16-1</i> (At3g55470)	SALK_003385	LBb1/16-1-RS	1.2 kb
	SALK_069424	LBb1/16-1-rt-F	0.3 kb
	WT	16-1-rt-F/16-1-RS	1.7 kb
<i>AtPP16-2</i> (At1g63220)	SALK_059900	LBb1/16-2-rt-R	0.6 kb
	WT	16-2-rt-F/16-2-rt-R	1.2 kb

To make double knock-out lines, the homozygous insertion lines for Salk_069424 and Salk_059900 were crossed. F2 plants were screened for double knock-out lines by genomic PCR. One candidate plant (#16) had T-DNA insertions in *AtPP16-1* and *AtPP16-2* and intact *AtPP16* was not observed (Figure 3). To examine whether the T-DNA insertion caused null allele or not, RT-PCR analysis was performed. In the #16 plant, no *AtPP16* RNA expression was observed (Figure 4). The #16 plant was grown on soil under normal conditions and no abnormal phenotype was observed (Figure 5).

To examine whether environmental conditions would affect the phenotype, the plants were grown under short-day conditions or low temperature conditions; however, no abnormal phenotype was observed (data not shown).

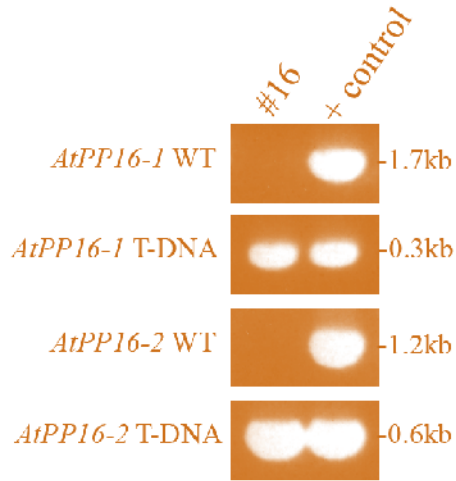


Figure 3. Genomic PCR was performed on leaf tissue from double homozygous candidate #16. T-DNA insertion points were detected; however, intact wild-type *AtPP16* genes were not detected. 40 cycles of PCR were performed.

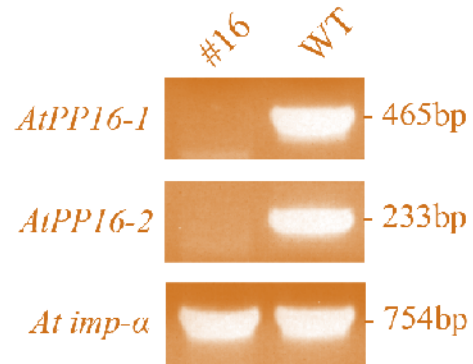


Figure 4. RT-PCR was performed on tissue collected from double homozygous candidate #16. mRNA was not detected in #16 plant for the *AtPP16* genes and was detected in wild-type plant. 40 cycles of PCR were performed.



Figure 5. A comparison of wild-type plant and double homozygous #16 plant shows no observable phenotype in #16 plant.

Discussion

Two genes in *Arabidopsis* showed the highest similarity to CmPP16 and were named *AtPP16-1* and *AtPP16-2*. These two genes are highly expressed in the stem and flower. The double knock-out plant for *AtPP16* genes showed no observable phenotype under normal, short-day and low temperature conditions.

The lack of an observable phenotype in the double KO plant grown under the three conditions shows that the loss of *AtPP16* genes is not detrimental to the plant. This could result from the *AtPP16* genes being involved in a stress response other than the ones tested. Follow-up experiments should be performed to test additional environmental conditions. CmPP16 is shown to have close homology with Rpp16 and Rpp17. These genes are also reported as Os-FIERG1 and Os-FIERG2 (*Oryza sativa* fungal elicitor immediate early responsive genes 1 and 2). This suggests that *AtPP16* genes may play a role in the response to pathogen infection, and thus should be tested under this condition.

The lack of a phenotype in double KO plants could also be a result of functional complementation by other redundant genes in *Arabidopsis*. A homology search for CmPP16 in the *Arabidopsis* genome shows that *AtPP16-1* and *AtPP16-2* are most closely related; however, other genes with lower similarity may have redundant functions. Phylogenetic tree analysis shows that another gene, At5g47710, is closely related to the clade of the CmPP16 subfamily (Figure 6). Thus, a follow-up experiment could be performed to knock out *AtPP16-1*, *AtPP16-2*, and At5g47710 simultaneously and to observe the phenotype.

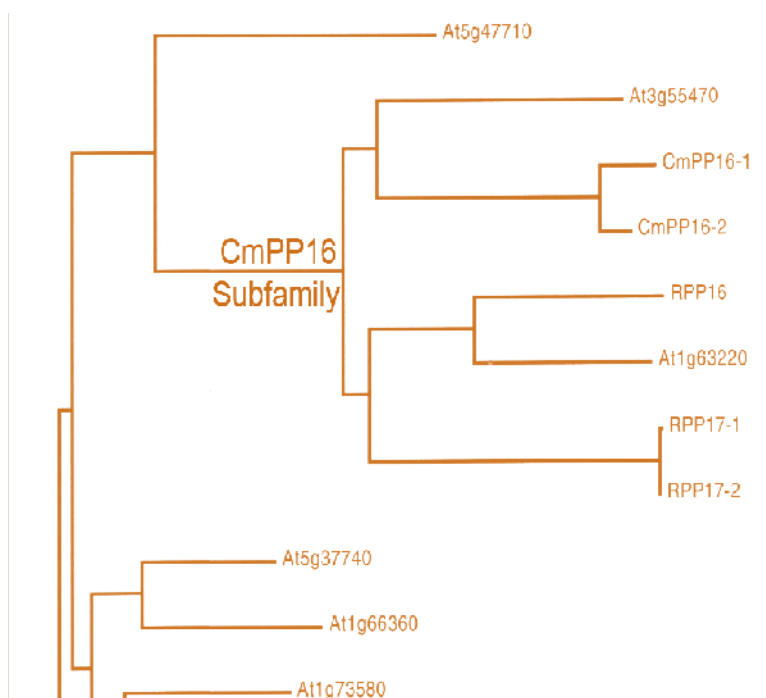


Figure 6. Phylogenetic tree analysis shows *AtPP16-1* and *AtPP16-2* within the CmPP16 Subfamily clade. At5g47710 is most closely related to the CmPP16 Subfamily clade.

Acknowledgments

I would like to thank Dr. William J. Lucas for providing the space, resources and guidance for my project, and Dr. Ken-Ichiro Taoka for providing a wealth of knowledge and advice throughout this learning experience.

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