

Chapter 2

Structure and Expression Pattern Analysis of *Arabidopsis PIP5K2*

Abstract Phosphatidylinositol monophosphate 5-kinase (PIP5K) which catalyzes the synthesis of phosphatidylinositol-4,5-bisphosphate by phosphorylation of phosphatidylinositol-4-phosphate at the 5' position of the inositol ring, is a key enzyme in phosphatidylinositol signaling pathway. In *Arabidopsis thaliana*, 15 genes encoding putative PIP5Ks have been identified. Among those, four are highly conserved with FAB1 from yeast and classified as type III PIP5K. The remaining 11 members can be further divided into two groups, group A and B according to protein structure. The *PIP5K2* is 2265-bp long and encodes a 754-amino acid peptide (~86.3 kDa). The protein contains seven MORN motifs at the N terminus and a C-terminal localized catalytic domain, which is similar to the structure of other type B PIP5Ks in *Arabidopsis*. Expression pattern studies reveal that *PIP5K2* is expressed in various tissues including roots, flowers, leaves, and seedlings. *PIP5K2* expression is especially strong during lateral root initiation and elongation and its expression is enhanced by exogenous auxin and NaCl treatment. GFP fusion study revealed that PIP5K2 is mainly localized on the plasma membrane.

Keywords *Arabidopsis thaliana* • Phosphatidylinositol monophosphate 5-kinase 2 • Expression pattern

2.1 Introduction

Phosphatidylinositol signaling pathway plays crucial roles in plant development and stress response [26, 31, 32]. In this pathway, phosphatidylinositol phosphate 5-kinase (PIP5K) is one of the key enzymes which catalyzes the synthesis of phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P₂]. PtdIns(4,5)P₂ is hydrolyzed to generate two important second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] [2, 19]. As a multifunctional molecule, the function of PtdIns(4,5)P₂ is far beyond just serving as the precursor of DAG and Ins(1,4,5)P₃. Researches in both animals and plants have demonstrated that PtdIns(4,5)

P₂ itself can be involved in different cell processes by binding with different protein partners, including actin-regulating proteins [5, 12, 28, 29], ion channels [14, 17, 27] and vesicle trafficking regulators [1, 3, 7, 8, 13].

Arabidopsis has 15 predicted PIP5Ks genome wide, among which four are highly conserved with FAB1 from yeast and classified as type III PIP5K. The remaining 11 members can be further divided into two groups, group A and B. Group B contains nine members all of which contain MORN motif at their N terminus [22]. Physiological functions of several PIP5Ks have been investigated and reported. PIP5K1 is considered to play a role in abscisic acid signaling since it is induced by water stress and abscisic acid treatment [20]. PIP5K3, which is strongly expressed in root hairs, plays a critical role in root hair formation and growth [10, 25]. PIP5K4 was first found to be critical in stomata opening [11]. Later it is found that three PIP5Ks, PIP5K4, 5, and 6 are all involved in pollen tube growth [9, 24, 33]. In addition, PIP5K9 which is the only *Arabidopsis* PIP5K with a nuclear localization signal peptide regulates root growth and is involved in sugar signaling [16].

PIP5K2 analyzed in this study belongs to group B and further in a subgroup with PIP5K1 and PIP5K3. Biochemical activity of PIP5K2 has already been reported. The recombinant PIP5K2 expressed in *E. coli* system showed substantial activity converting PtdIns4P to PtdIns(4,5)P₂ and also some activity converting PtdIns3P to PtdIns(3, 4)P₂, indicating PIP5K2 is an active enzyme that should be responsible for at least part of PtdIns(4,5)P₂ production in vivo.

The gene and protein structure, expression pattern, and subcellular localization of PIP5K2 are studied in this chapter. Part of this chapter is published on Cell Research [18].

2.2 Materials and Methods

2.2.1 Plant Material

Arabidopsis thaliana ecotype Columbia was used in the study.

2.2.2 Strains and Plasmids

2.2.2.1 Strains

XL1-Blue: *E. coli* strain used for plasmid transformation;

GV3101: *Agrobacterium* strain used for *Arabidopsis* transformation.

2.2.2.2 Plasmid Vectors

pMD18-T: plasmid vector from Takara, used for cloning;
pCAMBIA1300+pBI101: binary vector modified from pCAMBIA1300, used to generate promoter-GUS reporter construct for gene expression pattern analysis;
pA7-GFP: a gift from Prof. Bernd Mueller-Roeber, used for protein subcellular localization analysis.

2.2.3 Chemicals and Reagents

IAA: Indole-3-acetic acid, Sigma-Aldrich, Missouri, USA, Cat. No. I2886;
X-Gluc:5-Bromo-4-chloro-3-indolyl-D-glucuronide, Sigma-Aldrich, Cat. No. B3783;
Restriction endonucleases: all from MBI Fermentas;
DNA polymerases: from Takara;
RNA extraction reagent Trizol: from Invitrogen;
Reverse transcription kits and Real-time PCR kits: from Toyobo.

2.2.4 Methods

2.2.4.1 *Arabidopsis* Growth Conditions

Arabidopsis seeds were surface sterilized with 20 % (v/v) bleach and sown on Murashige and Skoog (MS) plates. One-week-old seedlings were transferred to soil and grown at 22 °C under a 16-h light/8-h dark photoperiod.

2.2.4.2 Agrobacterium-Mediated *Arabidopsis* Transformation

Arabidopsis transformation was performed using the floral dip method [4]. In detail, agrobacteria strain GV3101 harboring proper vector were cultured overnight at 28 °C. After centrifuging for 10 min at 4,500 rpm, the agrobacteria were resuspended in freshly prepared transformation buffer. The upper ground part of the *Arabidopsis* plants were dipped into the buffer for 10 s. Treated plants were kept in dark for overnight and then maintained under normal conditions for seed collection.

2.2.4.3 RNA Extraction, RT-PCR, and Quantitative Real-Time RT-PCR Analysis

Various tissues from wild-type *Arabidopsis* plants were sampled for RNA extraction. Plant materials (~100 mg) were ground in liquid nitrogen and transferred into 1.5 mL tubes. 1 mL Trizol was added to each sample, mixed well, and kept for 5 min at room temperature. After centrifuging for 10 min at 12,000 rpm, the supernatant was transferred to a new tube and mixed well with 200 μ L chloroform. After centrifuging for 10 min at 12,000 rpm, the supernatant was transferred into a new tube and mixed with 500 μ L isopropanol for RNA precipitation. Samples were centrifuged at 12,000 rpm for 10 min and then the precipitate was washed with 70 % ethanol, vacuum dried, and then dissolved in 20–50 μ L H₂O (RNase free).

First strand cDNA synthesis was performed using reverse transcription kit from Toyobo according to manufacturer's protocol. RT-PCR analysis was carried out using gene-specific primers to test the expression level of PIP5K2. The *Arabidopsis ACTIN2* gene was included as an internal control.

Real-time RT-PCR analysis was performed to study the transcription levels of PIP5K2 in response to exogenous auxin and NaCl treatment. Ten-day-old wild-type seedlings treated with IAA or NaCl by transferring them into liquid MS media containing 100 μ M IAA or 200 mM NaCl for 0, 1, 3, 6, and 12 h, were harvested for RNA extraction. PCR amplification was executed with the RotorGene 3000 system (Corbett Research) using the SYBR green detection protocol (Toyobo, Tokyo, Japan). The *Arabidopsis ACTIN7* gene was used as an internal control, and differences in product levels among the tested samples during the linear amplification phase were used to calculate differential gene expressions. Primers used are as follow PIP5K2 (5'-GAAGAATGAGTTGATTGTTGCGAC-3' and 5'-AGAT-AGATGCGGTGGTGTGGTC-3'), and ACTIN7 (5'-TTCCCGTTCTGCGGTAG TGG-3' and 5'-CCGGTATTGTGCTCGATTCTG-3').

2.2.4.4 Promoter-GUS Fusion Studies and Histochemical Analysis of GUS Activity

The ~1.9-kb PIP5K2 promoter region was amplified by PCR using primers PIP5K2p-1 (5'-CCCAAGCTTATCATTACCTCGTGCTCTTCA-3', added *Hind*III site underlined) and PIP5K2p-2 (5'-CGGGATCCGATTACGGATTAGGGTGA-3', added *Bam*HI site underlined) and *Arabidopsis* genomic DNA as the template. The amplified DNA fragment was subcloned into pCAMBIA1300+pBI101.1 [15] and the resultant construct was transformed into *Arabidopsis*. Positive transgenic plants were selected through hygromycin resistance screening, and independent lines of T2 and T3 homozygous progeny were used to detect GUS activity. Photography was performed using a Nikon microscope (SMZ800) with a digital camera (Nikon, Coolpix 4500).

2.2.4.5 Subcellular Localization Analysis

Full-length cDNA of *PIP5K2* was amplified by PCR using primers PIP5K2-1 (5'-GTCGACATGATGCGTGAACCGCTTG-3', added *SalI* site underlined) and PIP5K2-2 (5'-GTCGACGCCGTCCTTCGATGAAGATTCTG-3', added *SalI* site underlined) and cloned into pA7-GFP vector to generate the pA7-PIP5K2-GFP construct. Plasmids were delivered into onion cells by particle bombardment using PDS-1000/He Biolistic Particle Delivery System (BioRad, California, USA). The parameters were set as follows: 1,100 psi rupture disk; 27 inch Hg vacuum; 6 cm distance from the stopping screen to the target tissues. 1.5 µg plasmid DNA was used for each macrocarrier. Onion samples were kept in dark for 24 h at 25 °C after bombardment and then observed under a confocal laser-scanning microscope with an excitation wavelength of 488 nm (Zeiss LSM 510 META).

2.3 Results

2.3.1 Structural Analysis of *PIP5K2* Gene and Protein

The gene that encodes PIP5K2 in *Arabidopsis* genome is located on the first chromosome. The accession number is *At1g77740*. The full length of this gene is 3324 bp, which contains eight exons and seven introns. The encoding region is 2265-bp long (Fig. 2.1).

PIP5K2 encodes a 754-amino acid peptide with a molecular weight of about 86.3 kDa and a predicted isoelectric point at 8.5 (http://www.expasy.ch/tools/pi_tool.html). No hydrophobic transmembrane domain was predicted by hydrophobicity analysis (<http://www.expasy.ch/tools/protscale.html>) (Fig. 2.2).

Protein motif analysis was performed using SMART software [23]. The result showed that the protein contains seven MORN motifs at the N terminus (80–239 aa) and a C-terminal localized catalytic domain (382–571 aa) (Fig. 2.3). This structure is highly similar to other type B PIP5Ks in *Arabidopsis*.

The nine type B PIP5Ks in *Arabidopsis* can further be divided into three subgroups according to the evolutionary relationship. PIP5K1, 2, and 3 are in the same subgroup [21]. When comparing these three proteins at the amino acid level, it is

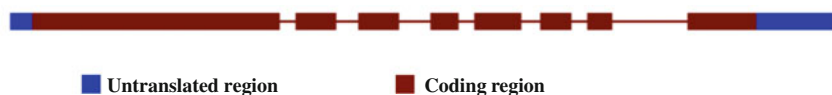


Fig. 2.1 Schematic map of the *PIP5K2* gene. Exons (brown boxes), introns (lines), and untranslated regions (blue boxes) are indicated. Reprinted from Mei et al. [18] @ Nature Publishing Group. Reprinted by permission (Color figure online)

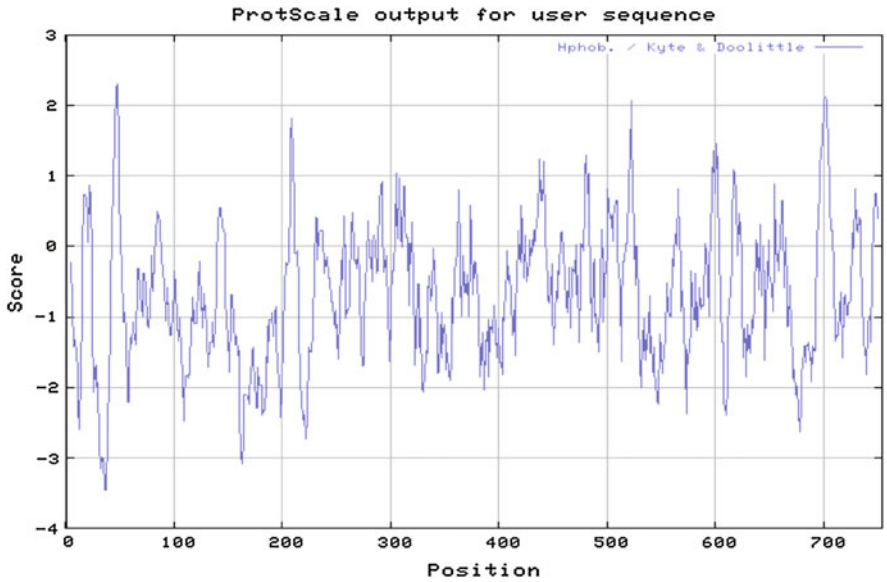


Fig. 2.2 Hydrophobicity prediction analysis of PIP5K2 protein

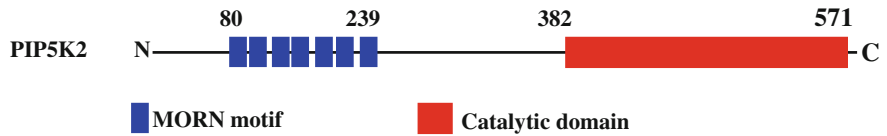


Fig. 2.3 Structure organization of PIP5K2 protein. MORN motifs (blue boxes) and catalytic domain (red box) are indicated. Reprinted from Mei et al. [18] @ Nature Publishing Group. Reprinted by permission (Color figure online)

found that PIP5K2 has the longest N terminus; the N-terminal MORN motif and C-terminal catalytic domain are very much conserved while it is relatively less conserved in the middle region (Fig. 2.4).

2.3.2 Expression Pattern Analysis of PIP5K2

To get clues for investigating the physiological functions of PIP5K2, the expression pattern of this gene was first analyzed. RT-PCR analysis was performed using gene-specific primers. The results showed that *PIP5K2* was expressed in various organs including roots, inflorescences, flowers, and leaves (Fig. 2.5).

Detailed expression pattern was investigated by promoter-reporter gene fusion studies. The promoter region of *PIP5K2* (~1.9 kb upstream of ATG) was obtained

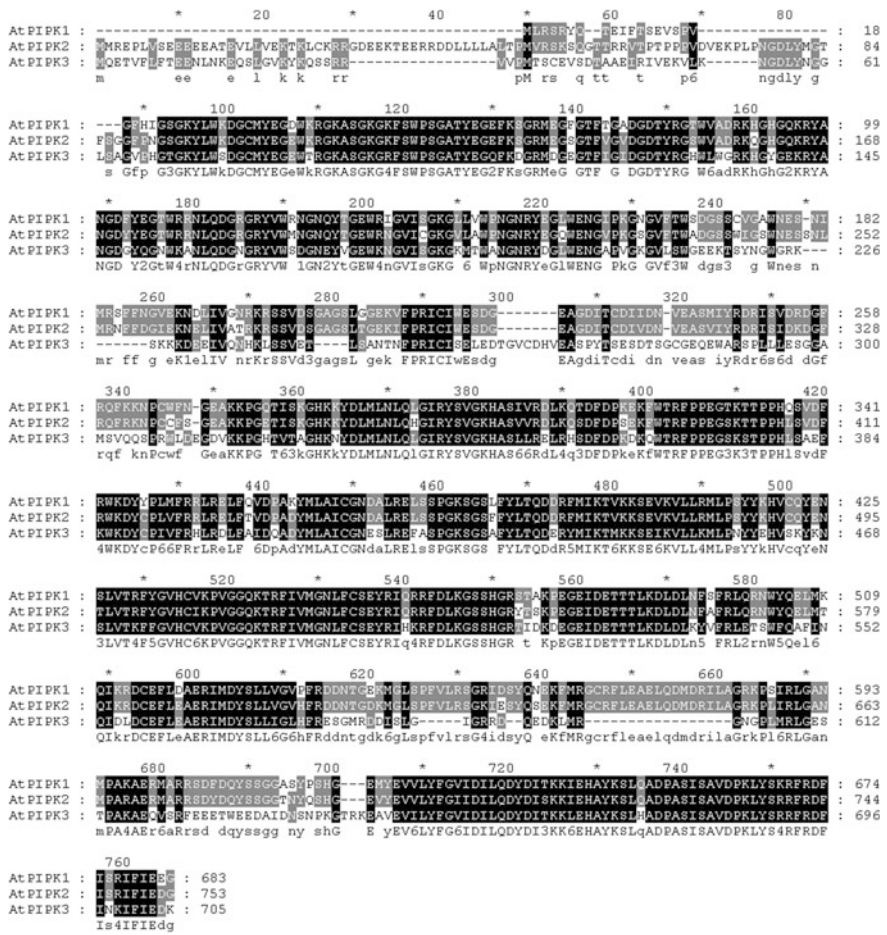
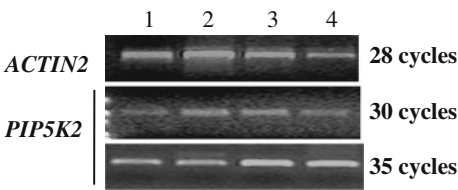


Fig. 2.4 Alignment of PIP5K2 with PIP5K1 and PIP5K3

Fig. 2.5 RT-PCR analysis showing *PIP5K2* expression in various tissues. 1 root, 2 inflorescence, 3 flower, 4 leaf



by PCR and cloned into promoter-GUS binary vector and transformed into *Ara-bidopsis*. After several generations of antibiotic screening, independent GUS-positive transgenic lines were obtained. GUS analysis was carried out on multiple

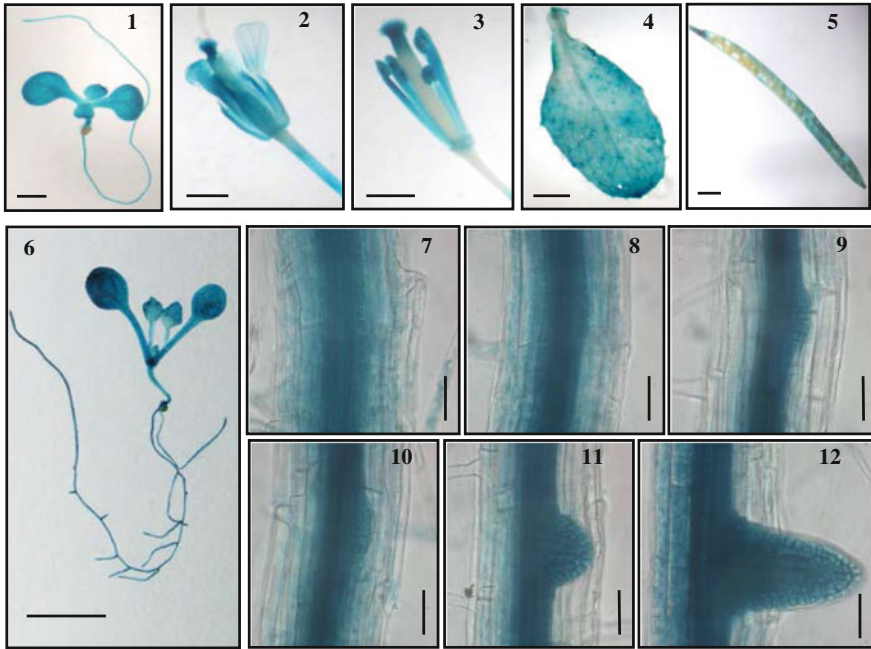


Fig. 2.6 Promoter-GUS fusion analysis of the expression of PIP5K2. Expression of PIP5K2 is detected in young seedlings (1), floral tissues (2, 3), leaves (4), and siliques (5). Strong expression of PIP5K2 is detected in the central cylinder, lateral root primordia, and emerged lateral roots at developmental stage A (7, 8), B (9–11), C (12), and D (6). Scale bar = 1 mm (1–5), 0.5 cm (6), or 50 μ m (7–12). Reprinted from Mei et al. [18] @ Nature Publishing Group. Reprinted by permission

independent lines. The results confirmed that the promoter region of PIP5K2 is able to drive GUS expression in cotyledons, hypocotyls, roots, leaves, flowers, and siliques but not in the developing seeds. In roots, strong expression is detected in lateral root primordia and emerged lateral roots of every developmental stage (Fig. 2.6).

PLACE software was used to scan the PIP5K2 promoter region and multiple *cis* elements that are associated with stress and disease response. ABA, GA, and auxin response were found in the promoter region, suggesting possible regulation of PIP5K2 expression by these factors. Further analysis using Real-time RT-PCR showed that PIP5K2 expression was significantly induced by 100 μ M IAA and 200 mM NaCl treatment for 1 h. The induction maintained during the 12 h treatment with a peak at 1 h (Fig. 2.7). These results suggest that PIP5K2 may be involved in an auxin-related process and the response to external salt stimuli.

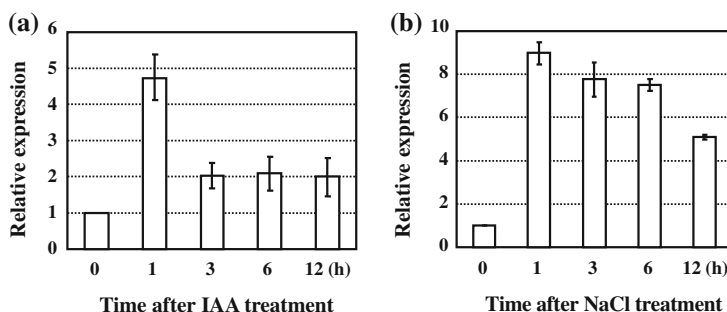


Fig. 2.7 *PIP5K2* expression is induced by exogenous IAA and NaCl treatment. Real-time RT-PCR analysis of *PIP5K2* expression after 100 μ M IAA (a) and 200 mM NaCl (b) for 0, 1, 3, 6, and 12 h. Expression level before treatment (0 h) is set as 1.0. $n = 3$

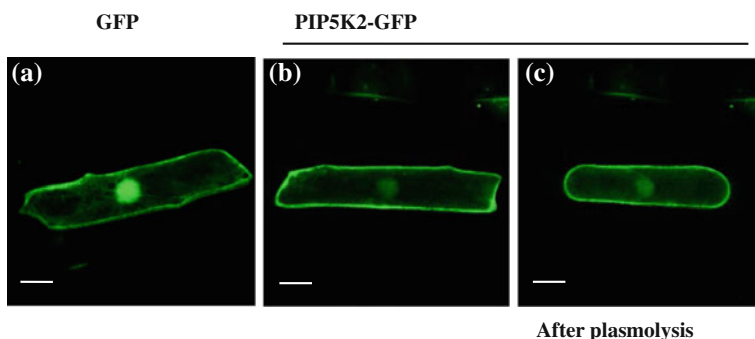


Fig. 2.8 Subcellular localization of PIP5K2. **a** Onion epidermal cell harboring pA7-GFP empty vector; **b**, **c** Onion epidermal cell expressing PIP5K2-GFP fusion protein; and **c**, Cell in **b** after plasmolysis. Bar = 50 μ m

2.3.3 *PIP5K2* Is Mainly Localized on the Plasma Membrane

To study the subcellular localization of PIP5K2, the full-length coding region was amplified by PCR and cloned into pA7 vector where it is fused to GFP. The obtained fusion expression vector was delivered into onion epidermal cells by particle bombardment for transient expression analysis. The empty vector was included as control. Here, the subcellular localization of PIP5K2 is reflected by GFP signal distribution. Observation under confocal microscopy revealed that the green fluorescence signal in control cells is dispersed in the cytoplasm, nuclei, and on the plasma membrane; however, green fluorescence in the cells expressing PIP5K2-GFP fusion protein is mainly detected on the plasma membrane, which is more obvious in the cells after plasmolysis (Fig. 2.8). This is similar to the subcellular localization of other PIP5Ks that are reported [10, 25]. It is worth noting that green

fluorescence is also detected in the nuclei, which indicates that PIP5K2 may be also responsible for the synthesis of PtdIns(4,5)P₂ in the nuclei.

2.4 Discussion

The activity of *Arabidopsis* PIP5Ks has been examined in either bacteria or insect cells. Recombinant PIP5K1 expressed from *E. coli* uses PtdIns3P and PtdIns4P as substrates producing PtdIns(3, 4)P₂ and PtdIns(4,5)P₂, respectively, and no further phosphorylation of PtdIns(3, 4)P₂ is observed [30]. However, when PIP5K1 is expressed in insect cells, PtdIns4P is converted into PtdIns(4,5)P₂. Besides, PtdIns(3, 4)P₂ is also phosphorylated at the 5'-OH generating PtdIns(3,4,5)P₃ [6]. The reason for this discrepancy is not clear.

The activities of other root-expressing PIP5Ks were tested using recombinant proteins expressed in *E. coli*. It is found that the activity of PIP5K2 converting PtdIns4P into PtdIns(4,5)P₂ is only lower than PIP5K3 and is much higher than other isoforms [25]. Expression pattern studies showed that *PIP5K2* is widely expressed in various tissues. In roots, *PIP5K2* is strongly expressed in central cylinder, lateral root primordia, and emerged lateral roots. This is very much different from the expression pattern of *PIP5K3* which is only strongly expressed in root epidermal cells and root hairs. In consistence with its strong expression in root hairs, PIP5K3 is proven to be essential for root hair development [25]. So it is reasonable to speculate that PIP5K2 may have functions in lateral root development and may also be involved in auxin-related process or response to salt stress based on the expression pattern revealed in this study.

Subcellular localization analysis demonstrates that PIP5K2 is mainly localized on plasma membrane but also in the nucleus, indicating PIP5K2 may be also responsible for the synthesis of PtdIns(4,5)P₂ in the nuclei. Indeed, although phosphatidylinositol pathway is mainly on plasma membrane, its existence in the nucleus has been reported in mammalian cells. *Arabidopsis* PIP5K9 is also reported to be partially localized in the nucleus with specific accumulation in nuclear speckles [16]. Whether PtdIns(4,5)P₂ is also produced in the nucleus of plant cells and the function of it in the nucleus are interesting questions to be investigated in the future. If this is proven true and PIP5K2 and/or PIP5K9 are involved in PtdIns(4,5)P₂ production in the nucleus, these two PIP5K2 might still have different functions based on different localization pattern.

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