Pak. J. Bot., 49(1): 143-146, 2017.

EXPRESSION AND THE SUBCELLULAR LOCALIZATION OF ATPUB19, A U-BOX E3 LIGASE IN *ARABIDOPSIS*

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Abstract

Plant U-box containing proteins (PUBs) belong to a new type of E3 ligase and have diverse roles in plant development and growth. In our previous study, we found that *PUB19* negatively regulated the ABA-induced stomata closure, stress-related genes expression and drought tolerance. To obtain more information on *PUB19*, the expression pattern and subcellular localization were analyzed. Northern blot and GUS staining were performed to detect the transcripts of *PUB19* in roots, stems, leaves, siliques and flowers. Both the distribution of GFP and cell fractionation assay displayed that PUB19 is a membrane-associated protein. All results showed some good findings to study the biological function of PUB19 deeply.

Key words: PUB19, Expression pattern, Subcellular localization, *Arobidopsis*.

Introduction

Ubiquitin-mediated pathway is involved in many biological process including lateral root formation, the response to hormone, phosphate starvation, biotic and abiotic stress (Xie *et al*., 2002; Gonzalez *et al*., 2006; Luo *et al*., 2006; Wang &Deng, 2011). Ubiquitination of substrates is done in presences of the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme(E2) and the ubiquitin ligase (E3)(Hatakeyama & Nakayama, 2003). In *Arabidopsis*, it is found that more than 1400 genes encode the ubiquitin ligase (E3), whereas there are 2 and 45 genes encoding E1 and E2, respectively (Smalle &Vierstra, 2004). According to subunits and mechanisms of action, E3 ligases were divided into four different groups including HECT, SCF, APC, and RING/U-box (Vierstra, 2009).

U-box containing proteins have been identified in *Arabidopsis*, rice, tobacco, yeast and human. 64 and 77 genes were found to encode U-box E3 ligases in *Arabidopsis* and rice, respectively. Such a larger number of U-box ubiquitin ligases in plants manifested the important role of this gene family in plant growth and development. ARC1in *Brassica napus* and PUB8 in *Arabidopsis thaliana* are associated with self-compatibility and Pseudo-self-compatibility (Stone *et al*., 2003; Liu *et al*., 2007). PUB44/SAUL was found to negatively regulated the stability of AAO3, which was very important to abscisic acid (ABA) biosynthesis (Raab *et al*., 2009; Drechsel *et al*., 2010). *SiPHOR1* is a positive regulator in GA pathway and down-regulation of it can generate GA-deficient phenotype (E. Monte *et al*., 2003; Amador *et al*., 2001). Many U-box containing proteins also play essential role in the response to pathogen in plants, such as SPL11, CMPG, ACRE276, PUB12, PUB13, PUB59 and PUB60 (Zeng *et al*., 2004; Yang *et al*., 2006; Monaghan *et al*., 2009; Lu *et al*., 2010). In addition to self-compatibility, the response to phytohormone and biotic stress, several PUBs were found to be involved in abiotic stress, such as PUB1 in pepper, PUB18, PUB19, PUB22, PUB23 in *Arabidops*is, and PUB15 in rice (Cho *et al*., 2006; Cho *et al*., 2008; Liu *et al*., 2011; Park *et al*., 2011; Berglerc & Hoth, 2011; Seo *et al*., 2012). All results above displayed that U-box containing ligases had diverse functions.

In our previous study, we showed that *PUB19* negatively regulated the ABA-induced stomata closure, stress-related genes expression and drought tolerance (Liu *et al*., 2011). Here, we discuss the expression pattern of *PUB19* and the subcellular localization of PUB19, which will enrich the understanding of this new member of U-box containing protein family.

Materials and Methods

Plant materials , RNA isolation and northern-blot: Wild-type *Arabidopsis* (*Arabidopsis thaliana*, ecotype Columbia-0) was used in this study. Seeds were surface-sterilized using 10% NaClO solution for 15 min and washed five times with sterilized water. The seeds were sown onto 1/2 MS medium with 1.5% (w/v) sucrose and 1% (w/v) agar, pH 5.8. The plates were then kept in the dark at 4°C for stratification for 3 days. The seeds were germinated at 22°C under a 16:8-h day:night light cycle. Ten days later, seedlings were transferred to soil and grown to maturity. Different organs were harvest at appropriate time for total RNAs extraction and GUS staining.

Total RNA was isolated using the hot phenol method (Xie *et al*., 1999), and 10 μg was applied in each lane for RNA gel analysis with the a-^{32}p-labeled *PUB19*-specific probe to check the expression level of *PUB19* in different organs. The *PUB19*-specific probe was amplified by PCR using primers: 5'-TGATCTTCGTTGTCCGATTTC-3' and 5'-CGAAGAATCTTCATCAACGATTCA-3'

Analysis of *PUB19* promoter activity: A 1700-bp promoter of the *PUB19* was amplified from genomic DNA of *Arabidopsis* by PCR with primers 5'-TGAAGCTTAGCTGAGTTGCTTAATAGAGG-3' (added *Bam*HI site underlined); Reverse, 5'-GCTCTAGATTAATAAAGATAAGATATGAG-3 (added *Pst*I site underlined). The fragment was cloned into *Bam*HI and *Pst*I sites of the pCAMBIA1300-221 binary

vector (Fig. 1a). Finally, *PUB19* promoter-GUS construct was introduced into the *Agrobacterium* strain EHA105 and transformed into wild type by the floral dip method. We obtained 15 independent lines of hygromycin-resistant transgenic plants. Mature T3 seeds were used for subsequent experiments. The expression of *GUS* driven by *PUB19* promoter in transgenic plants was detected by histochemical staining. The transgenic plants were immersed in staining solution(200 mM $NaH_2PO_4.H_2O$, 3.9mL; 200 mM $Na_2HPO_4.2H_2O$, 6.1 mL; 100 mM X-gluc, 200 µL) and incubated at 37°C for 12 h. Chlorophyll was cleared from the plant tissues by immersing them in 70% ethanol.

Subcellular localization of GFP-PUB19 fusion protein: To construct GFP-*PUB19* vector, the coding sequence of *PUB19* was cloned into pGDG vector which had GFP sequence at *Xho*I/*Apa*I sites with primers 5'-ACCTCGAGCTATGATCCATACACCAACCG-3' (added *Xho*I site underlined) and 5'-ACGGGCCCTCACCAGGCGTGGACAAACC-3' (added *Apa*I site underlined) (Fig. 2a). A truncated CDS of *PUB19* was amplified using primers 5'-ACTAGTATGGATGTGGCGGAGAGT-3'(added *Spe*I site and start codon underlined) and 5'-GGTACCCCAGGCGTGGACAAACC-3'(added *Kpn*I site underlined) without stop codon. After confirmed by sequencing, the truncated CDS was cloned into modified pCAMBIA1300-221 vector with GFP tag (Fig. 2a). Then the prepared vector was transferred into *Agrobacterium* strain EHA105. The cultures were diluted to OD_{600} =1.5 and infiltrated into the young tobacco leaves. For inducing transient expression of *GFP-PUB19* and *PUB19*$_{360-686}$*-GFP* fusion genes, tobacco plants were cultured for three days after infiltration. Then, the epithelial tissue of tobacco leaves were observed under a confocal laser microscope. Leaves infiltrated with *Agrobacterium* strains carrying the *GFP-PUB19* were digested into protoplasts. The protoplasts were analyzed by confocal microscope.

Cell fractionation assay: For cell fractionation analysis, tobacco leaves infiltrated with *Agrobacterium* carrying the *GFP-PUB19* were subjected to protein extraction followed by cell fractionation. Total protein extracts were obtained from leaves overexpressing a *GFP-PUB19* fusion gene by grinding in liquid nitrogen and then suspending in extraction buffer (50 mM Tris-MES, pH 8.0, 0.5 M sucrose, 1 mM $MgCl_2$, 10 mM EDTA, 5 mM DTT, and protease inhibitor cocktail) on ice (Kim *et al*., 2006). Total extract was centrifuged at 10,000 g to separate into the soluble fraction and the membrane fraction. The membrane fraction was further extracted with a detergent-free buffer and centrifuged at 100,000 g for the buffer-extracted fraction, and the pellet was suspended with SDS-containing buffer, then centrifuged at 100,000 g to separate the supernatant as the SDS-extracted fraction and the final membrane fraction. All fractions were separated by SDS-PAGE, and then GFP-PUB19 was detected using anti-GFP antibody.

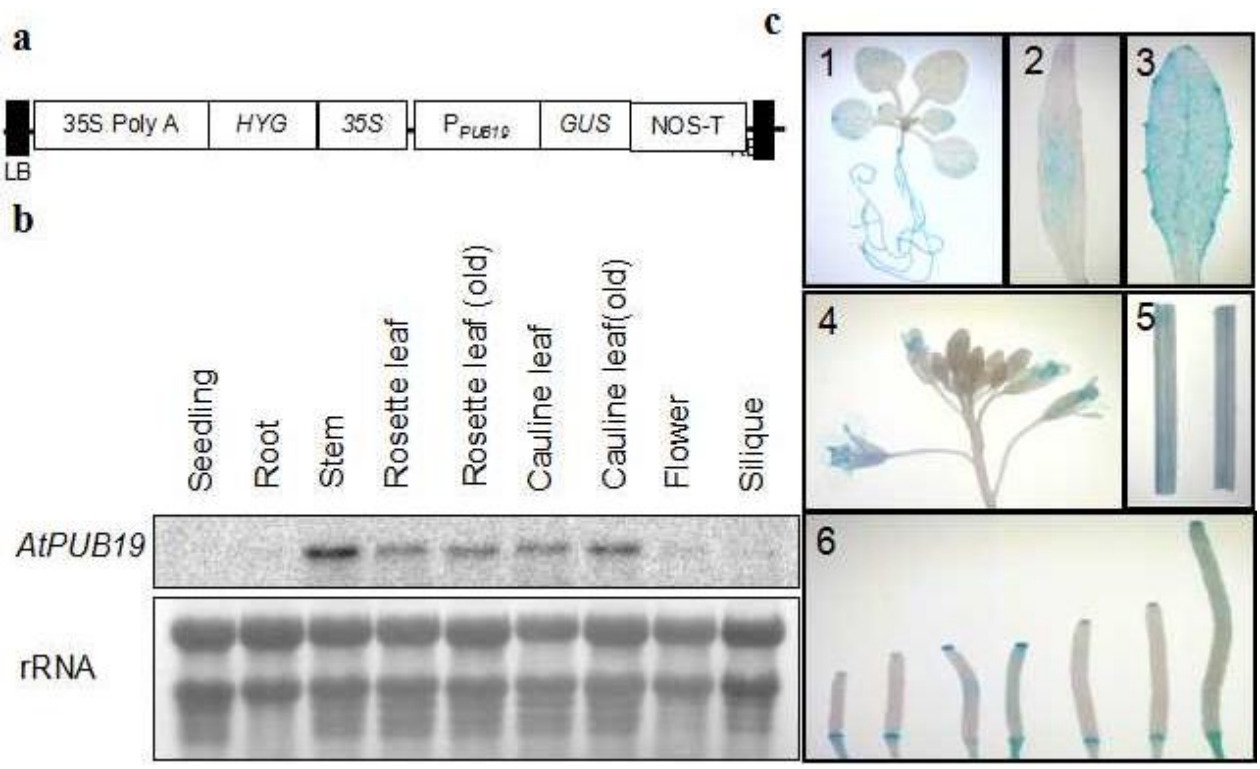

Fig. 1. The expression pattern of *PUB19* in different tissues.
a The schematic diagram of *PUB19* promoter-GUS construct. b The expression level of *PUB19* in different tissues by northern blot. RNA from different tissues including seedlings, roots, stems, young and old rosette leaves, young and old cauline leaves, flowers and siliques (up). rRNA as loading control (down). c *PUB19* promoter–GUS expression pattern in transgenic plants. (1) Whole seedling 14-dpg. (2) Cauline leaf. (3) A rosette leaf from a 4-week-old plant. (4) Flower. (5) Stem. (6) Siliques in different stage.

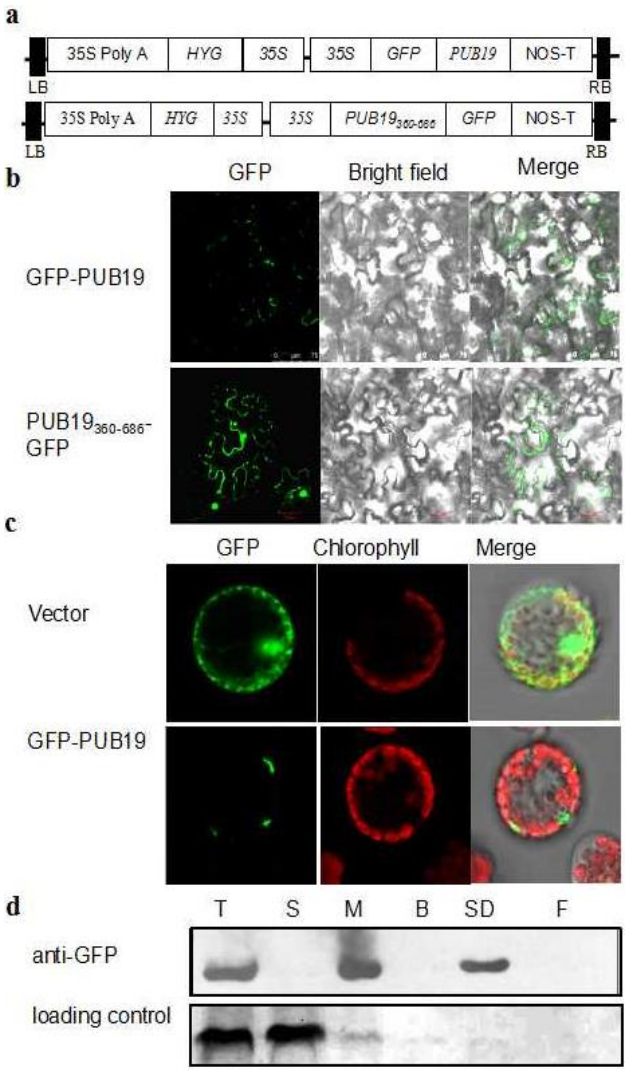

Fig. 2. Subcellular localization of PUB19.
a The schematic diagram of GFP-PUB19 and PUB19$_{360-686}$-GFP construct. b Subcellular localization of the PUB19 protein in tobacco leaves. c Subcellular localization of the PUB19 protein in protoplasts. d Cell fractionation assay of GFP-PUB19. Ponceau S staining of the RbcS is shown as a loading control (bottom panel). T, total extract; S, soluble fraction; M, membrane fraction; B, buffer-extracted fraction; SD, SDS-extracted fraction; F, final membrane fraction.

Result and Discussion

The expression pattern of *PUB19* in different tissues: To check the expression pattern in different tissues, northern blot was performed using *PUB19*-specific probe. As the result, the expression level of *PUB19* was high in stems and leaves of adult plants, but low in flowers, siliques and roots (Fig. 1b). We analysed the GUS activity in transgenic plants that harbored β-glucuronidase (GUS) CDS proven by *PUB19* promoter. The result was consistent with that of northern blot. Interestingly, histochemical staining showed that the *GUS* expression was detected in anthers and stigmas of siliques(Fig. 1c). *PUB19* may be involved in the development of pollen in *Arabidopsis*.

The subcellular localization of PUB19: *PUB19* encodes a protein of 686 aa with a predicted molecular mass of 76 kD. There is little other information on the function of PUB19 in database, which limited us to understand its biological role in plant. Subcellular localization provides us much more information on a protein and leads investigators to study its function. We generated GFP-PUB19 fusion protein driven by 35S promoter in leaves of tobacco by *Agrobacterium*-mediated infiltration. Under the confocal microscope, green fluorescence was distributed in patches at the plasma membrane (Fig. 2b). When the cells were digested into protoplasts, the similar result was observed (Fig. 2b).

PUB19 is a ARM-containing ligase and ubiquitinated itself (Liu *et al*., 2011). It is hard to generate GFP-PUB19 fusion protein in tobacco leaves efficiently. ARM-repeats in protein could interact with other proteins and determinate the subcellular localization, so ARM-repeats of PUB19 and GFP fusion protein(PUB19$_{360-686}$-GFP) was generated in tobacco leaves. Fusion protein without U-box domain was much richer than GFP-PUB19, which was also distributed in patches at the plasma membrane(Fig. 2b).

To ensure if PUB19 is localized on cell membrane, different fractions of cells were divided and analyzed using anti-GFP antibody. The signal was detected in total protein, SDS-extracted fraction and membrane fractions, but not in soluble fraction, buffer-extracted fraction and final membrane fraction(Fig. 2d). All results suggested that PUB19 was a membrane-associated protein.

The expression level was higher in old leaves than that in young ones(Fig. 1b). In addition, *PUB19* was involved in the response to salt (Berglerc & Hoth, 2011). In some microarray database, *PUB19* was classified into leaves senescence-associated gene. All results manifested that *PUB19* could function in senescence induced by abiotic stress. Several members of this family were identified to be associated with abiotic stress, but the mechanisms of their function were not clear. In *Arabidopsis*, *PUB22* and *PUB23* were two homologous genes and negatively regulated the drought tolerance in ABA-independent manner. Both of them encode two U-box proteins localized to the cytosol and ubiquitinated RPN12a (Cho *et al*., 2008). In our research, *PUB19* negatively regulated the response to drought and ABA (Liu *et al*., 2011). So the function of *PUB19* is different from that of *PUB22* and *PUB23*. Although PUB19 is anchored to the plasma membrane, no trans-membrane region was found in PUB19. This phenomenon was observed when transiently express GFP–PUB44 fusion protein in tobacco leaves (Drechsel *et al*., 2010). Not only PUB44, but also PUB42 and PUB43 are identified to localize at the membrane (Drechsel *et al*., 2010). Besides, the ARM repeats are essential for its subcellular localization and distribution (Drechsel *et al*., 2010). These proteins might be interact with some other membrane-associated proteins and were pulled to the plasma membrane. Further functional study of *PUB19* is important to enrich the ABA or drought tolerance of *Arabidopsis* mediated by *PUB19*.

Conclusions

In our study, we found that the expression level of *PUB19* was high in stems and leaves, but low in flowers, siliques and roots; Subcellular localization and cell fractionation assay showed that PUB19 is a membrane-associated protein. All our results will be helpful to elucidated the biological function of PUB19 in *Arabidopsis*.

Acknowledgments

This research was supported by the Doctor Funds of Xinjiang Bingtuan (2013BB003).

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(Received for publication 15 December 2016)