1 **Mutations in Exocyst Complex Subunit *SEC6* Gene Impaired Polar Auxin Transport and**
2 **PIN Protein Recycling in *Arabidopsis* Primary Root**

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7 *Key words:* Exocyst, SEC6, PIN proteins recycling, Polar auxin transport

8 *Abbreviations:* ABRC, *Arabidopsis* Biological Resource Centre; BFA, Brefeldin A; CLSM,
9 confocal laser scanning microscopy; DAG, days after germination; GUS, β -glucuronidase; IAA,
10 indole-3-acetic acid; NAA, 1-naphthalene acetic acid; PM, plasma membrane; TEM, transmission
11 electron microscopy; 2,4-D, 2,4-dichlorophenoxy.

Abstract

Polar auxin transport, which is critical for land plant pattern formation and directional growth, is largely depended on asymmetric distribution of PIN proteins at the plasma membrane (PM). Endocytosis and recycling processes play important roles in regulating PIN protein distribution and abundance at the PM. Two subunits (SEC8, EXO70A1) of exocyst, an octameric vesicle-tethering complex, have been reported to be involved in PIN protein recycling in *Arabidopsis*. However, the function of exocyst complex in PIN protein recycling and polar auxin transport remains incompletely understood. In this study, we utilized two *SEC6* down-regulation mutants (*PRsec6-1* and *PRsec6-2*) to investigate the role of exocyst subunit SEC6 in the primary root development, polar auxin transport and PIN proteins recycling. We found that in *PRsec6* mutants: 1. Primary root growth was retarded, and lateral root initiation were compromised. 2. Primary roots were sensitive to exogenous auxin 1-napthalene acetic acid (NAA) but not 2,4-dichlorophenoxy (2,4-D). 3. Recycling of PIN1 and PIN2 proteins from the Brefeldin A (BFA) compartment to the PM was delayed. 4. Vesicles accumulated in the primary root tip cells, especially accumulated in the cytosol closed to the PM. These results further demonstrated that the exocyst complex plays an important role in PIN protein recycling and polar auxin transport in *Arabidopsis* primary root.

1. Introduction

In land plant, auxin indole-3-acetic acid (IAA) is a key regulator that modulates various developmental processes, such as embryogenesis, organogenesis, vascular tissue formation and tropic responses [1, 2, 3]. An auxin gradient has been shown to play a central role in the root development [4], which is mainly established by auxin polar transport between cells, where IAA moves acropetally (towards the root apex) through the central cylinder and basipetally from the root apex towards the base [5]. Although local IAA biosynthesis also contributes to the establishment and maintenance of the gradient [6, 7]. The polar transport of auxin from cell to cell is mainly relied on the polar localization of auxin transporters at the PM [5], such as influx carriers AUXIN RESISTANT1/LIKE AUX (AUX/LAX) [8], and efflux carriers PIN-FORMED (PIN) proteins [9, 10]. Particularly, the localization and abundance of PIN proteins at the PM are critical for polar auxin transport, which are tightly regulated by vesicle trafficking pathways, especially endocytosis and recycling [11]. In general, PM localized PIN proteins internalize to the early endosomes via clathrin-dependent pathway, followed by either recycling to domains at the PM or further sorting into the late endosomes for their ultimate degradation in the lytic vacuole [11, 12]. The recycling process mediated by the ARF GTPase guanine nucleotide exchange factor (ARF-GEF) GNOM is sensitive to BFA [13]. In addition, recycling of PIN proteins from the early endosomes to the PM for polarized exocytosis is controlled by the coordinated action of proteins such as small GTPases, tethering factors, SNARE proteins and regulatory syntaxin binding proteins [14, 15].

Regulation of polar exocytosis in the yeast and mammalian cells often involves the exocyst complex (consists of SEC3, SEC5, SEC6, SEC8, SEC10, SEC15, EXO70 and EXO84) that tethers secretory vesicles to the specific sites on the PM and facilitates their exocytosis [16, 17]. *Arabidopsis* contains homologues for all exocyst subunits, of which, SEC6 and SEC8 are encoded by single gene, others are encoded by multiple genes [18, 19, 20]. In according with its expected role in exocytosis, the exocyst complex plays important roles in regulation cell wall formation [21, 22], cytokinesis [23, 24, 25], polar cell growth [26, 27], and pathogen defense [28, 29]. In addition, recently reports showed that the plant exocyst complex plays an essential role in Exocyst-Positive Organelle (EXPO) formation, which mediate an unconventional exocytosis from cytosol to cell wall [30, 31]. The exocyst also participate in an autophagy-related Golgi-independent membrane

trafficking into the vacuole [32]. In addition, the exocyst complex has also been implicated in auxin polar transport. SEC3A interacts with adaptor protein ICR1 [33], which is an ROP GTPases effector that regulates polar auxin transport [34], these data indicated a potential linkage between exocyst and auxin. The trafficking dynamics of PIN proteins in *exo70a1* and *sec8* mutant root cells is altered, which is thought to underlie the compromised polar auxin transport [35]. Recently, Cole *et al* failed to identify aberrant auxin signaling and transport as primary drivers for reduced root growth in exocyst mutants [36]. Therefore, the function of the exocyst in polar auxin transport and root growth has not been completely understood so far.

In this study, we showed that in two pollen rescued *sec6* loss-of-function *Arabidopsis* mutants (*PRsec6-1* and *PRsec6-2*), the primary root growth was retarded, and lateral root initiation were compromised, the primary root were sensitive to exogenous auxin NAA but not 2,4-D. In addition, in the root cells, PIN protein recycling from the BFA compartment to the PM was delayed, and the TEM data showed that majority of the vesicles accumulated closed to the PM in the cytosol. Taken together, our data showed that the exocyst complex plays a role in polar auxin transport, and it might facilitate PIN protein polar exocytosis during recycling in the primary root cells.

2. Materials and Methods

2.1 Plant materials and growth condition

The *Arabidopsis* Columbia (Col), the T-DNA insertion mutants *sec6-1/+* (SALK_078235) and *sec6-2/+* (SALK_072337) (Columbia background) were obtained from the *Arabidopsis* Biological Resource Centre (ABRC). The T-DNA insertion lines were back-crossed to wild-type plant for four times. Conditions for plant growth were followed as previously reported [24]. Briefly, the surface-sterile seeds were germinated on the Murashige and Skoog (MS) medium supplemented with 3% sucrose, kept at 4°C for two days, and then moved into a growth chamber with a 16-h-light and 8-h-dark cycle. The *PRsec6-1* and *PRsec6-2* plants were generated as described [24]. Seeds of *ProDR5::GUS*, *ProPIN1::PIN1-GFP*, *ProPIN2::PIN2-GFP* and *ProDR5::GFP* transgenic *Arabidopsis* plants were obtained from Dr. Jian Xu.

2.2 β -glucuronidase (*GUS*) staining and phenotype characterization

For GUS staining, the 3-d-old seedlings transformed with *ProDR5::GUS* were stained in 5mM EDTA, 0.1% Triton X-100, 5mM $K_4Fe(CN)_6$, 0.5mM $K_3Fe(CN)_6$, and 1mg mL⁻¹ X-Gluc for 4h, and washed in a solution of ethanol : acetic acid at 3:1 for 30min.

For primary root growth observation, 3 days after germination (DAG), 5 DAG and 7 DAG seedlings were collected and photographed. The root lengths were measured by the Image J software.

2.3 BFA treatment

3 DAG seedlings were transferred from the MS plates into tubes containing half-strength MS liquid medium supplemented with 50 μ M BFA (50 mM stock in DMSO; Sigma-Aldrich) and incubated under light conditions in a growth chamber for 2h. The seedlings were then taken out and washed.

2.4 Auxin treatment

To observe primary root growth response to auxins, seeds of wild-type and *PRsec6* plants were germinated on the MS plates without auxin, at 3 DAG, seedlings were moved onto the MS plates supplemented with 0.3 nM auxin (NAA or 2,4-D) and grew vertically for 4 days and photographed. The primary root lengths were measured by the Image J software.

To observe lateral root initiation response to auxin, seeds of wild-type and *PRsec6* plants were germinated on the MS plates without auxin, at 3 DAG, seedlings were moved onto the MS plates

without NAA or supplemented with 10 nM or 100 nM NAA.

2.5 Transmission electron microscopy

The primary roots of 3 DAG seedlings were incubated in a fixation solution containing 5% (v/v) glutaraldehyde, 0.1 M sodium cacodylate, pH 7.0, washed in water for 3 times, and further fixed in 1% osmium tetroxide for 3h, dehydrated in a series of acetone solutions [25, 50, 75 and 100 % (v/v)], followed by a resin/acetone dilution series [25, 50, 75 and 100 % (v/v)], and embedded in epoxy resin. After polymerization at 65°C for 18h, serial sections were prepared and examined with a JEM1230 transmission electron microscope (Hitachi, Tokyo, Japan).

3. Results

3.1 Primary root growth and lateral root initiation are retarded in *PRsec6* mutants

SEC6 is a single copy gene in *Arabidopsis*, T-DNA insertions in the gene (*sec6-1* and *sec6-2*) resulted in a severe pollen tube growth defect and a complete male gametophytic sterility [27]. In our previous study, a construct containing *SEC6* coding sequence driven by the pollen-specific *LAT52* promoter was transformed into *sec6-1/+* and *sec6-2/+* plants. The constructs rescued the pollen tube growth defects in two *sec6* mutants respectively, allowing the generation of seedlings homozygous for both mutations, which were named as *Pollen Rescued sec6* mutants (*PRsec6-1* and *PRsec6-2*). RT-PCR assays further indicated that *SEC6* gene is dramatically down-regulated in both mutants [24]. The *PRsec6* mutants exhibited multiple development phenotypes during sporophyte development, including dwarfism, retarded primary roots and cytokinesis defects in the embryo and the leaf [24].

At 3 DAG, the length of the primary roots in *PRsec6-1* was 0.69 ± 0.17 mm, dramatically shorter than that of wild-type (3.49 ± 0.29 mm) (Fig. 1A and 1C). The length of the primary roots in *PRsec6-1* was 0.69 ± 0.17 mm at 5 DAG, 0.91 ± 0.27 mm at 7 DAG, in contrast to 5.73 ± 0.45 mm at 5 DAG and 9.68 ± 0.67 mm at 7 DAG in wild-type (Fig. 1B and 1C). *PRsec6-2* showed a similar root growth defect (Fig. 1A-C). In addition, lateral root initiation in *PRsec6* mutants was analyzed. Compared to wild-type seedlings, the lateral root initiation was delayed in both *PRsec6* mutants in a time course study (Fig. 1D), and the number of lateral roots was much less than that of wild-type plant at 7 DAG (Fig. 1E). When both *PRsec6* mutants were treated with 10 nM or 100 nM NAA, the lateral root initiation can be enhanced by exogenous auxin NAA (Fig. 2E). These data indicated a critical role of *SEC6* gene in the primary root development and lateral root initiation.

3.2 Auxin activity is reduced in *PRsec6* primary roots

The primary root growth and lateral root initiation defects in *PRsec6* mutants suggested that auxin activity might be affected in *PRsec6* roots. *DR5* is a synthetic promoter that contains an auxin response element whose expression provides reliable information concerning auxin activity in roots and other tissues [37]. In 3 DAG primary root tips, *GUS* staining and GFP signals were much weaker in *PRsec6* than in the wild-type (Fig. 2A and 2B). These results indicated that auxin activity is reduced in the primary root of *PRsec6* mutants.

3.3 *PRsec6-1* mutant is sensitive to exogenous auxin NAA but not 2,4-D

To understand whether the auxin activity reduction in *PRsec6* primary root is caused by auxin biosynthesis, transport or signaling, the sensitivity of *PRsec6-1* mutant to two types of exogenous auxins was examined. One is NAA, a diffusible auxin which is able to cross the PM without the help of facilitators, the other is 2,4-D, which requires facilitators [38]. Wild-type and *PRsec6-1* seeds germinated on the MS plate were moved to the plates supplemented with 0.3 nM NAA or 0.3 nM 2,4-D at 3 DAG. The growth rate of wild-type primary root on plate supplemented with 0.3 nM NAA or 2,4-D is ~3.5 mm/day, obviously higher than the rate (~1.7 mm/day) of wild-type roots grown on MS plates (Fig. 3A-C). In the case of *PRsec6-1* mutant, the primary root growth rate is ~1.2 mm/day on the plate supplemented with 0.3 nM NAA, which is obviously higher than that (~0.31 mm/day) on the MS plate (Fig. 3A-C). Therefore, *PRsec6-1* is sensitive to NAA. In contrast, the primary root growth rate of *PRsec6-1* on plates supplemented with 2,4-D is ~0.36 mm/day, comparable to that grown on the MS plate (Fig. 3B and 3C), indicating that *PRsec6-1* is insensitive to 2,4-D. These data demonstrated that auxin polar transport was compromised in *PRsec6-1* mutant, which contribute to the root growth retardation.

3.4 Transport of PIN proteins from the BFA compartment to the PM was delayed in the PRsec6 mutant

The polar distribution and dynamics of PIN protein are critical to establish and maintain auxin polar transport [10]. Therefore, *ProPIN1::PIN1-GFP* and *ProPIN2::PIN2-GFP* were introduced into *PRsec6-1* plants. At 3 DAG, we could not find obvious differences in the steady state PIN1-GFP and PIN2-GFP localization in *PRsec6-1* primary root under the confocal laser scanning microscopy (CLSM).

To understand if PIN protein recycling was altered by the mutation in *SEC6*, *ProPIN1::PIN1-GFP* and *ProPIN2::PIN2-GFP* transgenic wild-type and *PRsec6-1* seedlings were treated with 50 μ M BFA. At 2h of the treatment, PIN1-GFP and PIN2-GFP internalized into the BFA compartments in both *PRsec6-1* and wild-type root cells (Fig. 4A and 4B). After washout BFA, PIN1-GFP and PIN2-GFP started moving to the PM, as a result, the GFP labeled BFA compartments begin to decrease or disappear in some cells. We analyzed the average numbers of BFA compartments in the root cells (n =70 in each background, cells were picked randomly, cell without BFA compartments were also counted and the number of BFA compartment was regarded as zero). The average number of BFA compartments in the counted *PRsec6* root cells is comparable to that in

wild-types. After wash out at 30 and 90 min, the average number of BFA compartments in the counted root cells is obviously higher than that in wild-types (Fig. 4A-D). Percentages of cells who harbors BFA compartments were also assessed. As shown in Fig. 4E and 4F, at 30 min after wash out, ~45% of wild-type cells contained the BFA compartments, in contrast to ~70% of *PRsec6-1* cells. After 90 min of washout, BFA compartments could be observed in only 4% of wild-type cells, whereas they were present in about 35% of *PRsec6-1* cells. This observation indicated that PIN proteins recycling from the BFA compartment to the PM is delayed in *PRsec6-1* primary root cells.

3.5 Vesicles accumulated in *PRsec6* primary root cells

The delay of PIN protein recycling from the endosomes to the PM in *PRsec6* primary root cells suggested that during the recycling process, the exocytosis of PIN proteins might be delayed. Cells in the division zone of primary roots in 3 DAG wild-type and *PRsec6-1* plants were observed by TEM. Vesicles were noticed to accumulate in the cytosol of *PRsec6-1* root cells with an average diameter of 100 nm (n>300) (Fig. 5B and 5D), a similar size to plant exocytosis vesicles reported [39], while the accumulation was not seen in wild-type cells (Fig. 5A and 5C). When the field of images was taken randomly and analyzed, vesicle density was about 4.24 vesicles per μm^2 in *PRsec6-1* root cells, which is obviously higher than that (0.77 vesicles per μm^2) in wild-type ones (Fig. 5B, 5D and 5F). Interestingly, the vesicle density in the area within 500 nm to the PM in *PRsec6-1* root cells was 10.81 vesicles per μm^2 , much higher than the same area in wild-type root cell (0.89 vesicles per μm^2) (Fig. 5F). These data suggested that tethering of exocytic vesicles to the PM is disturbed in *PRsec6* primary root cells.

4. Discussion

4.1 Auxin polar transport alteration contributes to root growth defect in *PRsec6* mutants

In this study, we demonstrated that the primary root growth was severely retarded in both *PRsec6* mutants. This phenotype is similar to what has been observed in *exo70a1*, *sec8*, *exo84b* single and *sec5a exo70a1*, *sec8 exo70a1* double mutants [26, 36]. Auxin activity and transport were investigated in this study. We found that in *PRsec6* mutant primary roots, *ProDR5::GUS* and *ProDR5::GFP* signals were weaker than that in wild-type, lateral root initiation were compromised, indicating that auxin activity was reduced in *PRsec6* roots. Furthermore, we found that *PRsec6-1* was sensitive to exogenously applied auxin NAA, which can cross the PM without facilitators and overcome the transport defect in root, but not to 2,4-D, which requires facilitators. These data indicated that auxin polar transport is compromised in *PRsec6*, and this alteration contributes to the root growth retardation in the mutants. Our previous study showed that the cytokinesis defect in *PRsec6* roots might also contribute to the root growth retardation [24]. In addition, other potential causes that lead to the root growth defects in *PRsec6* mutants is currently being investigated, which could be helpful to understand the molecular and developmental roles of the exocyst complex.

4.2 Exocyst complex might facilitate exocytosis of PIN proteins during recycling

Polar auxin transport in plant is largely depended on the dynamics of PIN proteins at the PM. The BFA treatment assay indicated that PIN protein trafficking from the BFA compartment to the PM is delayed in *PRsec6-1* root tip cells. The TEM data showed that large amount of vesicles accumulated in the cytosol of *PRsec6-1* root cells, with majority of them localizing close to the PM, which further indicated exocytosis defects during recycling. Recently, visualization of the dynamics of the exocyst subunits SEC6, SEC8, EXO70A1 and EXO84b in root epidermal cells showed that these subunits dynamically dock and undock at the PM, potentially creating sites for vesicle tethering and exocytosis [40]. In *PRsec6-1* primary root cells, although vesicles were shown to accumulate near the PM at the TEM level (Fig. 5), no obvious deviation of PIN-GFPs distribution at the PM was observed by CLSM, which could be because PIN proteins localized on the vesicles accumulated very close to the PM, at a distance below the resolution power of the CLSM. However, this speculation should be verified by immuno-EM to show that the accumulated vesicles nearby PM harbors PIN proteins.

223 Interestingly, other than PIN proteins, recycling defects of the brassinosteroid receptor BRI1 were
224 also observed in the roots of *exo70a1* mutant [35]. BRI1 is a transmembrane protein that
225 constitutively cycles between endomembrane compartments and the PM through BFA-sensitive
226 trafficking [41]. BRI1 trafficking might be affected in *PRsec6* as well, as membrane protein
227 recycling from the BFA compartments is defective there. However, this hypothesis should be
228 verified in the future. In short, our work indicated mutation in *SEC6* gene leads to defects in polar
229 auxin transport and PIN proteins recyclings, further confirmed that exocyst complex plays a
230 important role in PIN proteins exocytosis during its recycling.

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Legends of figures

Fig. 1. Primary root growth in wild-type (WT) and *PRsec6* seedlings.

(A) Wild-type (WT) and *PRsec6* seedlings at 3 DAG;

(B) Wild-type (WT) and *PRsec6* seedlings at 5 DAG;

(C) Statistics of primary root length of wild-type (WT) and *PRsec6* seedlings at 3, 5 and 7 DAG.

(D) Lateral root initiation of *PRsec6* was delayed compared to that of wild-type.

(E) Average number of lateral root primordia per seedling of 7 DAG wild-type and *PRsec6*, respectively, grown on MS medium supplemented with different concentrations of NAA. Initiation of lateral roots was retarded in *PRsec6* mutants. (10 *PRsec6-1*, 10 *PRsec6-2* and 20 wild-type seedlings were counted, respectively). Bars = 1 mm in (A) and (B).

Fig. 2. Auxin activity is reduced in primary root of *PRsec6* mutants.

(A) *ProDR5::GUS* staining in wild-type (WT) and *PRsec6* primary roots;

(B) *ProDR5::GFP* signals in wild-type (WT) and *PRsec6* primary roots;

Bars = 50 μ m in (A) and (B).

Fig. 3. *PRsec6-1* mutant was sensitive to exogenously applied auxin NAA but not 2,4-D

(A) Wild-type (WT) and *PRsec6-1* seedlings on MS plate (top row), supplemented with 0.3 nM NAA (middle row) and 0.3 nM 2,4-D (bottom row).

(B) Statistics of root length of wild-type (WT) and *PRsec6-1* seedlings with or without auxin treatment.

(C) Statistics of root growth rates of wild-type (WT) and *PRsec6-1* seedlings with or without auxin treatment.

(Con) represent controls without auxin treatment, (NAA) represents 0.3 nM NAA treatment, (2,4-D) represents 0.3 nM 2,4-D treatment, n = 15 for each sample, Bars = 1cm in (A).

Fig. 4. Recycling of PIN1 and PIN2 proteins from the BFA compartment to the PM was impaired by *PRsec6* mutation.

(A) and (B) PIN1-GFP (A) and PIN2-GFP (C) localization in wild-type (WT) and *PRsec6-1* seedlings before and 2 h after BFA treatment, 30 and 90 min after BFA washout;

(C) and (D) Average numbers of PIN1-GFP (C) and PIN2-GFP (D) labeled BFA compartments per cell in wild-type and *PRsec6-1* roots, 70 cells were counted in each sample.

(E) and (F) Percentage of epidermal cells with BFA compartments after 2 h BFA treatment, 30 and 90 min after BFA washout in wild-type (WT) and *PRsec6-1* seedlings expressing PIN1-GFP (E) and PIN2-GFP (F). Bars = 10 μ m in (A) and (B)

Fig. 5. Vesicles accumulated in *PRsec6* primary root cells.

386 (A, B) TEM images of primary root cell in wild-type (A) and *PRsec6-1* mutant (B);
387 (C, D) Magnified images of (A) and (B) respectively. ER, endoplasmic reticulum; G, Golgi
388 apparatus; M, mitochondrion; N, nucleus;
389 (E) Statistics of vesicle density in random field of root cells in wild-type (WT) and *PRsec6-1*
390 (Indicated as WT and *PRsec6-1*), and in cytosol closed to the PM (distance to PM <500 nm)
391 (Indicated as WT# and *PRsec6-1*#). n = 10 in each sample. The data are represented as the mean $\pm$
392 SE, ** $p < 0.01$ by *student's t* test. Bars = 500 nm in (A) and (B), 100 nm in (C) and (D);

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5