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Project Summary: The long-term objective of this work is to understand the molecular events and mechanisms involved in secretory membrane trafficking and organelle biogenesis, which are crucial for normal plant growth and development. Our studies have suggested a vital role for the cytosolic chaperone Cdc48p/p97 during cytokinesis and cell expansion which are highly dependent upon secretory membrane trafficking. Localization studies have shown that the plant Cdc48p/p97, AtCDC48, and the Arabidopsis ortholog of the ER- and Golgi-associated SNARE, syntaxin 5, (referred to as SYP31) are targeted to the division plane during cytokinesis. In addition, AtCDC48 and SYP31 were shown to interact *in vitro* and *in vivo*. To characterize further the function of AtCDC48 and SYP31 we have utilized affinity chromatography and MALDI-MS to identify several plant-specific proteins that interact with SYP31 and/or modulate the activity of AtCDC48 including two UBX (i.e. ubiquitin-like) domain containing proteins, PUX1 and PUX2 (Proteins containing UBX domain). These proteins define a plant protein family consisting of 15 uncharacterized members that we postulate interact with AtCDC48. Biochemical studies have demonstrated that PUX2 is a novel membrane adapter for AtCDC48 that mediates AtCDC48/SYP31 interaction and is likely to control AtCDC48-dependent membrane fusion. In contrast, PUX1 negatively regulates AtCDC48 by inhibiting its ATPase activity and by promoting the disassembly of the active hexamer. These findings provide the first evidence that the assembly and disassembly of the CDC48/p97complex is actually a dynamic process. This new unexpected level of regulation for CDC48/p97 was demonstrated to be critical *in vivo* as *pux1* loss-of-function mutants grow faster than wild-type plants. These studies suggest a role for AtCDC48 in plant cell cycle progression including cytokinesis and/or cell expansion. The proposed studies are designed to: 1) characterize further the localization and function of AtCDC48 in membrane trafficking and organelle biogenesis during plant cytokinesis and cell expansion, 2) to analyze the subcellular localization and function of two members of the SYP3 t–SNARE family, SYP31 and SYP32, and 3) to determine the role of select members of the PUX protein family and the distinct biochemical pathways to which they target the chaperone activity of AtCDC48 to. The integration of genetic, morphological, and biochemical data from these studies is expected to contribute significantly to both an understanding of the function and organization of the plant secretory pathway and its role in plant cell morphogenesis.

Specific Aims: The overall goal of this project was to begin to define the biochemical mechanisms that control membrane trafficking and fusion during plant cytokinesis and cell expansion. In particular we proposed to use a multidisciplinary approach involving microscopy, biochemistry and reverse genetics to characterize the function and regulation of the membrane fusion chaperone AtCDC48 and the t-

SNARE, SYP31 which are localized to the division plane during plant cytokinesis. The specific aims of the project included:

1. The identification of plant cell-specific components that interact with AtCDC48 and SYP31
2. To clone and biochemically characterize the function of AtCDC48/SYP31 interacting proteins
3. To isolate and characterize *Atcdc48*, *syp3*, and AtCDC48/SYP31 interacting protein gain of function and loss-of-function mutants

Introduction: Plant cytokinesis and cell expansion, which are fundamental process required for plant growth and development, are dependent upon membrane trafficking and fusion for the formation of the cell plate and cell plate associated-ER membranes. CDC48/p97 is a conserved and essential hexameric AAA-ATPase that functions as a molecular chaperone in numerous diverse cellular activities. CDC48/p97 activity is recruited to specific functions through its interaction with adapter proteins. Our working hypothesis is that AtCDC48 and the *Arabidopsis* homolog of syntaxin5/Sed5p, SYP31, mediates events important for plant cytokinesis and cell expansion.

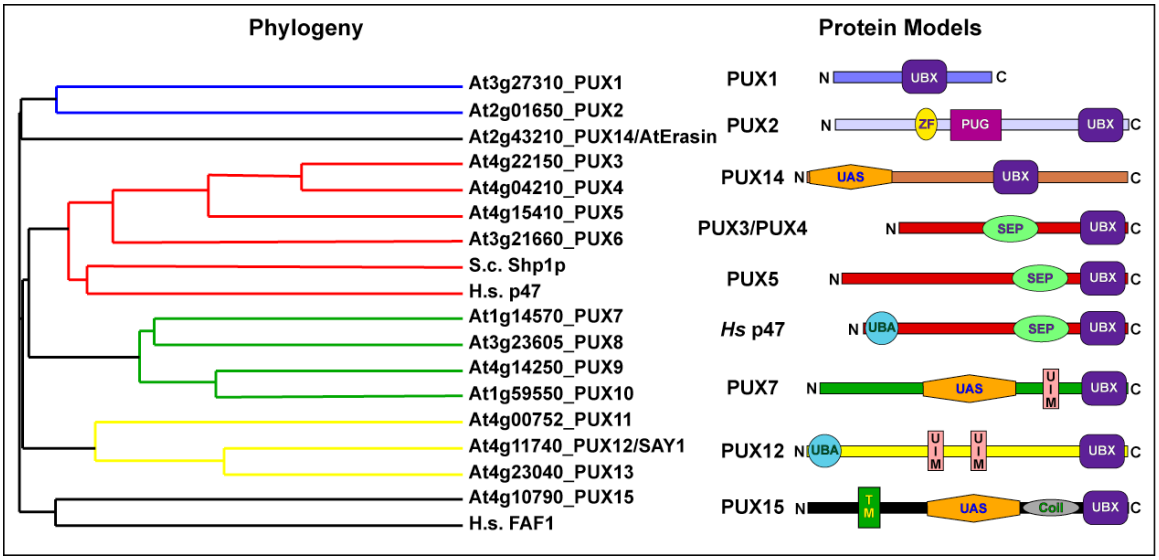

Figure 1: Cladogram of PUX protein family and protein domain organization of *Homo sapiens* p47 adapter protein and the Arabidopsis PUX proteins family. The proteins domains presented include UBX (ubiquitin-like protein fold; InterPro domain: IPR001012), UBA (ubiquitin associated domain; InterPro domain: IPR000449), Zf (C2H2 zinc finger; Pfam domain: PF00096), PUG (domain in protein kinases, N-glycanases, and other nuclear proteins; SMART domain: SM00580), and SEP (domain found in *S.c.* Shp1p, *D.m.* eyes closed gene (eyc), and p47; SMART domain:SM00553), UIM (ubiquitin interacting motif; SMART domain:SM00726)

Aim1: Identification of AtCDC48/SYP31 adapter proteins:

In the homotypic fusion of ER and mitotic mammalian Golgi membrane fragments a p97/Cdc48p complex containing the adapter protein p47, interacts with syntaxin 5. Thus one approach to understanding the role of AtCDC48 and SYP31 in plant cytokinesis is to identify and characterize the function of putative AtCDC48/SYP31 adapter proteins. To do this we utilized affinity

chromatography and MALDI-TOF mass spectrometry to identify proteins that bound selectively to the cytosolic domain of SYP31. We have identified two uncharacterized UBX-domain containing proteins, PUX1 and PUX2 (see Figure 1), which interact with AtCDC48. UBX-domains are ubiquitin-like protein folds that function as interaction domains for CDC48/p97.

With the exception of the UBX domain PUX1 and PUX2 show only limited sequence identity with mammalian p47. In addition, PUX1 is distinct from either PUX2 or p47 in which the UBX domain, is located at the C-terminus of the protein. In PUX1, the UBX domains are centrally located within the protein (Figure 1). Cytosolic PUX1 binds to the N-terminus and negatively regulates AtCDC48 by inhibiting its ATPase activity and by promoting the disassembly of the active hexamer. This new unexpected level of regulation for CDC48/p97 was demonstrated to be critical *in vivo* as *pux1* loss-of-function mutants grow faster than wild-type plants (Rancour et al 2004). To examine the role of ATP binding and hydrolysis in PUX1-mediated p97 we have generated by site directed mutagenesis ATP hydrolysis defective His_6-T7-AtCDC48 mutants containing single mutations in the D1 (E308Q) and D2 (D581N) and in both D1 and D2 ATPase modules of *His_6-T7-AtCDC48* by site-directed mutagenesis. Both binding and hydrolysis mutant CDC48 assembled into hexamers however PUX1 disruption of these oligomers has block even though binding of PUX1 UBX domain was unaffected (Park et al 2007).

PUX2 is a peripheral membrane protein that interacts with AtCDC48 *in vitro* and co-fractionates with membrane-associated AtCDC48 *in vivo*. Biochemical reconstitution and immunolocalization data suggest that PUX2 facilitates the interaction of SYP31 and AtCDC48 during interphase and cytokinesis, thereby regulating an AtCDC48 membrane-associated function. In contrast to other UBX domain containing proteins, PUX2 interacts with AtCDC48 through a novel PUG domain. Furthermore, PUX2 binds AtCDC48 through the C-terminal D2 domain, an unconventional interaction for CDC48/p97 adapters. Loss-of-function *pux2-1* Arabidopsis plants displayed altered endoplasmic reticulum distribution and a corresponding increase in cellular chloroplast content. Ongoing experiments are aimed at defining the contact site(s) between AtCDC48 and PUX1 (Rancour et al 2007).

Aim 2: Identification of CDC48 interacting proteins

Molecular analysis of the PUX protein family:

In addition to PUX1 and PUX2 the Arabidopsis genome encodes 13 more potential gene products containing UBX domains that are conserved in other plant species (see Figure 1). With the exception of PUX3-6, other members of the PUX protein family display limited sequence homology with proteins in the yeast and mammalian sequence databases. Our working hypothesis is that PUX proteins function as AtCDC48 adapters, to target its chaperone activity to distinct plant-specific biochemical pathways. Indeed all PUX proteins tested so far interact with AtCDC48 suggesting this to be an AtCDC48 regulatory protein family (see table 1).

Based on phylogeneic and amino acid sequence comparison analysis, 13 of the PUX proteins can be organized into distinct clades with two or more potentially functionally redundant members, whereas PUX 14 and PUX15 appear to be unique (Figure 1). Characterization of the morphogenesis of all available *pux* T-DNA insertion lines (Table 1) is in progress.

PUX3-6 display significant sequence identity over the entire length of the predicted polypeptide with the mammalian p97/Syntaxin 5 adapter protein p47 (Figure 1). One significant difference however, is that PUX3-6 lack the p47 N-terminal UBA domain which has been shown to be critical for mono-ubiquitin binding and the regulation of p97-mediated mitotic Golgi fusion. PUX5, which has a high degree of sequence identity to PUX3 and PUX4 has been reported to be a regulatory

subunit of Arabidopsis protein phosphatase. The biochemical function and role of PUX3-5, PUX7-PUX15 in plant cell morphogenesis remains to be determined.

Aim 3: Isolation and characterization of *Atcdc48* and *syp3* mutants

Characterization of AtCDC48 function in plant growth and development:

Figure 2. 6 days old-wild-type and homozygous *Atcdc48a-1 (arrowhead)*. Scale bar is 2mm

The biochemical pathways regulated by p97/CDC48 in plants remains to be determined. In order to gain insight into the *in vivo* role of this critical chaperone in plants we have we have used complementary approaches of reverse genetics, visualization of functional YFP-AtCDC48, and an ethanol-inducible dominant negative expression system to elucidate the role of AtCDC48 *in planta* (Park and Bednarek, Submitted). The homozygous *Atcdc48* T-DNA insertion mutants are not viable (Figure 2) and show pleiotropic developmental arrests points. *Atcdc48* allele showed significantly reduced male transmission efficiency and the analysis of heterozygous *AtCDC48/Atcdc48* in a *quartet (qrt)* background indicates that the mutation greatly affects pollen tube growth. YFP-AtCDC48, a fusion protein that functionally complements the insertion mutant defects, localizes in the nucleus, cytoplasm and is recruited to the division midzone during cytokinesis. The pattern of nucleus localization differs due to the stage of the cell cycle and differentiation state. Inducible expression of AtCDC48 ATPase mutant results in root trichoblast differentiation defects apparent in excessive root hair emergence and hallmark cytokinetic defects of cell wall stubs and incomplete cell separation. These data provide strong evidence that AtCDC48 plays critical roles in cell division, expansion, and differentiation.

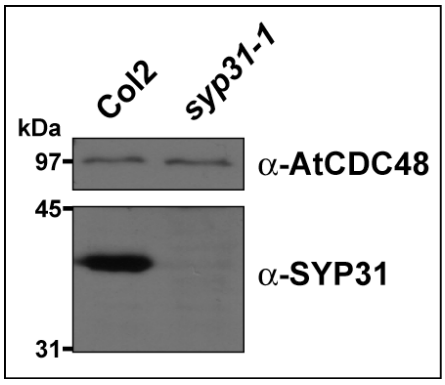

Figure 3: *syp31* mutant plants do not express SYP31 protein. AtCDC48 and SYP31 immunoblot analysis of total protein extracts from wild-type (Col2) and homozygous mutant *syp31-1* 7-day old seedlings.

Identification of *syp31* mutants: To characterize the plant ortholog of Syntaxin 5/SED5, SYP31 during cytokinesis and cell expansion we have identified Arabidopsis, *syp31-1* and syp32-1 lines containing T-DNA insertions within coding region of *SYP31* and *SYP32*. In contrast to *Atcdc48* mutants, homozygous *syp31-1* plants did not display any obvious growth or developmental phenotypes (data not shown). Immunoblot analysis of total protein extracts prepared from wild-type and isogenic *syp31-1* seedlings confirmed that expression of SYP31 was abolished in the mutant (Figure 3). These results suggested that the second Arabidopsis Sed5p/syntaxin 5 ortholog, SYP32, is likely to be functionally redundant with SYP31. SYP31 (37kD) and SYP32 (39kD) share ~50% amino acid sequence identity. We have cloned the SYP32 cDNA and have generated and expressed GST-tagged SYP32ΔTM fusion protein constructs lacking the C-terminal transmembrane anchor. Purified GST- SYP32ΔTM was used to generate anti-SYP32 specific antibodies for proposed localization and *syp32* mutant characterization experiments. Heterozygous loss-of-function *syp32-1*

display severe defects in female gametogenesis. To further analyze the role of SYP32 in plants we have generated inducible RNAi *syp32* lines, which display growth and developmental defects during seedling morphogenesis. We are in the processes of further characterizing *syp32-1* and *syp32*^{RNAi} lines for defects in cytokinesis and cell expansion.

Key Personal:
These studies were primarily conducted by a graduate student, Ms. Sookhee Park, a post-doctoral fellow, Dr. David Rancour, and myself.

Publications whose studies are related directly to the aims of proposal DE-FG02-99ER20332 that acknowledge DOE Energy Biosciences support:

1. Park, S., Rancour, D.M. and **Bednarek, S. Y.** (2008). *In planta* analysis of the cell cycle-dependent localization of AtCDC48A and its critical roles in cell division, expansion, and differentiation. *Plant Physiol.* **148,** 246-258

2. Park, S., Rancour, D. M., and **Bednarek, S. Y.** (2007). Protein domain-domain interactions and requirements for the negative regulation of Arabidopsis CDC48/p97 by the plant UBX-domain containing protein, PUX1. *J. Biol. Chem.* **282**, 5217-5224

3. Rancour, D. M., Sookhee, P. and **Bednarek, S. Y.** PUX2 and the PUX protein family constitute a diverse set of CDC48 interacting factors in Arabidopsis. Manuscript in prep.

4. Rancour, D. M. and **Bednarek, S. Y.** Functional characterization of the early secretory pathway syntaxins, SYP31 and SYP32. Manuscript in prep.

Other publications generated by the PI that acknowledge DOE Energy Biosciences support:

1. Rancour, D.M., Backues, S.K., and **Bednarek, S.Y.** (**in press**). Protein antigen expression in E. coli for antibody production. In *Immuno-electron Microscopy: Methods and Protocols*, S.D. Schwartzbach and T. Osafune, eds. (Humana Press).

2. Han, B.-W., Bingman C. A., Mahnke, D. K., Bannen, R. M., **Bednarek, S. Y.**, Sabina, R. L. and Phillips, G. N. (2006) Membrane association, Mechanism of Action, and Structure of Arabidopsis *EMBRYONIC FACTOR 1* (*FAC1*). *J. Biol. Chem.* **281,** 14939-14947

PUX designation	AGI Locus	cDNA	*E. coli* expression	Interaction w/ AtCDC48?	Plant Mutants (Status)
PUX1	At3g27310	Yes	Yes	Yes	Yes (LoF)
PUX2	At2g01650	Yes	Yes	Yes	Yes (LoF)
PUX3	At4g22150	Yes	Yes	Yes	YES (LoF)
PUX4	At4g04210	Yes	Yes	Yes	Yes [T-DNA (NO), amiRNA (YES)]
PUX5/B' PP2A	At4g15410	Yes	Yes	Yes	Yes (LoF)
PUX6	At3g21660	NE	NA	NA	Have seed, Not Analyzed
PUX7	At1g14570	Yes	Yes	Yes	Yes (TBD)
PUX8	At3g23605	Yes	Yes	TBD	Yes (TBD)
PUX9	At4g14250	NE	NA	NA	Have seed, Not Analyzed
PUX10	At1g59550	NE	NA	NA	Have seed, Not Analyzed
PUX11	At4g00752	Yes	Yes	Yes	Yes (TBD)
PUX12/SAY1	At4g11740	Yes (both PUX12 and SAY1)	Yes	YES and NO, respectively	Yes (TBD)
PUX13	At4g23040	Yes	Yes	Yes	WIP
PUX14/AtErasin	At2g43210	Yes	Yes	Yes	Yes (TBD)
PUX15	At4g10790	Yes	No	TBD	Have seed, Not Analyzed

Key	
NE	Not expressed
NA	Not applicable
TBD	To be determined
LoF	Loss-of-function
WIP	Work in progress

Table 1