PEROXISOME ASSOCIATED PROTEOLYTIC PROCESSES IN ARABIDOPSIS

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ABSTRACT

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Peroxisomes are small single-membrane-bounded organelles that play key roles in development and metabolism in most eukaryotic organisms. Peroxisome functions encompass ß-oxidation of fatty acids, detoxification of hydrogen peroxide and the metabolism of a range of biochemical compounds including glyoxylate, glycolate, urate, polyamines, benzoate, phylloquinone, bile acids and plasmalogen to name a few, as well as synthesis of plant hormones such as jasmonic acid (JA) and indole-3-butyric acid (IBA). Peroxisomes exhibit great functional diversity largely because of a plastic proteome that varies greatly depending on the environmental condition, tissue type or developmental stage of the specific organism. Proteins in peroxisomes are imported by the actions of conserved machinery that includes several proteins known as peroxins that are important for peroxisome biogenesis. Proteins destined for the peroxisome matrix contain peroxisome targeting signal (PTS) enabling their recognition by cytosolic receptor proteins that transport them to the peroxisome. Although we have an increased understanding of how peroxisomal protein import is accomplished, we know little about how proteins in peroxisomes are degraded. In this research, I provide evidence that RING domains of three peroxisomal membrane proteins AtPEX2, AtPEX10 and AtPEX12 have E3 ligase activity. I further show that AtPEX2 specifically interacts with two homologous ubiquitin receptor proteins, DSK2a and DS2Kb that have been implicated as adapters linking ubiquitination and 26S proteasome-based degradation

events. *DSK2* amiRNA lines lacked obvious plant growth phenotypes and were not compromised in peroxisome functions, suggesting that functional redundancies exist among ubiquitin receptor proteins. My results indicate that Arabidopsis RING peroxins and DSK2s can together form a peroxisome membrane associated degradation system.

I also explored the role of a predicted ovarian tumor-like cysteine protease (OCP1) in Arabidopsis. OCP1 was found to be a novel plant specific peroxisomal protein with a canonical C-terminal PTS1 and a novel N-terminal PTS2. Analysis of mutant lines revealed that OCP1 influences IBA metabolism in the peroxisome. Further, *ocp1* mutants show retarded degradation of two transiently expressed seedling peroxisome enzymes, isocitrate lyase (ICL) and malate synthase (MS) suggesting that OCP1 has a role in the timely removal of ICL from seedling peroxisomes. In summary, these studies add significantly to our knowledge of proteolysis in plant peroxisomes and open up several avenues for future investigations that may have ramifications in agriculture and biomedical applications.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

β-gal	Beta-galactosidase
Δ	Deletion
35S	Cauliflower Mosaic Virus 35S promoter
AA	Amino acid
ABRC	Arabidopsis Biological Resource Center
AD	Activation domain
Ala	Alanine
AmiRNA	Artificial microRNA
ARC	Accumulation and Replication of Chloroplasts
ATP	Adenosin-5'-Triphosphate
BD	DNA binding domain
BLAST	Basic Local Alignment Search Tool
C	Celsius
CFP	Cyan fluorescent protein
Col-0	Columbia ecotype 0
C-terminal	Carboxy terminal
Cys	Cysteine
DsRed	*Discosoma* species red fluorescent protein
DTT	Dithiothreitol
DUB	Deubiquitinase
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum

ERAD	Endoplasmic reticulum associated degradation
GFP	Green fluorescent protein
GST	Glutathione-S-transferase
IBA	Indole 3-butyric acid
IPTG	Isopropylthio- β -galactoside
JA	Jasmonic acid
KDa	Kilo Dalton
Lys	Lysine
MAD	Mitochondrial associated degradation
MBP	Maltose binding protein
NM	Nanometer
N-terminal	Amino terminal
PEX	Peroxin
PIM	Peroxisome import machinery
PMSF	Phenylmethylsulfonyl fluoride
PTS1	Peroxisomal targeting signal 1
PTS2	Peroxisomal targeting signal 2
RING	Really interesting new gene
SD	Synthetic dropout
SD/-HUT	SD media lacking histidine, uracil, and tryptophan
SD/-HUTL	SD media lacking histidine, uracil, tryptophan, and leucine
Ser	Serine
TBST	Tris-buffered saline with Tween 20

Thr	Threonine
Tyr	Tyrosine
T-DNA	Transfer DNA
Ub	Ubiquitin
UBC	Ubiquitin conjugating enzyme
UBQ	Ubiquitin
WT	Wildtype
YFP	Yellow fluorescent protein

CHAPTER 1

Literature Review

Peroxisomes and peroxisomal-associated proteolysis

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Peroxisomes

Overview

Peroxisomes are single membrane-bounded organelles found ubiquitously in all eukaryotes. Though predominantly associated with β-oxidation of fatty acids and hydrogen peroxide (H_2O_2) catabolism, peroxisomes also mediate an array of other biochemical processes that vary depending on the organism, *per se* plasmalogen biosynthesis in mammals, photorespiration in plants, methanol oxidation in some yeasts and glycolysis in trypanosomes. Partly through an expanded proteome, plant peroxisome functions have diversified and are known to contribute to several essential metabolic pathways such as those pertaining to glyoxylate cycle, parts of jasmonic acid (JA), benzoate, phylloquinone, biotin and ispoprenoid biosynthesis, as well as indole-3-butyric acid (IBA), polyamine, sulfite and urate metabolism (Tanabe et al., 2011; Hu et al., 2012; Klempien et al., 2012; Qualley et al., 2012; Widhalm et al., 2012). Over the last few years there is also growing cognizance that peroxisomes serve as platforms for countering or launching innate immune responses in both plants and mammals (Lipka et al., 2005; Bednarek et al., 2009; Clay et al., 2009; Dixit et al., 2010; Lazarow, 2011; Rojas et al., 2012) . Strong defects in peroxisome biogenesis or core peroxisome metabolic functions lead to fatal disorders in humans and embryonic lethality in plants, underscoring the biological necessity of functional peroxisomes (Schrader and Fahimi, 2008; Kaur et al., 2009).

Peroxisome assembly, division and inheritance are controlled by an eclectic set of proteins called peroxins (PEX) that are vital for peroxisome biogenesis and function (Distel et al., 1996). Reflective of their functional heterogeneities, peroxisomal

dysfunction mutants in various organisms have very specific phenotypes. Yeast null mutants in all components of peroxisomal protein import are viable but often lack morphologically distinguishable peroxisomes and are unable to metabolize oleate/methanol (Ma et al., 2011). In mammals, impaired PEX gene functions are manifested in form of pernicious diseases catalogued under peroxisome biogenesis disorders such as Zellweger syndrome spectrum and rhizomelic chondrodysplasia punctata type 1 (Nagotu et al., 2012). In Arabidopsis, while null mutants in several PEX genes are unviable, most of the partial loss-of-function mutants in *PEX* genes are weak and pale, with seedlings that often display resistance to auxin precursor IBA-mediated root inhibition and sucrose dependence (Kaur et al., 2009).

Peroxisome protein import

In the absence of a genome, the entire peroxisomal protein complement is comprised of proteins that are nuclear encoded and translated on cytosolic ribosomes prior to import into the organelle. Further, peroxisomes are distinguished from other organelles by their ability to import fully folded and even oligomeric proteins into the organelle matrix (Rucktaschel et al., 2011). Distinct groups of peroxins are devoted to sorting peroxisomal membrane and matrix proteins; an overview of only matrix protein import is provided here.

The basic architecture of the import machinery and order of events in the translocation of matrix proteins is essentially conserved in all organisms (Rucktaschel et al., 2011). Although several advances in dissecting protein import have been made, our understanding of mechanistic details is far from complete. Peroxisomes are amazingly malleable, adopting structurally similar but metabolically diverse forms depending on

cell type, developmental cues and prevailing environmental conditions. This dynamic remodeling is accomplished through the peroxisomal matrix protein import machinery (PIM/importomer). Background on the matrix protein import process is vital to understanding how degradation operates in the peroxisome, and thus is provided below. Nomenclature in use - PMP for peroxisome membrane protein, Pexp for yeast/fungi, Pex for mammals, AtPEX for Arabidopsis proteins and PEX when referring to proteins common to all three kingdoms.

Peroxisome targeting signals (PTSs) and PTS receptors

The targeting of peroxisomal matrix proteins is determined by the presence of peroxisome targeting signals (PTSs) that are recognized by circulating receptor proteins. The C-terminal tripeptide PTS1, which is usually comprised of Ser-Lys-Leu (SKL) or variants thereof, is used by the majority of known peroxisomal matrix proteins. A small fraction of matrix proteins use PTS2, an N-terminally located nonapeptide, RLx5HL, for import into peroxisomes (Schrader and Fahimi, 2008).

As the receptor for PTS1-containing proteins, PEX5 is composed of two clearly demarcated regions, a natively unfolded N-terminal half and a 6 tetratricopeptide repeat (TPR) domain half that binds the PTS1 peptide of cargo proteins (Carvalho et al., 2006). Multiple diaromatic repeats (WXXXF/Y) span the N-terminal half and are important for interactions with PEX14 on the peroxisome membrane (see the docking complex section below). A small number of peroxisomal proteins, which do not have PTS, are also bound by the N-terminal domain of Pex5p and pass into the peroxisomal matrix. The role of the N-terminal domain supposedly extends beyond trafficking of non-PTS

proteins, because its high conformational flexibility is implicated in enwrapping PTS1 proteins too (Schliebs et al., 1999; Otera et al., 2002; Grou et al., 2009b).

PEX7, a member of the β-transducin related domain (WD-40) family protein, is the receptor for PTS2-containing proteins. PEX7 cannot act autonomously and taps accessory proteins to ferry PTS2 proteins to peroxisomes (Platta and Erdmann, 2007; Rucktaschel et al., 2011). Three PEX7 co-receptors exist in fungi. Pex18p and Pex21p are partially redundant proteins that provide ancillary functions in PTS2 import in *Saccharomyces cerevisiae*. Apart from *S.cerevisiae,* all other fungi use Pex20p as an auxiliary protein for Pex7p mediated PTS2 import (Titorenko et al., 1998; Otzen et al., 2005; Leon et al., 2006b). In general, all PTS2 co-receptors are shuttling proteins that co-operate with Pex7p in the transport of PTS2 cargo and associate transiently with the PIM. In some fungal species, the co-receptor independently translocates the cargo to the peroxisome, but in others the translocation requires Pex7p (Titorenko and Rachubinski, 1998; Otzen et al., 2005; Leon et al., 2006a).

In a parsimonious evolutionary step, a PEX7 interaction motif was appended to PEX5 in plants and mammals, endowing two functions to the PTS1 receptor (Dodt et al., 2001; Einwachter et al., 2001). Arabidopsis uses just the single receptor for both PTS1 and PTS2 import, but in humans and rice a long isoform (Pex5L) serves as the PEX7 co-receptor (Otera et al., 2000; Hayashi et al., 2005; Woodward and Bartel, 2005; Lee et al., 2006).

The docking complex

PEX14 and PEX13 comprise the docking complex in mammals and plants; a

third protein, Pex17p is additionally found in this complex in fungi. PEX5-mediated interactions with PEX13 and PEX14 at the docking complex facilitate cargo translocation at the peroxisomal membrane (Rucktaschel et al., 2011). PEX5 is an unusual protein that adopts a transmembrane topology that allows it to traverse the peroxisomal membrane during the import process (Gouveia et al., 2000; Gouveia et al., 2003). These unique biochemical properties and interactions with PEX14 open a transient channel in the peroxisome membrane, which acts as a portal for the entry of the receptor-cargo complex (Meinecke et al., 2010).

Post docking protein complexes: RING peroxins and PEX4/PEX22

Current knowledge suggests that the Really Interesting New Gene (RING) domain-containing integral PMPs (PEX2, PEX10, PEX12) facilitate translocation of the receptor-cargo complex (Platta and Erdmann, 2007). Because omission of any of the RING peroxins is not conducive for import, events taking place after docking are not very well understood and deconstructing the *in vivo* functions for the RING peroxins has proved singularly challenging. That RING peroxin functions are fundamental for import is indisputable, but why and how, remain open questions. At some point after docking, the cargo proteins are released into the peroxisomal matrix by an uncharted mechanism. The interaction of a fungal specific intraperoxisomal bridging protein (Pex8p) with cargo-loaded Pex5p was proposed to disengage the Pex5p-cargo interaction (Agne et al., 2003). In the absence of compelling evidence for this hypothesis and lack of Pex8p homologs in plant and mammals, the discharge mechanism remains obscure. Pex4p is a member of the E2 family of ubiquitin

conjugation enzymes (Wiebel and Kunau, 1992; Koller et al., 1999). It is a peripheral membrane protein that is affixed to the membrane by a scaffolding protein, Pex22p (Koller et al., 1999). AtPEX22 is structurally and topologically similar to the corresponding proteins in fungi but shares very weak sequence similarities with their counterparts therefore it could only be identified through interaction by AtPEX4 (Zolman et al., 2005). While orthologs are present in plants, mammals seem to lack this complex (Grou et al., 2009b).

The dislocation complex

Two AAA ATPases (ATPases associated with various cellular activities), PEX1 and PEX6, comprise part of the peroxisome protein import machinery and are believed to play important roles in the dislocation of the receptor proteins to the cytosol, a process driven by ATP hydrolysis (Thoms and Erdmann, 2006). Pex15p in yeast, Pex26 in mammals and APEM9 in plants are the anchor proteins that tether the ATPases to the peroxisomal membrane (Goto et al.; Elgersma et al., 1997; Birschmann et al., 2003; Matsumoto et al., 2003). These three proteins are structurally similar but share low sequence identities, a mark of species-specific evolutionary divergence.

In sum, newly synthesized cargo proteins are recognized by cytosolic receptors and escorted to the peroxisomal docking complex. Post-docking the cargo is released into the lumen. The translocon, which is comprised of the RING peroxins, PEX4/22 and the AAA complex, aids in the extrication of the receptor from the peroxisomal membrane to the cytosol, where a new cycle of import is initiated (Figure 1.1).

Organellar proteolysis through the ubiquitin proteasome system (UPS)

Cells invest in diverse quality control (QC) methods, such as the ubiquitin-proteasome system (UPS), proteases, and autophagy, to orchestrate timely, effective and specific degradation of proteins, a central tenet of events guiding development in all organisms. Compartmentation affords cells the liberty of spatially controlled proteolysis, thus adding more dimensions to cellular proteostasis mechanisms (Pines and Lindon, 2005). Peroxisome biogenesis and functions are also regulated through the use of all these three modes of QC.

The ubiquitin proteasome system is the hub of proteolytic response within the cell. It is initialized by the covalent attachment of ubiquitin moieties to Lys residues in the substrate proteins through the consecutive actions of E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme; UBC), and E3 (ubiquitin ligase) (Dye and Schulman, 2007; Komander and Rape, 2012). Polyubiquitinated proteins are dispatched to the 26S proteasome, where they are subsequently dismantled (Ub chains), denatured and degraded (Bochtler et al., 1999). A growing body of work has begun to challenge the paradigm that UPS degradation is restricted to the cytosol. Endoplasmic reticulum-associated degradation (ERAD), mitochondrial-associated degradation (MAD) pathways and Protein QC system in the nucleus, all exemplify non-cytosolic functions of the UPS associated degradation (Gardner et al., 2005; Smith et al., 2011; Taylor and Rutter, 2011). Thus, organelle-associated UPS exist and are obviously far more pervasive than initially realized.

Endoplasmic reticulum-associated degradation (ERAD)

ERAD is especially fascinating and merits elaboration due to the startling parallels with PIM. Comparisons with the ER are also inevitable because evolutionarily the peroxisome biogenesis proteins are derived from ER and at least in yeasts, it is clear that the ER is the progenitor of peroxisomes (Gabaldon; Hoepfner et al., 2005; Tam et al., 2005; Schluter et al., 2006). Briefly, ERAD functions as a conserved eukaryotic QC and regulatory pathway that removes misfolded proteins from the ER (Vembar and Brodsky, 2008; Smith et al., 2011). Elimination of proteins requires retrograde transport across the ER membrane and is dependent on the cytosolic proteolytic machinery. Dedicated surveillance complexes at the ER membrane specifically recognize and degrade proteins resident in the diverse geographical regions of the ER. These varied pathways converge at the level of substrate dislocation and are completed with the help of an AAA ATPase that conveys the extracted proteins to the proteasome by way of accessory factors. A combination of proteins with different functional specialties is used in ERAD. These proteins include a TPR-containing membrane receptor protein, three peripheral UBCs, two multispanning integral membrane E3 RING ligases, an AAA ATPase, and several adapter proteins, all of which find resonance with components of the PIM. This raises the possibility that analogous to ERAD, PIM could regulate peroxisome proteolysis by explicitly targeting peroxisomal proteins for degradation. This idea has gained acceptance with the discovery of ubiquitination of the peroxisomal receptor proteins by the PIM, (Purdue and Lazarow, 2001; Kiel et al., 2005b; Kragt et al., 2005; Leon et al., 2006b; Platta et al., 2007; Hensel et al., 2011) as detailed in the following sections.

Ubiquitination of peroxisomal receptor and co-receptor proteins

Ubiquitination of the yeast Pex5p

The inherent instability of Pex5p was well known, but the molecular basis of this phenomenon remained a mystery (Koller et al., 1999; Zolman and Bartel, 2004; Kiel et al., 2005b). The persistence of Pex5p and presence of its higher molecular weight entities in late peroxin mutants (Pex4p, Pex22p, Pex1p, Pex6p, Pex15p) was indicative of the occurrence of post-translation modification (PTM) of this receptor (Platta et al., 2004; Kiel et al., 2005b; Kragt et al., 2005). Further investigations revealed that Pex5p was modified by ubiquitination. In agreement with this, degradation resistant Pex5p forms were seen in proteasomal mutants and upon treatment with proteasomal inhibitors (Platta et al., 2004; Kiel et al., 2005b; Kiel et al., 2005a; Kragt et al., 2005). Lack of peroxisomes, defects in docking machinery and absence of RING peroxins abrogated the ubiquitination of Pex5p, underscoring that import was a necessary facet of this process (Platta et al., 2004; Kiel et al., 2005b). Genetic analysis defined the type of ubiquitination on Pex5p, with monoubiquitination predominating in *Δpex4p* or *Δpex22p* but polyubiquitination in the dislocation complex mutants' *Δpex1p*, *Δpex6p*, or *Δpex15p* (Platta et al., 2004; Kiel et al., 2005b; Kragt et al., 2005). Introducing mutations of various yeast *UBCs* to *Δpex1* or *Δpex4* background further clarified that Ubc4p oversaw the polyubiquitination and monoubiquitination was reliant on Pex4p (see section titled Ubc4p-meidated polyubiquitination) and 1.3.4) (Platta et al., 2004; Kiel et al., 2005b; Kragt et al., 2005; Williams et al., 2007). Sequential deletions of Pex5p identified that the N-terminal domain was sufficient for ubiquitination (Williams et al., 2007); further systemic elimination of lysines in this region found that Lys^{18} and Lys^{24}

were the sites for polyubiquitination (Kragt et al., 2005; Platta et al., 2007). However, peroxisomal functions were unaffected by a block in Pex5p polyubiquitination (Williams et al., 2007). A Pex5p variant mutated for $Lys^{18/24}$ was still monoubiquitinated (Williams et al., 2007). A conserved Cys^{6} residue was ascertained to be the recipient for monoubiquitination; altering this residue had direct impacts on the ability of the yeast to metabolize oleate (Williams et al., 2007). Further, it was demonstrated that ubiquitinated Pex5p was stalled in the peroxisomal membranes and could not relocate to the cytosol in the absence of terminal import peroxins, for example, Pex4p, Pex22p, Pex1p, Pex6p and Pex15p (Platta et al., 2007). These results proffer an explanation for the dynamic localization of Pex5p wherein import is a function of early acting peroxins while the departure is regulated by ubiquitination and late acting peroxins (Kiel et al., 2005b; Platta and Erdmann, 2007).

Ubiquitination of mammalian Pex5

Previous experiments had established the dependence of Pex5 export on the presence of ATP and Cys^{11} on Pex5 (Oliveira et al., 2003; Carvalho et al., 2007b). Using the export deficient ^{35}S-labeled Pex5C11S and GST-Ub, *in vitro* import experiments proved that Pex5 is monoubiquitinated (Ub-Pex5) (Carvalho et al., 2007a). Interestingly, in case of the ^{35}S-labeled Pex5, Ub-Pex5 could only be identified under non-reducing conditions. Taken together, it was evident that an Ub thiol ester-conjugated Pex5 was generated normally and the variant Pex5C11S underwent covalent Ub linkage. Further, it was determined that Cys^{11} was the site for

monoubiquitination of Pex5 and that post-ubiquitination Ub-Pex5 resided in the soluble fraction (Carvalho et al., 2007a). Consistent with this, substitution of Cys^{11} with Ala or Arg abolished ubiquitination, trapping Pex5 in the peroxisome membrane (Grou et al., 2009a). Intriguingly, replacing Cys^{11} with Lys (Pex5C11K) did not change the properties of the receptor, as Pex5C11K was ubiquitinated and both import/export processes were unperturbed. These results suggest that the presence of Ub is mandatory for export of Pex5 but Cys residue and thiol ester bond are not obligatory for the process (Grou et al., 2009a). Corroborating evidence obtained from *in vivo* studies also prove that tagging Pex5 with Ub marks it for export by the dislocases (Okumoto et al., 2011b).

It is worth mentioning that PEX5 sequence alignments show that the Cys residue (Cys^{6} in *S.cerevisiae* and Cys^{11} in humans) is evolutionarily conserved. The N-terminal domains of PEX5 from *S. cerveisiae* and human are interchangeable, implying that the mechanisms underlying PTS1 receptor recycling are shared in all organisms (Carvalho et al., 2007a; Williams et al., 2007). Although ubiquitination of PEX5 has not been demonstrated in plants, the lower AtPEX5 levels in the *Atpex6* mutant, conservation of the import machinery across kingdoms, and the conserved Cys recycling residue on AtPEX5 suggest that plant PEX5 may be modified in a similar fashion (Figure 1.2A) (Zolman and Bartel, 2004). A model showing known factors involved in yeast and mammalian PEX5 monoubiquitination and yeast receptor polyubiquitination processes is shown in Figure 1.2 B, C and D respectively.

Ubiquitination of Pex20p in Pichia pastoris

Pex20p, the PTS2 co-receptor in *P. pastoris*, is normally partitioned between the cytosol and peroxisomes, where the RING peroxins are indispensable for the cytosolic relocation and Pex14p for the peroxisomal localization. The steady state levels of Pex20p are grossly compromised in late step import mutants (Pex4p, Pex1p, Pex6p) and polyubiquitination at Lys^{19} is the source of this destabilization (Leon et al., 2006b). Further, a conserved N-terminal Cys^{8} residue and the presence of Ub Lys48 polyubiquitination were deemed necessary for the egress of Pex20p (Leon et al., 2006b; Leon and Subramani, 2007). Cumulatively, this has spawned a model where during the normal course of events Pex14p supports Pex20p import while the Cys^{8} and RING peroxins are essential for its export. In situations where export is blocked, a QC system kicks in to ubiquitinate Pex20p for its degradation. The involvement of combined Pex4p-RING peroxin activities and an uncharacterized UBC in Pex20p ubiquitination are invoked. This pathway has been termed RADAR (Receptor recycling and degradation in the absence of recycling) (Leon et al., 2006b).

Ubiquitination of Pex18p in S. cerevisiae

Pex18p, the PTS2 co-receptor in *S. cerevisiae*, is a short-lived protein that undergoes constitutive turnover but is stabilized in mutants of proteins that can be sorted into 4 categories: (i) import peroxins such as Pex13p, Pex14p, Pex4p, Pex1p and Pex6p, (ii) PMP receptor Pex3p, (iii) Ubc4p and Ubc5p, and (iv) proteasome related proteins Cim5p (regulatory subunit of 26S proteasome) and Doa4p (deubiquitinase) (Purdue and Lazarow, 2001; Hensel et al., 2011). From these findings it was inferred

that ubiquitination of Pex18p needs the proteasome, E2s, presence of intact peroxisomes and the peroxisome import machinery. Additionally, ubiquitination of Pex18p was demonstrated and linked to residues Lys^{13}, Lys^{20} and Cys^{6}. Like Pex20p, the $Pex18pK^{13/20}$ was determined to be important for the RADAR pathway and the $Pex18pC^{6}$ was vital for recycling of Pex18p. The relevance of recycling is emphasized by the fact that the $Pex18pC^{6}$ species arrests on peroxisome membranes and fails to complement the *Δpex18pΔpex21p* mutants (Hensel et al., 2011).

So far, all three of the identified ubiquitinated peroxisomal receptors/co-receptors have conserved Cys and Lys residues in their N-terminal regions and their peroxisomal dynamics employ strikingly similar mechanisms (Hensel et al., 2011). Collectively, it seems apparent that peroxisomes preferentially use Cys (Ub) to channel receptors for recycling to propagate further rounds of import. The lability of the thioester bond seems to offer a facile and energy efficient strategy to reuse receptors and accelerate import kinetics. On the other hand, the peroxisome QC machinery evokes the conventional Lys (Ub) in consigning dysfunctional receptors to the UPS (Ma et al., 2011).

Ubc4p-mediated polyubiquitination

As previously mentioned, Ubc4p is responsible for the bulk of polyubiquitinated Pex5p and Pex18p (Purdue and Lazarow, 2001; Platta et al., 2004; Kiel et al., 2005b; Kragt et al., 2005). Ubc4p is a cytosolic E2 that has partially overlapping roles with Ubc5p and Ubc1p (Seufert and Jentsch, 1990; Seufert et al., 1990). Consistent with this functional redundancy, polyubiquitination of Pex5p is observed in *ΔUbc4p*, *ΔUbc4p*

ΔUbc5p and *ΔUbc4p ΔUbc1p* backgrounds (Platta et al., 2004). Since polyubiquitination resistant Pex5p ($Lys^{18/24}$ mutant) can functionally replace Pex5p, these enzymes are expected to be expendable; however, there are discrepancies about their reported contribution to peroxisome metabolism (Platta et al., 2004; Kragt et al., 2005).

Pex4p-mediated monoubiquitination

The peroxisomal localization and an intact catalytic site of Pex4p are crucial for Pex5p monoubiquitination (Platta et al., 2007). *In-vitro* experiments conducted with recombinant Pex4p show that it monoubiquitinates Pex5p and also possesses autoubiquitinating activity (Platta et al., 2007). Pex22p acts as a non-canonical E2 co-activator, stimulating the transfer of ubiquitin by Pex4p (Williams et al., 2012). Monoubiquitination of Pex5p primes it for dissociation from the peroxisome membrane by the AAA complex (Platta et al., 2007). The kinetic stability of Pex5p in *ΔPex4* is species-specific as enumerated by the extremely rapid turnover in *P. pastoris*, enhanced depletion in *Hansenula polymorpha,* and extended stability in *S. cerevisiae* (Koller et al., 1999; Platta et al., 2004; Kiel et al., 2005b; Kiel et al., 2005a; Kragt et al., 2005). Seemingly irreconcilable, these differences arise from the varying efficiency of proteasomal degradation of stalled Pex5p by the organism in question (Williams et al., 2007). Lastly, since Pex4p precedes the dislocases in peroxisomal import, dual modifications by Ubc4 as well as Pex4p cause the pronounced Pex5p polyubiquitination in AAA complex mutants (Williams et al., 2007). Whether AtPex4 also has similar biochemical activity is not yet known.

Mammalian E2D family proteins carry PEX4 like functions

Devoid of apparent PEX4 homologs, the cytosol of mammalian cells was shown to harbor the UBC activity responsible for ubiquitination of Pex5. Biochemical fractionation of rat liver cytosol isolated five potential UBCs, three of which were members of the E2D family (UbcH5a/b/c) (Grou et al., 2008). Purified peroxisomes incubated with exogenous E1 and recombinant E2Ds stimulated the production of Ub-Pex5, validating that the E2D family proteins implement PEX4-like function in mammals (Grou et al., 2008). This E2D-mediated ubiquitination took place after docking and translocation but prior to export, reinforcing the idea that receptor ubiquitination is prerequisite for export to occur (Figure 1.2C) (Grou et al., 2009b).

The role of the RING peroxins as E3 Ligases

RING domain proteins are part of the E3 ligase superfamily that connect E2 to the substrate, completing the ubiquitin circuit for tagging proteins for degradation by the 26S proteasome (Deshaies and Joazeiro, 2009). The necessity of RING peroxins for receptor ubiquitination, the presence of an E2 (Pex4p) in the peroxisome, and the physical interaction of RING peroxins with Pex5p as well as Pex4p made the three RING peroxins (i.e., PEX2, PEX10 and PEX12) strong candidates for E3s (Chang et al., 1999; Okumoto et al., 2000; Albertini et al., 2001; Eckert and Johnsson, 2003; Kragt et al., 2005). *In vitro* assays confirmed that all three RING peroxins from yeast exhibit ubiquitin ligase activities and allocated Pex12p for monoubiquitination and Pex2p and Pex10p for polyubiquitination activities (Williams et al., 2008; Platta et al., 2009). It is also conjectured that perhaps the RING peroxins act as multimeric complexes with

varying stoichiometries. Whether RING peroxins bestow substrate specificity for receptor ubiquitination or act in the QC for non-receptor peroxisomal proteins remains unresolved. Whether RING peroxins essay E3 ligase activities in mammals and plants has not been explored.

Deubiquitinating enzymes (DUBs) mediate receptor regeneration

In yeast, using the peroxisomal AAA-complex (Pex1p/Pex6p) as bait pulled down a protein with DUB activity that was traced to ubiquitin specific protease 15 (Ubp15p). Ubp15p partially colocalizes with peroxisomes, interacts with first AAA domain of Pex6p and is able to cleave ubiquitin from Pex5p. Mutant strains show clustered peroxisomes, higher levels of ubiquitinated Pex5p and stress induced PTS1 defects (Debelyy et al., 2011). Removal of ubiquitin from Pex5p could have multiple effects, such as being necessary for the pre-export recognition by AAA complex, to evade proteasomal degradation and extend the stability of the protein or for interaction(s) with PTS1 proteins in the cytosol. Together with the ATPases, Ubp15p-mediated hydrolysis of ubiquitinated Pex5p is a key element in the export and recycling of the PTS1 receptor (Debelyy et al., 2011).

In mammals, ubiquitin specific protease 9X (USP-9X) is responsible for removing Ub from Pex5. Enhanced detection of Ub-Pex5 in UbaI (DUB inhibitor) supplemented samples indicated that a DUB actively regulates the level of the exported Ub-Pex5 (Grou et al., 2009a). In line with the soluble nature of Ub-Pex5, the ATP-dependent dislocation of Ub-Pex5 species and its subsequent deubiquitination were determined to be uncoupled events occurring at the peroxisome membrane and cytosol respectively.

Subjecting rat liver cytosol to size exclusion chromatography and monitoring eluates for DUB activity against Ub-Pex5 narrowed down to a candidate enzyme. MS analysis of this candidate protein band revealed that USP-9X encoded the relevant activity profile, hydrolyzing thioester, peptide and isopeptide bond-linked Ub-Pex5 (Grou et al., 2012). However, suppression of USP-9X in HeLA cells failed to divulge impaired peroxisomal import or have any effect on PEX5 steady state levels. This contradictory result may be rationalized through the occurrence of compensatory activities of alternative USPs, non-enzymatic breakdown of Ub-PEX5 or incomplete silencing of USP9X (Grou et al., 2012). Further, considering the inherent weakness of thioester linkages, it was also found that physiological levels of GSH (glutathione) were sufficient to disrupt the Ub-PEX5 molecule (Grou et al., 2009a). Given that Ub derivitization of PEX5 does not hamper its PTS1 cargo binding/transporting capabilities and the non-durable nature of Ub-PEX5 conjugate, the exact benefit of USP-9X in receptor recycling remains to be elucidated (Grou et al., 2009a).

Arabidopsis peroxisome-associated degradation (PexAD)

A previous study demonstrated that during the developmental transition of seedling peroxisomes to leaf peroxisomes, mutants of *AtPEX4*, *AtPEX22*, *AtPEX6* and *AtPEX5* have stabilized glyoxylate cycle enzymes, i.e., ICL (isocitrate lyase) and MS (malate synthase). In addition, elevated or diminished levels of peroxisome-generated H_2O_2 expedite or impede the degradation of ICL and MS. Collectively, this study suggests that these four Arabidopsis peroxins have novel roles in the removal of

damaged or obsolete peroxisome matrix proteins, a process termed PexAD (Lingard et al., 2009).

Role of a RING ligase in inter-organellar traffic

It has been noted in mammalian cells that some small cargo-loaded vesicles that bud off from the mitochondria ultimately coalesce with a subset of peroxisomes (Neuspiel et al., 2008). Remarkably, these mitochondrial-derived vesicles (MDVs) are loaded with a RING ligase named MAPL (Mitochondrial-Anchored Protein Ligase). MAPL is a small ubiquitin like modifier (SUMO) E3 ligase that promotes the activity of the mitochondrial fission protein, dynamin related protein 1 (Drp1) by sumoylation (Braschi et al., 2009). Since Drp1 is known to be ubiquitinated by mitochondrial ligases (Nakamura et al., 2006; Yonashiro et al., 2006; Horn et al., 2011; Wang et al., 2011), SUMOylation probably blocks the ubiquitination sites on Drp1, enhancing the stability of the protein. Peroxisomal fission also requires Drp1 (Schrader et al., 2012). Although the role of the MDV-delivered MAPL in peroxisomes is presently unclear, it is an interesting avenue for further research.

Peroxisomal chaperones and proteases

Cells employ the collective efforts of chaperones and proteases to circumvent the deleterious effects of misfolded, unfolded, or aggregated proteins. Most subcellular organelles host a battery of Hsps and molecular chaperones in their lumen to assist in the folding of newly imported proteins or refolding of denatured polypeptides (Leidhold and Voos, 2007). Although the primary role of proteases is to purge deleterious or

denatured proteins from the cellular milieu, there is growing awareness that they also play regulatory roles in cells through modulating the function of substrate proteins (Lopez-Otin and Bond, 2008). Given the irreversible nature of degradation, protease actions must be fine-tuned to ensure that the right substrate is processed at the correct temporal or developmental juncture (Lopez-Otin and Bond, 2008). Compartmentalization is an additional regulatory mechanism to maintain the specificity of protease action. Studies on peroxisomal proteases are fairly incipient and the fate of obsolete or damaged peroxisomal proteins is not very well understood. The following sections summarize the current state of knowledge.

Chaperones

The import of folded substrates by peroxisomes seems to preclude the need for chaperone activity within the organelle. Although *in vitro* import assays have shown that cytosolic Hsp70 (heat shock protein) and Hsp90 chaperone activities are associated with and enhance peroxisomal protein import in pumpkin (Crookes and Olsen, 1998), little is known about protein (re-) folding within peroxisomes. Considering the high production rate of reactive oxygen species in peroxisomes, it is likely that peroxisomes have evolved a mechanism to safeguard matrix proteins from denaturation. Knowing that chaperones are prerequisites for luciferase renaturation, a study conducted on mammalian cultured cells used it as a reporter to assess the effect of heat stress and thermotolerance by targeting it to various organelles. Peroxisomes mirrored the kinetics and efficiency of luciferase refolding observed for other organelles. It was concluded that peroxisomes must therefore possess constitutive as well as heat inducible factors

that aid in protein refolding in their matrix. The involvement of non-canonical chaperones that execute the stress response was also considered a possibility (Hageman et al., 2007). In mammalian cells, a recent study points at heat shock factor 1 (HSF1) as a major regulator of protein refolding in peroxisomes (Heldens et al., 2012).

Although mammals and yeast seem to lack intraperoxisomal matrix chaperones, two matrix targeted small Hsps from Arabidopsis complement the corresponding yeast *hsp* mutant, hinting at the functional conservation of this type of Hsps as chaperones across kingdoms (Ma et al., 2006). Analysis of loss-of-function mutants of the sHsps will be instrumental to defining the physiological function of these proteins in greater details. Small Hsps lack ATP-hydrolyzing activity and are dependent on other Hsps/ATPases for protein renaturation. Various Hsp70 isoforms and DnaJ homologues have been reported to be present in peroxisomes in other plant species (Preisig-Muller et al., 1994; Corpas and Trelease, 1997; Wimmer et al., 1997; Diefenbach and Kindl, 2000); their orthologs could conceivably be present in Arabidopsis. These proteins, in conjunction with the identified sHsps, may help to alleviate stress-induced protein aggregation and denaturation in the peroxisomal matrix.

Proteases

Mammalian Trypsin containing domain 1 (Tysnd1) protease and Arabidipsis DEG15 protease

A prominent feature for PTS2 protein import is the post-import cleavage of the N-terminal signal peptide. In mitochondria and chloroplasts, cleavage of the transit peptide is mandatory for folding or further targeting of proteins to organellar sub-structures

(Adam and Clarke, 2002; Gakh et al., 2002). Traditionally, peroxisomes of developing oilseeds have been termed glyoxysomes due to the presence of isocitrate lyase and malate synthase, enzymes that operate in the glyoxylate cycle (Nishimura and Beevers, 1979). A glyoxysomal processing protease (GPP) belonging to the Deg/HtrA [high temperature requirement A] family of Ser proteases was purified from watermelon and shown to harbor PTS2 processing activity. Further biochemical characterization of GPP revealed that GPP exists in equilibrium between a general degrading monomeric form and the dimeric processing protease. GPP exhibited activity maxima in the alkaline range of pH 8-9; temperature of 45°C and the presence of Ca^{2+} ions shifted the equilibrium to the dimeric form (Helm et al., 2007). *Atdeg15* (Arabidopsis homolog of GPP) plants display mild resistance to the IBA- mediated root inhibition and loss of PTS2 processing of several peroxisomal proteins (Schuhmann et al., 2008). Considering that PTS2 protein processing seems to be restricted to higher eukaryotes and the absence of PTS2 processing has no effect on either enzyme activity or protein import, it is not surprising that *Atdeg15* has no significant defect in peroxisome metabolism or plant physiology. These observations have also led to the suggestion that there seems to be no specific evolutionary benefit associated with the retention of PTS2 processing in higher eukaryotes. However, only a nominal number of proteins in yeasts deploy the PTS2 import pathway compared to plants, where the PTS2 repertoire has undergone manifold expansion in number (19 in Arabidopsis vs. 3 in yeast) as well as type (7 PTS2 in Arabidopsis vs. 1 in yeast). The sheer diversity of PTS2 signals prompts the hypotheses that (i) alternate cleavage activities that act redundantly with AtDEG15 may be present in plant peroxisomes and (ii) effects of PTS2 non-cleavage on

import efficiency and enzyme activity have been examined for only a small subset of PTS2 proteins (2 out of 19), and might not hold true for all PTS2 enzymes (Kato et al., 1996; Kato et al., 1998). It is also plausible that PTS2 processing may be important not for the import but rather for retention of the proteins in peroxisomes. Furthermore, transit peptide processing of mitochondrial proteins is vital for their stability, because uncleaved precursor proteins are subject to rapid degradation (Millar et al., 2008). It is not inconceivable that PTS2 cleavage bestows enhanced stability to substrate enzymes within the peroxisome. Lastly, several organellar protease activities are vital contributors to the assembly of multienzyme complexes that facilitate metabolic channeling, thus DEG15 may play an analogous role in peroxisomes (see below and section titled co-operative activities of AtDEG15/Tysnd1 and AtLON2/PsLon).

The mammalian counterpart of AtDEG15, Tysnd1 was indentified through directed PTS1-based bioinformatics searches. In a departure from norm, a handful of PTS1 enzymes in mammals undergo regulated cleavage by an undefined protease (Osumi et al., 1980; Mori et al., 1991; Leenders et al., 1994). In addition to PTS2, these PTS1 enzymes turned out to be substrates for Tysnd1's Ser endopeptidase activity (Kurochkin et al., 2007). As all Tysnd1 substrates are part of the β-oxidation enzyme spiral, it was proposed that Tysnd1 promotes the arrangement of supramolecular-enzyme complexes that streamline β-oxidation in peroxisomes (Kurochkin et al., 2007).

Tysnd1 is subject to auto-catalytic regulation, where self-cleavage inhibits its' protease activity. Depletion of Tysnd1 led to the accumulation of premature/unprocessed proteins, elongated peroxisomes and a significant reduction in β-oxidation of very long chain fatty acids (VLCFAs). Though the PTS1 substrate

proteins are easily coimmunoprecipitated with the catalytically inactive Tysnd1, the same could not be demonstrated for PTS2 precursor proteins, indicative of the complexities that govern substrate recognition by proteases. Tysnd1 also interacts with itself to form oligomers and binds to another peroxisomal protease, PsLon (see section on mammalian PsLon and section titled co-operative activities of AtDEG15/Tysnd1 and AtLON2/PsLon) (Okumoto et al., 2011a).

Phylogenetic analysis conducted on the Deg family of proteases found that the family is divided into four distinct groups, reflective of their protein domain structure. Notably, all the peroxisomal paralogues of AtDEG15, contrary to other DEGs, have a C-terminal protease domain and are characterized by the absence of recognizable PDZ (postsynaptic density of 95 kDa, Discs large and zonula occludens 1) domains (Helm et al., 2007). PDZ domain(s) are often known as protease cofactors since they have dual responsibilities, i.e., substrate recognition and activation of the protease domain (Clausen et al., 2011). AtDEG15 and Tysnd1 lack PDZ domains; how they accomplish substrate recognition is not well understood. The protease domain of AtDEG15 is also atypical, with a ~60-amino acid stretch separating the catalytic His from the rest of the catalytic residues. Removing this loop from recombinant AtDEG15 caused a slight reduction in substrate processing, however the significance (if any) of this stretch is currently unknown (Schuhmann et al., 2008).

Long form radiation sensitive (Lon) protease

Lon are Ser proteases that belong to the evolutionarily conserved AAA+ ATPase family. Lon assembles into a hexameric ring that is equipped with a central catalytic

cavity. This architectural module ensures that only unfolded substrates are subject to degradation. While the ATP hydrolysis powers substrate unfolding and translocation, proteolysis occurs within the confines of the chamber. Each subunit of the hexamer is composed of an N-terminal, an AAA+ ATPase and a protease domain. The exposure of aromatic and hydrophobic residue-rich segments in unfolded or misfolded proteins enables substrate recognition by Lon; such segments/regions have been termed degrons (Sauer and Baker, 2011). Lon has a prominent and well-established role in clearance of unfolded and oxidized protein in bacterial species and mitochondria. Lon proteases may also act as chaperones facilitating re-folding of proteins or assembly of protein complexes (Suzuki et al., 1997; Janska et al., 2010). In this aspect, the degron sequence seems to be a key regulatory element that dictates whether Lon opts for the protease or the chaperone activity (Gur and Sauer, 2009). The known functions of peroxisomal Lon isoforms are presented in the following paragraphs.

Fungal Peroxisomal Lon (pLon)

pLon is conserved among all fungi barring *S. cerevisiae* and *Candida albicans.* Absence of *H. polymorpha* pLon was associated with increased intracellular ROS, decreased cell viability, a modest increase in peroxisomal numbers and partial stabilization of an intrinsically unfolded protein. The characteristics of pLon mutant gave rise to the speculation that the protein functions in elimination of ROS-damaged peroxisomal proteins (Aksam et al., 2007).

Mammalian Peroxisomal Lon (PsLon)

PsLon was discovered through proteomics conducted with peroxisomes isolated from rat hepatocytes (Kikuchi et al., 2004). Subsequent studies revealed that PsLon interacted with a host of β-oxidation enzymes but was only responsible for the specific processing-based activation of acyl CoA oxidase (AOX). HEK293 cell lines expressing a dominant negative isoform of PsLon had defects in AOX cleavage, reduced β-oxidation and aberrant distribution of catalase, while overexpression of PsLon caused enlargement of peroxisomes (Omi et al., 2008). However, this AOX processing feature of PsLon was not reproducible in HeLa cells in which PsLon was knocked down *via* siRNA. Instead, PsLon appears to process the self-cleavage products of Tysnd1 and in accordance with this observation, Lon degron segments are present in both Tsynd1 fragments strengthening the notion that PsLon degrades Tysnd1 fragments (Okumoto et al., 2011a). PsLon is induced as an aftermath of treatment with a peroxisome proliferator, remarkably reaching an expression maxima following discontinuation of treatment while other resident peroxisome proteins are turned over (Yokota et al., 2008). These results were interpreted to signify that the persistent PsLon was involved in actively degrading the complement of induced peroxisome matrix enzymes. Together with 15-LOX (see section titled pexophagy in mammals), PsLon is believed to contribute ~ 20-30% of peroxisome degradation in mammals.

Plant Peroxisomal LON2 (AtLON2)

The Arabidopsis *lon2* mutants are compromised in assorted peroxisome related functions, ranging from marked reduction in lateral root production, missorting of peroxisomal matrix proteins such as catalase, hydroxypyruvate reductase and malate

dehydrogenase (CAT, HPR and MDH), accelerated degradation of thiolase, to subtle IBA resistance and somewhat mild sucrose dependence in seedlings. The mutant plants also exhibit progressive exacerbation of aforementioned matrix protein mislocalization and an overall retarded growth phenotype (Lingard and Bartel, 2009). The maize peroxisomal ZmLON2 partially complements the yeast mitochondrial Lonp mutant (*pim1*), suggesting that the proteins have functional compatibility (Barakat et al., 1998). Moreover, mutants of the mitochondrial Lon isoforms in both Arabidopsis and humans also show age-related phenotypes, lending credence to the idea that proteolytic activity of AtLON2 is instrumental in timely removal of proteins that impede sustained matrix protein import (Lingard and Bartel, 2009; Rigas et al., 2009b; Rigas et al., 2009a; Ugarte et al., 2010).

Cooperative activities of AtDEG15/Tysnd1 and AtLON2/PsLon

The interaction between Tysnd1 and PsLon alongwith the role of PsLon in degrading Tysnd1 fragments has led to a model where these enzymes act in a concerted fashion to regulate peroxisomal β-oxidation. Constitutive processing of β-oxidation pathway enzymes by Tysnd1 is proposed to promote their arrangement into multiprotein complexes. Meanwhile, a subset of the existing Tysnd1 pool undergoes autocatalysis, resulting in downregulation of its activity. The peptide fragments generated through Tysnd1 self and substrate cleavages are proteolyzed by PsLon. Alternatively, part of self-cleaved Tsynd1 could retain protease activity, and degradation by PsLon may render it inactive (Figure 1.3) (Okumoto et al., 2011a). In Arabidopsis, combining the *Atdeg15* and *Atlon2* mutations accentuate growth defects observed in

Atlon2, providing genetic support for the coordinated actions of these two enzymes (Lingard and Bartel, 2009).

Insulin degrading enzyme (IDE)/ peroxisomal peptidase M16 (AtPXM16)

The mammalian IDE, the first peroxisomal protease identified, is classified as a metalloendoprotease because of the presence of a unique Zn binding motif (Authier et al., 1994). IDE was initially implicated in proteolysis of the thiolase preleader sequence. Subsequent characterizations revealed that IDE catalysis is limited to niche substrates < 7 kDa, with oxidized lysozyme and hemoglobin comprising the only exceptions. IDE is evolutionarily conserved. Although most homologs contain PTS1 sequences, a major fraction of the enzyme in mammalian cells is in the cytosol, imparting the eponymous insulin degrading activity (Kurochkin, 2001). *AtPXM16* encodes for the Arabidopsis homolog of mammalian IDE; examinations of T-DNA insertion alleles have so far failed to uncover a function for this peptidase in plants (Lingard and Bartel, 2009).

Other Proteases

Studies in pea have found up to seven endoprotease activities associated with senescent peroxisomes, and these activities were speculated to play roles in turning over not only peroxisomal proteins during early senescence but also proteins from multiple subcellular compartments during advanced stages of senescence (Distefano et al., 1997; Distefano et al., 1999). Molecular identification of these proteases is still lacking. Several prospective peroxisomal proteases have also been identified through plant peroxisome proteomics and bioinformatics studies (Reumann et al., 2004; Reumann et al., 2009; Lingner et al., 2011).The validation and characterization of these

putative peroxisomal proteases is ongoing and may shed light on the precise contribution of each to peroxisomal endoproteolytic activity and plant metabolism.

Pexophagy

Pexophagy refers to the selective degradation of peroxisomes by the autophagic machinery of the cell in response to environmental cues. Pexophagy shares several components of the classical autophagy machinery (ATGM) but additionally also requires specificity factors that recruit the ATGM to exclusively degrade peroxisomes (Johansen and Lamark, 2011; Weidberg et al., 2011). Peroxisomes are targeted for degradation by two distinct pathways, which vary both genetically and in their morphological progression (Figure 1.4). Macropexophagy is the engulfment of an individual peroxisome by a double membrane structure known as pexophagosome, which subsequently undergoes heterotypic fusion with either lysosomes (mammals) or the vacuole (yeasts). In micropexophagy, a cluster of peroxisomes is almost completely surrounded by an extended vacuolar membrane and capped by a specialized double membraned structure known as microphagy specific membrane apparatus (MIPA). Membrane fusion events between the MIPA and the vacuolar arm deliver the trapped peroxisomes to the vacuole lumen for degradation. Till date micropexophagy has only been observed in *P. pastoris*. The phagophore assembly site (PAS) is prerequisite for macropexophagy and micropexophagy, serving as an initiation site that promotes nucleation of the membrane for both the pexophagosomes as well as the MIPA (Manjithaya et al., 2010; Till et al., 2012).

Pexophagy in yeasts

Methylotrophic yeasts have proved to be an ideal model system to study and decipher pexophagy pathways. Simple shifts in nutritional media cause a rapid surge in peroxisome numbers, accounting for ~ 40% of the cell volume. Conversely, switching to media, which causes repression of peroxisome proliferation/biogenesis, triggers pexophagy. For instance, in *P. pastoris,* oleate or methanol induces peroxisome proliferation. Transferring cells from oleate to glucose or methanol to ethanol causes macropexophagy, while switching from methanol to glucose results in micropexophagy (Manjithaya et al., 2010). Apart from nutritional clues, cellular ATP homeostasis is an important determinant of whether micropexophagy (high ATP) or macropexophagy (low ATP) occurs (Till et al., 2012). As previously mentioned, though the core ATGM is shared with pexophagy, it is the selectivity factors that confer specificity to the response. Mutants of selectivity factors only show phenotypes for pexophagy. Atg30p is the peroxisomal cargo receptor, which tags peroxisomes for degradation. Atg30p is activated *via* phosphorylation, resulting in binding to PMPs such as Pex3p and Pex14p on the peroxisomes while simultaneously interacting with PAS organizing components, Atg11p and Atg7p (Farre et al., 2008). Atg11p and Atg17p serve as scaffolds responsible for recruiting other constituents of ATGM (Figure 1.5). Atg26p is a steroyl glucosyltranferase that mediates elongation of the membrane at the PAS (Yamashita et al., 2006). Furthermore, Atg26p, along with Atg30p, Atg11p as well as Atg17p, are essential for degradation of large peroxisomes, possibly through mediating expansion of enveloping membranes (Nazarko et al., 2009). Atg35p contributes only to micropexophagy and is required for MIPA formation (Nazarko et al., 2011). Finally,

Atg24p was shown to act in the final steps of pexophagy possibly by regulating vacuolar membrane fusion (Ano et al., 2005).

Pexophagy in mammals

It is well known that treatment with hypolipidemic drugs promotes peroxisome proliferation in mammals. It had also been noted that withdrawal of drugs resulted in dramatic decrease of the proliferated liver peroxisomes, though this observation had received scant attention until recently (Moody and Reddy, 1976). Combining proliferation-based recovery models with ultrastructural studies and administration of leupeptin to block lysosomal proteases, researchers observed peroxisomes within autophagosome-like double-membraned structures (Kovacs et al., 1982; Yokota et al., 1993). Several studies also showed that peroxisomal populations were stabilized in the presence of an autophagy inhibitor, 3-methyl adenine (Luiken et al., 1992; Kondo and Makita, 1997; Huybrechts et al., 2009). Further, the persistence of excess peroxisomes in autophagy deficient ($ATG7^{-/-}$) mice provided evidence for the direct involvement of the autophagic machinery in peroxisome degradation in mammals (Iwata et al., 2006). So far, PEX14, LC3-II (processed and lipidated microtubule associated protein 1 light chain 3), along with intact microtubules, are the only known components of the mammalian pexophagy response (Hara-Kuge and Fujiki, 2008). Interaction of PEX14 with LC3-II occurs at the same site as does PEX14-PEX5 binding, suggesting that competitive binding discerns the fate of peroxisomes, i.e. degradation vs. preservation (Oku and Sakai, 2010).

p62, an autophagy adapter protein, facilitates the recognition and autophagic turnover of polyubiquitinated protein aggregates in mammals. A recent study showed that in mammalian cells, artificially fusing an ubiquitin moiety to the cytosolic side of ectopically expressed PMPs, PMP34 and PEX3, was sufficient to induce degradation of peroxisomes in a p62- and ATG12-dependent manner (Kim et al., 2008). However, the physiological PMP receptor for this kind of degradation remains to be identified.

15-Lipoxygenase (15-LOX) is a cytosolic enzyme that can peroxidize membrane lipids. It has been shown that 15-LOX can specifically associate with peroxisome membranes, resulting in autolysis of the organelle. In this case, 15-LOX dissolves the peroxisomal membrane, exposing peroxisomal proteins to the cytosolic proteases. Addition of 15-LOX inhibitors reduced the focal disruptions of peroxisome membranes (Yokota et al., 2001). These results suggest that 15-LOX activities contribute to a minor extent to the turnover of peroxisomes in mammalian cells (Yokota and Dariush Fahimi, 2009).

Pexophagy in plants

There have been no reported incidences of pexophagy occurring in plants (Liu and Bassham, 2012). Although the core ATGM seems to be conserved from yeast to plants, none of the selective ATG factors appear to have any cognate orthologs in Arabidopsis (Meijer et al., 2007). The significantly higher number of peroxisomes per cell coupled with a lack of reliable peroxisome proliferators and quantification methodologies make studying pexophagy in plants extremely challenging. As mentioned in 1.4.2.1, during the remodeling of glyoxysomes to peroxisomes, ICL and

MS are degraded in a temporal fashion and this degradation is retarded in several peroxin import mutants (Lingard et al., 2009; Monroe-Augustus et al., 2011). Although degradation of ICL and MS is delayed in peroxin mutants, it is not completely abolished suggesting that perhaps the entire organelle might undergo regulated turnover. Thus, this developmental transition might serve as a good starting point to investigate pexophagy in plants.

Physiological importance of pexophagy

Organelle populations and homeostasis are the collective outcome of the biogenesis, proliferation and degradation processes. Since pexophagy has been mainly studied in the context of introduction and subsequent withdrawal of peroxisome proliferators in yeasts and mammals, until recently, the primary role attributed to pexophagy was elimination of obsolete/superfluous organelles in order to salvage and conserve cellular resources (Manjithaya et al., 2010). Using HaloTag® technology, which enables visualization of tagged marker proteins or organelles under physiological conditions, it has been shown that 30% of the normal cellular peroxisomes in mammals undergoes pexophagy per day, indicating that it is a regular constitutive response (Huybrechts et al., 2009). In addition, constitutive degradation of peroxisomes has been observed in *H. polymorpha*, even under conditions that promote peroxisome proliferation (Aksam et al., 2007).

Peroxisome accumulation has been reported in aging human cells and in autophagy mutants of yeast, a phenotype accompanied by a decrease in cell viability (Legakis et al., 2002; Aksam et al., 2007). Increased peroxisome population has also

been correlated to increased reactive oxygen species (ROS) in both organisms, due to decreased activity of catalase (*H.p*) or reduced import capacity of peroxisomes (humans) (Legakis et al., 2002; Aksam et al., 2007). These data suggest that there is a direct link between peroxisome turnover, redox status and cellular ageing/viability.

Surprisingly, pexophagy seems to be an important event that determines the infectivity of both plant and human pathogenic fungi. In the absence of Atg26p-mediated pexophagy, the cucumber anthracnose fungi*, Colletotrichum orbicular*, fails to penetrate and consequently to invade the host plant (Asakura et al., 2009). In *Arabidopsis,* a recent study demonstrated that both autophagy and pexophagy are necessary for the growth and survival of *Candida boidinii* cells at the phyllopshere (Kawaguchi et al., 2011). Likewise, the human opportunistic fungus, *Candida glabrata*, exploits the pexophagy response to survive when being internalized by circulating macrophages; loss of pexophagy specific Atg11p results in a substantial loss of parasite survival in macrophages (Roetzer et al., 2010). Thus, it seems apparent that in pathogenic fungi, peroxisomes serve as an easily mobilizable resource to meet the cellular demands imposed during host pathogenesis and parasitic virulence.

Aims of the thesis research

Although various components of plant peroxisome matrix protein import machinery had been identified, prior to the research undertaken in this thesis, little was known about proteolytic processes that occur in plant peroxisomes. Ubiquitination of PEX5 in various yeast species had just been reported and parallels to ERAD were being noted. Previous research in Arabidopsis had established that homozygous

mutants in RING peroxins were embryo lethal. Work presented in Chapter 2 thus used in-vitro approaches to circumvent the technical challenges of working with embryo lethal mutants and sought to answer whether RING peroxins of plant peroxisomes had E3 ligase activities. Additional experiments were aimed at identifying and characterizing interactor proteins to provide insights into the mechanims governing peroxisome associated degradation processes in plants. In Chapter 3 I focused on a putative peroxisomal protease and present data on the identification and characterization of this protein which seems to be involved in overseeing degradation of transiently expressed glyoxylate cycle enzyme, ICL. Finally in Chapter 4, general conclusions derived from this body of work are presented along with directions for future research.

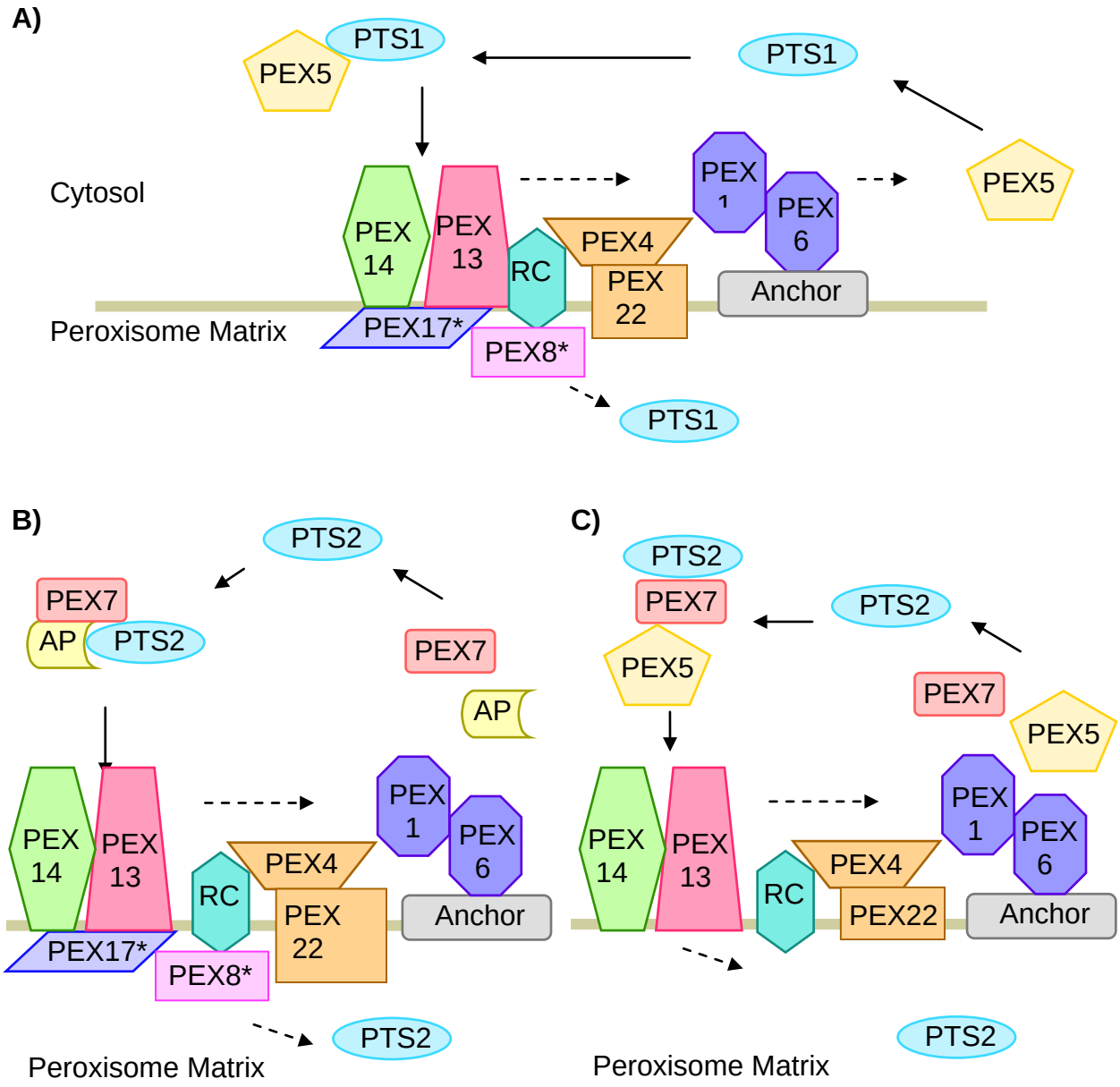

Figure1.1 Model for matrix protein import into peroxisomes.

A) Import of PTS1 proteins: PEX5 recognizes and binds PTS1-containing proteins in the cytosol. The receptor-PTS1 protein complex then traffics to the peroxisome where PEX5 associates with the docking complex proteins, PEX14 and PEX13, on the peroxisome membrane. Interactions between PEX5 and PEX14 open a transientchannel in the peroxisome membrane that allows the import of the receptor PTS1 protein complex into the peroxisome. Subsequently, the PTS1 protein is

Figure1.1(Cont'd)

dissociated from PEX5 and released into the peroxisomal matrix by an unknown mechanism. The export of the receptor to the cytosol is facilitated by the consecutive actions of the RING complex including PEX2, PEX10 and PEX12 (RC in the figure) and PEX4/PEX22 complex (absent in mammals) followed by ATP-driven dislocation mediated by the peroxisomal AAA ATPases, PEX1 and PEX6. The AAA complex is tethered to the peroxisome membrane by Pex15p in yeast, Pex26 in mammals and APEM9 in plants (represented by Anchor in the figure). Two components are unique to yeast (marked by asterisk); Pex17p in the docking complex and Pex8p that serves as an intraperoxisomal organizer.

B) Import of PTS2 proteins in fungi: PEX7 recognizes and binds PTS2-containing proteins in the cytosol. The PEX7-PTS2 protein complex binds coordinately with an accessory protein (AP) and is ferried to the peroxisome docking complex. The identity of AP varies depending on the fungal species (Pex18p and Pex21p in *S. cerevisiae* or Pex20p in *P. pastoris*). The subsequent steps of import are assumed to be similar to PTS1 import. The events facilitating the release of PEX7 and PTS2 protein are not well known.

C) Import of PTS2 proteins in plants and mammals: PEX5 serves as the accessory protein in import of PTS2 proteins in plants and mammals. All other steps are proposed to follow similar pathways as PTS1 import.

For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.

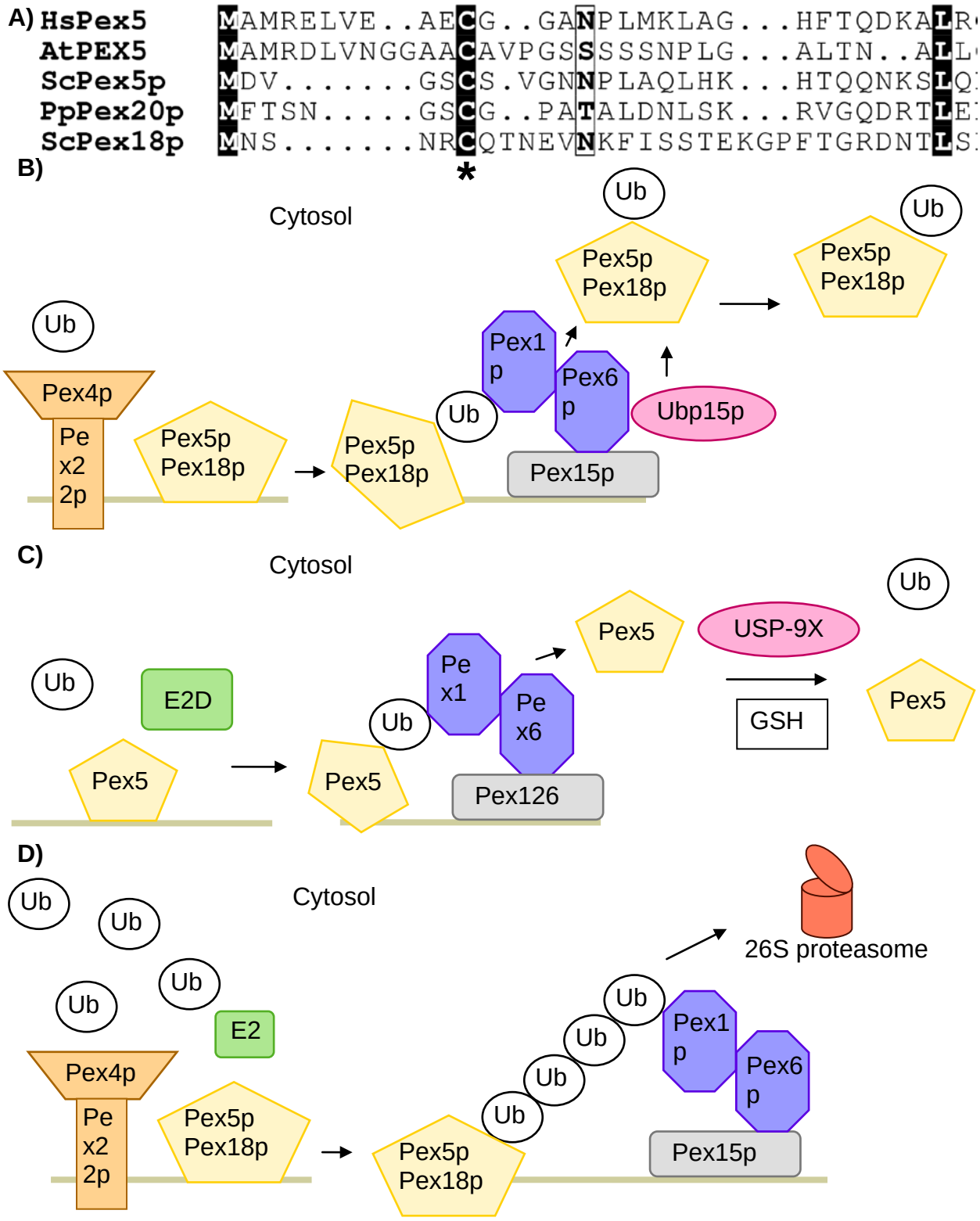

Figure1.2 Ubiquitination of peroxisome receptor proteins.

A) Schematic showing the sequence alignment of N-terminal regions of PTS1 receptor

Figure1.2(Cont'd)

(PEX5) and co-receptors (Pex18p, Pex20p) from various organisms. An asterisk indicates the conserved cysteine residue present in all evolutionary lineages. Fifty amino-acid regions from human Pex5 (HsPex5), *Arabidopsis thaliana* PEX5 (AtPEX5), *S. cerevisiae* Pex5p (ScPex5p), *P. pastoris* Pex20p (PpPex20p) and *S. cerevisiae* Pex18p (Pex18p) were aligned using ClustalW.

B) Yeast receptor proteins (Pex5p and Pex18p) undergo monoubiquitination on a conserved Cys residue in a Pex4p dependent manner. Monoubiquitination facilitates export of the receptor from the peroxisome membrane by the dislocation complex. Peroxisome associated deubiquitinase, Ubp15p, cleaves the ubiquitin from the receptor completing the receptor recycling pathway.

C) Mammalian Pex5 is monoubiquitinated on Cys^{11} by the activities of the cytosolic E2D family of ubiquitin conjugating enzymes. Addition of ubiquitin enables the export of Pex5 to the cytosol by the dislocation complex. In the cytosol, ubiquitin is removed from Pex5 either by a deubiquitinase (USP-9X) or by disruption of the Ub-Pex5 thio-ester bond by glutathione (GSH) present in the cytosol. Recycled Pex5 re-initiates the import pathway.

D) A block in export or precocious stalling of receptor proteins (Pex5p, Pex18p, Pex20p) on the peroxisome membrane triggers a quality control mechanism that polyubiquitinates yeast receptor proteins. Polyubiquitination occurs on Lys residues and requires the activities of Ubc4p, Ubc5p and Ubc1p (depicted by E2). Polyubiquitinated receptors are degraded *via* the 26S proteasome. Pex18p is not shown in the figure.

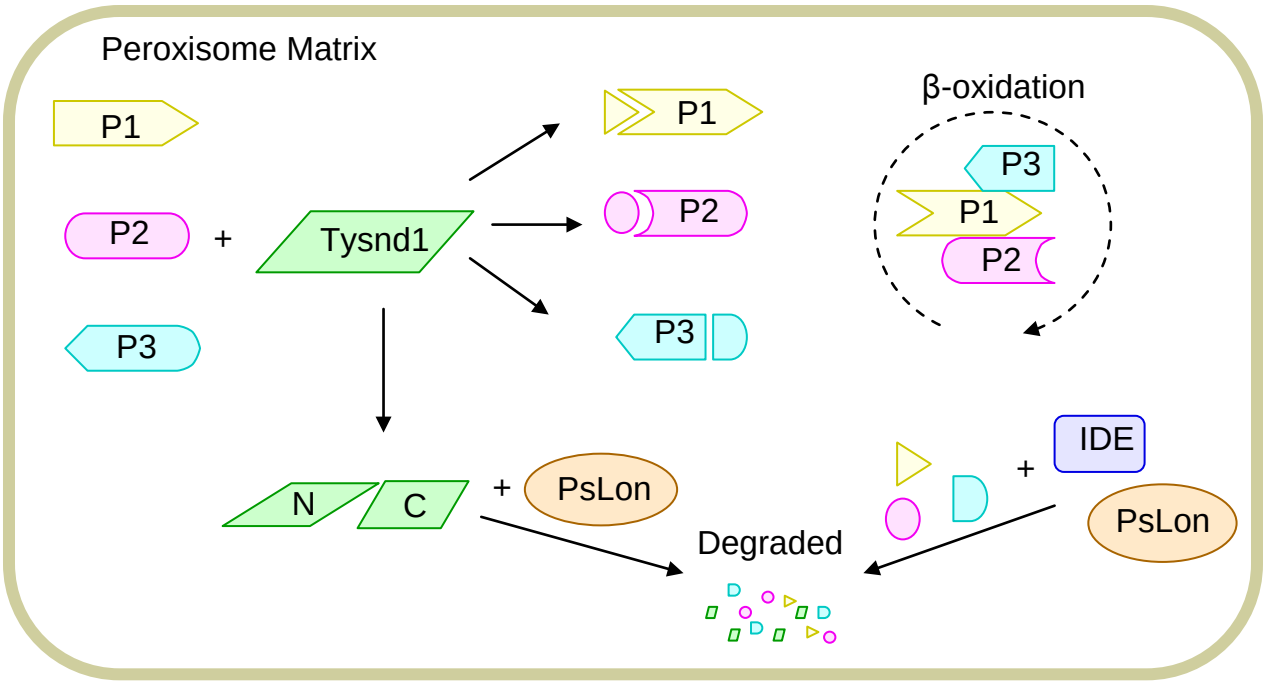

Figure1.3 Role of peroxisomal proteases.

Tysnd1 processes enzymes in the β-oxidation pathway by either removing PTS2 peptide (P1, P2) or by regulated cleavage of PTS1 proteins (P3). Processing facilitates the organization of enzymes into multiprotein complexes resulting in efficient β-oxidation. The enzyme segments cleaved by Tysnd1 are probably further degraded by PsLon or IDE. Tysnd1 is inactivated by autocatalysis generating two fragments, N and C. N and C expose degron sequences resulting in their proteolysis by PsLon.

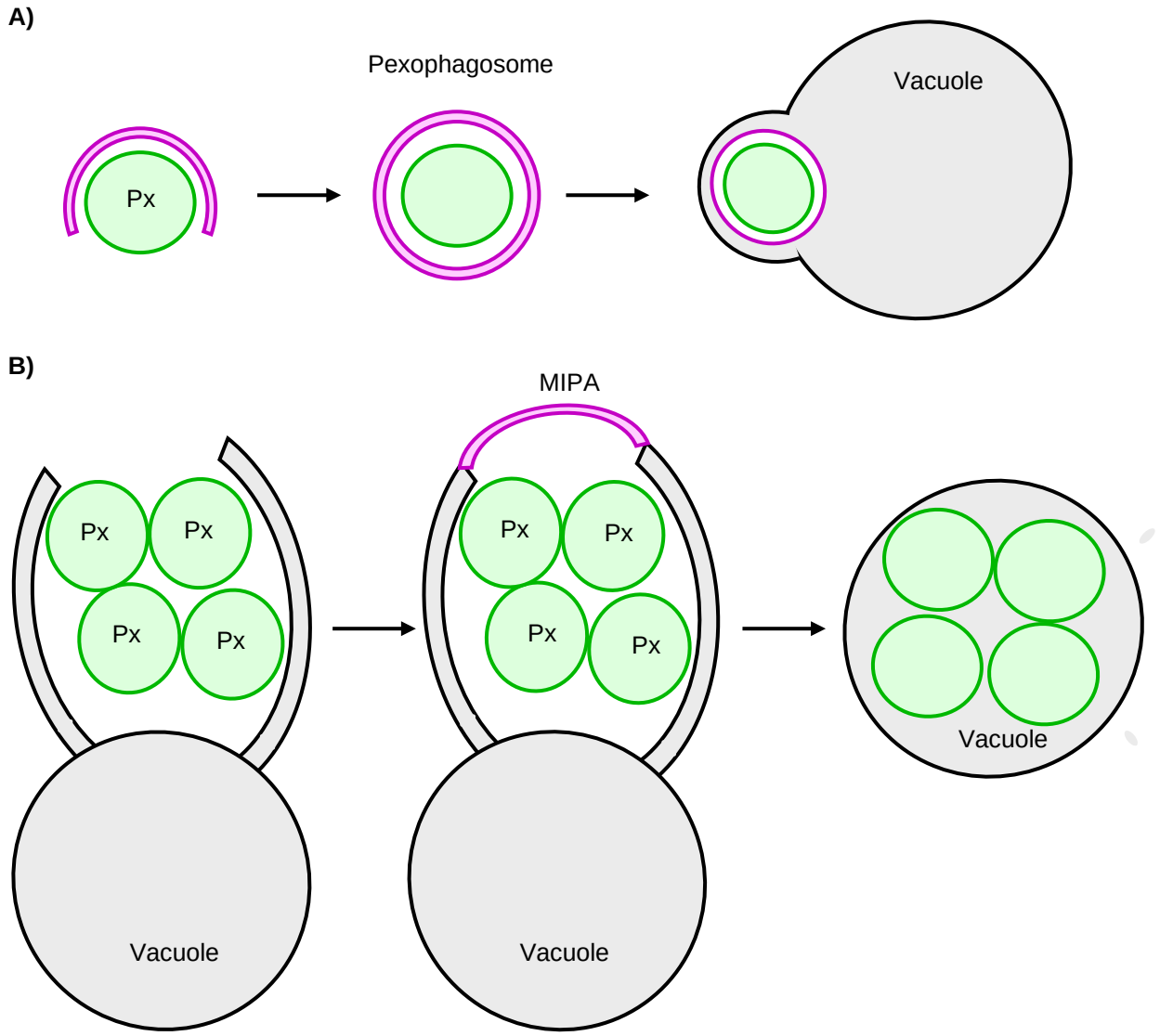

Figure1.4 Modes of Pexophagy.

A) Macropexophagy: An individual peroxisome (Px) is surrounded by a double membraned structure called the pexophagosome that delivers the peroxisome to the vacuole for degradation. The PAS is the progenitor of the pexophagosome membrane (not shown).

B) Micropexophagy: A cluster of peroxisomes are engulfed by sequestering membranes originating from the vacuole and capped by the microphagy specific membrane apparatus (MIPA).

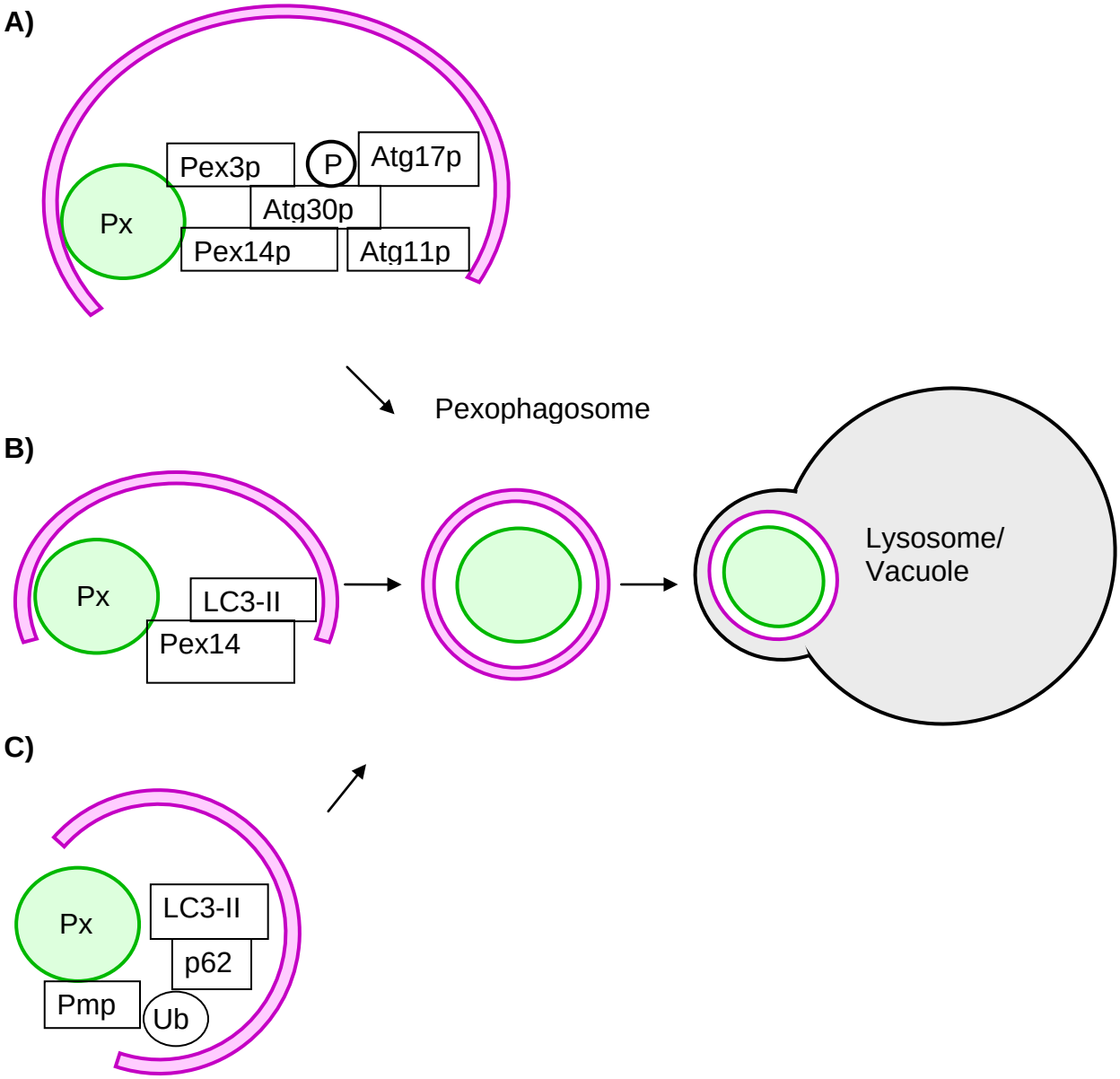

Figure1.5 Comparison of selectivity factors involved in pexophagy.

A) Pexophagy in fungi: Atg30p acts as the receptor for pexophagy. Interaction of Atg30p with Pex3p and Pex14p enables it localization at the peroxisome (Px) membrane. Phosphorylation of Atg30p (shown as P) facilitates its interaction with ATGM scaffold proteins, Atg11p and Atg17p. Synergistic contributions of some other selectivity factors (not shown) and ATGM components results in the formation of the pexophagosome that subsequently fuses with the vacuole.

Figure1.5 (Cont'd)

B) Pexophagy in mammals: Interactions between Pex14 and LC3-II determine the onset of pexophagy in mammals. Microtubules are also necessary for the process (not shown).

C) Ubiquitin mediated pexophagy in mammals: p62 acts as the autophagy receptor for ubiquitinated aggregates. Artificial fusion of ubiquitin to Pmps (Pex3 or Pmp34) facilitates recognition by p62 and selective autophagic degradation.

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CHAPTER 2

Arabidopsis RING Peroxins are E3 Ubiquitin Ligases that Interact with Two Homologous Ubiquitin Receptor Proteins

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ABSTRACT

Peroxisomes are essential eukaryotic organelles that mediate various metabolic processes. Peroxisome import depends on a group of peroxisome biogenesis factors called peroxins, many of which are evolutionarily conserved. PEX2, PEX10 and PEX12 are three RING finger-domain-containing integral membrane peroxins crucial for protein import. In yeast, RING peroxins act as E3 ligases, facilitating the recycling of the peroxisome import receptor protein PEX5 through ubiquitination. In plants, RING peroxins are essential to plant vitality. To elucidate the mode of action of the plant RING peroxins, we employed in vitro assays to show that the Arabidopsis RING peroxins also contain E3 ligase activities. We also identified a PEX2-interacting protein, DSK2b, which is a member of the ubiquitin receptor family known to function as shuttle factors ferrying polyubiquitinated substrates to the proteasome for degradation. DSK2b and its tandem duplicate DSK2a are localized in the cytosol and the nucleus, and both interact with the RING domain of PEX2 and PEX12. *DSK2* amiRNA lines did not display obvious defects in plant growth or peroxisomal processes, indicating functional redundancies among Arabidopsis ubiquitin receptor proteins. Our results suggest that Arabidopsis RING peroxins can function as E3 ligases and act together with the ubiquitin receptor protein DSK2 in the peroxisomal membrane-associated protein degradation system.

INTRODUCTION

Peroxisomes are single membrane-bounded organelles found ubiquitously in eukaryotes. They mediate an array of biochemical reactions and are crucial players in lipid metabolism and detoxification reactions (Islinger et al., 2010). In plants, peroxisome functions have also been implicated in glyoxylate cycle, photorespiration, hormone metabolism (IBA, JA), and other metabolic functions (Kaur et al., 2009; Hu et al., 2012). The significance of peroxisomes is underscored by the numerous human diseases and lethal plant phenotypes caused by peroxisomal deficiencies (Steinberg et al., 2006; Hu et al., 2012).

Peroxisomes are metabolically plastic, remodeling their constituent enzyme complement depending on cell type, developmental cues and prevailing environmental conditions (Islinger et al.; Hayashi and Nishimura, 2006). The changes in peroxisome proteome are accomplished by virtue of the dynamic import machinery, which comprises of a set of proteins called peroxins (PEX), many of which are conserved from fungi, animals and plants (Distel et al., 1996; Hu et al., 2012). Peroxisome matrix proteins, most of which are distinguished by the presence of a peroxisome targeting signal (PTS), are encoded in the nucleus and imported into the peroxisome post-translationally. There are two kinds of PTS: PTS1 is a C-terminal tripeptide, while PTS2 is an N-terminal cleavable nonapeptide (Platta and Erdmann, 2007). PEX5 and PEX7 are cytosolic receptors for PTS1- and PTS2-containing proteins respectively. In plants, the PTS2-bound PEX7 protein further binds co-operatively with PEX5 to form an import competent cargo-receptor complex (Hu et al., 2012). The cargo-receptor complex is then ferried to the membrane-docking complex consisting of PEX14 and PEX13. This

event is followed by translocation and finally export of the receptor, which is then ready for another round of protein import (Rucktaschel et al., 2011).

Translocation is accomplished by the consecutive activities of the RING finger complex (PEX2, PEX10, PEX12), ubiquitin-conjugating enzyme (UBC) complex (PEX4, PEX22) and two AAA ATPases (PEX1, PEX6). Although the post docking events are not very well understood, it is known that the three RING peroxins are vital for peroxisome import to occur (Rucktaschel et al., 2011). These RING peroxins are thought to form an importomer to aid matrix protein import, but their exact mode of action remains undefined. In both yeast and mammals, the recycling of the PTS1 receptor PEX5 is contingent upon monoubiquitination (Platta et al., 2004; Kiel et al., 2005; Kragt et al., 2005; Carvalho et al., 2007; Grou et al., 2009; Okumoto et al., 2011). In yeasts, the E2 activity is imparted by PEX4, while in mammals the E2D family of UBCs catalyzes PEX5 monoubiquitination (Platta et al., 2007; Grou et al., 2008). Furthermore, if recycling is compromised, the yeast PEX5 undergoes polyubiquitination and proteasomal based degradation (Platta et al., 2004; Kiel et al., 2005; Kragt et al., 2005). In this context, the yeast RING peroxins have been demonstrated to have ubiquitin ligase activity, wherein PEX12 functions as a monoubiquitin ligase while PEX2 and PEX10 contain polyubiquitinating activities (Williams et al., 2008; Platta et al., 2009).

So far, there is no direct evidence to support the notion that PEX5 in plants has to be ubiquitinated prior to export. It has been noted that the PEX5 Cys residue that undergoes ubiquitination is conserved in plants and that the import mutant, *pex6*, has

lower steady state levels of PEX5, hinting at similar mechanisms for PEX5 recycling in plants (Zolman and Bartel, 2004; Hu et al., 2012). Similarities such as the presence of UBCs, RING ligases and AAA ATPases, have also been drawn between the peroxisome import machinery and Endoplasmic Reticulum Associated protein Degradation (ERAD), a quality control system that removes misfolded proteins from the ER (Schluter et al., 2006). In support of this model, Arabidopsis *pex5*, *pex4 pex22* double and *pex6* mutants show enhanced stability of two transiently expressed enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase, during the developmental transition of seedling peroxisomes to leaf peroxisomes (Lingard et al., 2009). This study suggests that these peroxins may have a role in the regulated removal of obsolete or damaged proteins from within the peroxisome.

In yeast, null mutants of the RING peroxins are viable but the peroxisomes are not import competent (Platta and Erdmann, 2007). In mammals, mutations in any of the RING peroxins results in fatal genetic diseases such as the Zellweger spectrum disorders (Steinberg et al., 2006). In *Arabidopsis,* null mutants of the RING peroxins have embryo lethal phenotypes, implying the essential functions of these proteins for survival (Hu et al., 2002; Schumann et al., 2003; Sparkes et al., 2003; Fan et al., 2005). It is also speculated that the plant RING peroxins have novel functions apart from their role in matrix protein import. For example, a gain-of-function allele of *PEX2* (*ted3*) suppresses the phenotypes of the photomorphogenic mutant *det1*, while RNAi lines of *PEX10* have defects in cuticular wax accumulation (Hu et al., 2002; Kamigaki et al., 2009). In addition, plants overexpressing a dysfunctional PEX10 contained deformed peroxisomes with reduced contact with chloroplasts and exhibited impaired

photorespiration. This change in peroxisome morphology was linked to the TLGEEY motif in the N terminus of PEX10, and the RING domain was implicated in mediating inter-organellar contact (Schumann et al., 2007; Prestele et al., 2010).

To elucidate the molecular function of the RING peroxins in plants, we first employed in vitro assays to demonstrate that all three Arabidopsis RING peroxins act as ubiquitin ligases. Further, using a yeast two-hybrid screen with the RING domain of PEX2 as bait, we identified DSK2b, a ubiquitin receptor protein that links substrate ubiquitination process and proteasomal degradation events. The interaction was confirmed with in vitro pull down assays and structural motifs responsible for the interaction were dissected. Both DSK2b and its closely related paralog DSK2a could interact with PEX12 in addition to PEX2, and both are localized to the cytosol as well as the nucleus. Reducing the expression of the *DSK2* genes by artificial microRNA (amiRNA) did not cause obvious defects in peroxisome functions, suggesting functional redundancy between members of the ubiquitin receptor family in Arabidopsis.

RESULTS

The Arabidopsis RING peroxins possess E3 ligase activities

The majority of the RING proteins, including the yeast RING peroxins, function as E3 ligases (Deshaies and Joazeiro, 2009). To investigate if the Arabidopsis RING peroxins possess E3 ligase activity, we cloned, expressed and purified MBP fusions of the PEX2/10/12 RING domains, all of which reside at the cytoplasmic C terminus of the proteins (Figure 2.1A and 2.1B). The cellular ubiquitination cascade was reconstituted using an in vitro ubiquitination assay that comprises of a wheat E1 ubiquitin activating

enzyme, the human E2 ubiquitin conjugating enzyme UBCH5b, recombinant RINGs, and His-ubiquitin (Xie et al., 2002; Zhang et al., 2007). In the presence of E1, E2 and ubiquitin, all of $PEX2^{RING}$, $PEX10^{RING}$ and $PEX12^{RING}$ exhibited monoubiquitination activity, whereas no activity was observed with MBP alone or in the absence of E1 or E2 (Figure 2.1C, 2.1D and 2.1E).

In parallel, we used another in vitro system with a rabbit E1 and another human E2 (UBCH5c) to test for E3 ligase activity. We also observed monoubiquitination activity of $PEX2^{RING}$ and $PEX10^{RING}$, and in case of $PEX12^{RING}$ a possibly enhanced E3 activity was detected (Figure 2.2A).

The RING motif forms a cross brace structure, in which eight metal ligands, usually cysteine and histidine, co-ordinate two Zn ions (Deshaies and Joazeiro, 2009). While PEX2 and PEX10 have all the eight conserved metal ligands, PEX12 only has five of the eight conserved residues for zinc binding (Figure 1A), which has been shown to bind a single zinc ion (Zn) in yeast (Koellensperger et al., 2007). Thus, to further test the importance of the RING structure to the E3 activity of $PEX2^{RING}$ and $PEX10^{RING}$, we used a Zn chelator, *N*,*N*,*N′*,*N′*-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN), to disrupt the RING domain of PEX2 and PEX10, and tested whether the disrupted RING domains still possess E3 activity using a previously published in vitro E3 assay system that employs a yeast E1 and an Arabidopsis E2 (UBC8) (Stone et al. 2005). The well characterized E3 ligase COP1-Interacting Protein 8 (CIP8; Hardtke et al., 2002) was used as a positive control. In the presence of E1, UBC8 and bovine ubiquitin, CIP8, $PEX2^{RING}$, and $PEX10^{RING}$ showed E3 ligase activity (Figure 2.2B and 2.2C).

Depletion of Zn by TPEN treatment abolished ubiquitin ligase activity for the E3s, whereas the lost E3 activity of TPEN-treated samples could be restored by incubation with zinc chloride (Figure 2.3A). Immunoblotting of the samples with MBP antibodies confirmed that $PEX2^{RING}$ and $PEX10^{RING}$ undergo Zn-dependent autoubiqutination (Figure 2.3B).

Taken together, our data demonstrate that the RING domain of the three Arabidopsis RING peroxins, PEX2, PEX10, and PEX12, contain E3 ubiquitin ligase activity. At least for PEX2 and PEX10, the E3 activities are dependent on the binding to Zn ions.

Identification of ubiquitin receptor family proteins DSK2a and DSK2b as PEX2-interacting proteins

The detection of E3 ligase activity for all three Arabidopsis RING peroxins prompted us to search for their functional partners and potential substrates. We began this effort by trying to identify PEX2-interacting proteins in a yeast two-hybrid (Y2H) screen, using the RING domain of PEX2 ($PEX2^{RING}$) as bait against an Arabidopsis seedling cDNA library with the GAL4 Y2H system. Sequencing of positive clones revealed a potential interacting protein, DSK2b (At2g17200).

DSK2b belongs to the ubiquitin-like (UBL) and ubiquitin-associated (UBA) domain-containing ubiquitin receptor proteins that mediate recognition of ubiquitinated substrates for ubiquitin-proteasome based degradation (Fu et al., 2010). These proteins bind to ubiquitinated substrates *via* the UBA domain and the proteasome subunits through the UBL domain, thus serving as shuttle factors that couple polyubiquitination to

proteasomal degradation (Fatimababy et al., 2010). In Arabidopsis, DSK2b has a tandemly duplicated and highly similar paralog, DSK2a (At2g17190), which shares 82% sequence identities with DSK2b at the protein level. The DSK2 proteins each consist of an N-terminal UBL domain, four chaperonin domains in the middle and a C-terminal UBA domain (Figure 2.4A). Given the high sequence similarities between DSK2a and DSK2b, we tested both proteins in a LexA matchmaker Y2H system for interaction with the full-length PEX2 and PEX2^{RING}. Positive interactions, revealed by the LacZ reporter activity (blue color) in the colonies, were observed only between the two DSK2s and PEX2^{RING}, in addition to the positive control (Figure 2.4B), suggesting that both DSK2a and DSK2b are capable of interacting with PEX2^{RING}. The expression of the fusion proteins in yeast was verified by immunoblotting (Figure 2.4D). The absence of interaction with the full-length PEX2 could be due to mistargeting or misfolding of the proteins, or steric constraints that obscured the RING domain.

We then used in vitro pull down assays to validate the Y2H interaction results by expressing recombinant DSK2a and DSK2b as 6xHis-tagged fusion proteins and PEX2^{RING} as a MBP fusion protein, and assessed the ability of Ni-NTA-bound His-DSK2 to retain or pull down MBP-PEX2^{RING} in bacterial lysates. Both DSK2a and DSK2b were capable of pulling down MBP-PEX2^{RING} (Figure 2.4C). We conclude that DSK2a and DSK2b indeed interact with the RING domain of PEX2.

Previous studies on DSK2 showed that the N–terminal UBL domain interacts with the proteasome subunit RPN10 through the UIM1 (ubiquitin interaction motif) motif and also weakly interacts with the RPN13 subunit via a PRU (pleckstrin-like receptor of ubiquitin) domain (Fatimababy et al., 2010; Lin et al., 2011). The UBA domain associates in high affinity with K48-linked ubiquitin and is required for interaction with ubiquitinated proteins (Lin et al., 2011). To delineate which domain is responsible for interacting with the RING peroxins, we made truncated constructs with UBL, chaperonin 1 and 2, chaperonin 3 and 4, and UBA domains respectively deleted, and tested the ability of the truncated proteins to interact with $PEX2^{RING}$ in Y2H. Our data showed that the chaperonin domains 3 and 4 play a strong role in the interaction with $PEX2^{RING}$, and chaperonin domains 1 and 2 and the UBL domain are also involved in the interaction, albeit to lesser degrees (Figure 2.5A).

The RING domain of PEX12 also interacts with DSK2a and DSK2b

RING domain proteins have been implicated in a diverse range of biological processes and make up one of the largest protein families in the *Arabidopsis* proteome (Budhidarmo et al., 2012). Hallmark of the RING domain is the presence of eight Cys/His residues that co-ordinate two Zn ions. On the basis of the Cys/His residues present in the RING domain and the number of amino acids between them, the 477 *Arabidopsis* RING proteins are classified into two major classes, H2 and HC, of which HC is further subdivided into HCa and HCb (Stone et al., 2005). The RING peroxins PEX2, PEX10, and PEX12 belong to the HCa type of RING proteins.

To determine whether DSK2s also interact with the other two RING peroxins and promiscuously with other types of RING proteins, we tested the interaction between the DSK2s (bait) and othe RING proteins (prey) in Y2H assays. In addition to PEX10 and PEX12, ARI8 (At1g65430, an HCb), and the protein encoded by At2g44330 (H2) were also included. Considering that PEX10 and PEX12 are integral membrane proteins, and that only $PEX2^{RING}$ interacted with DSK2s, we also used the RING domains of the two peroxins in the assay. Since ARI8 has two RING domains in the N-terminal region of the protein (Mladek et al., 2003), we also made a truncated construct that expressed only the RING domains ($ARI8^{RING}$). Our results demonstrated that the DSK2s interacted strongly with $PEX12^{RING}$, but very weakly with $PEX10^{RING}$ and not with the other two RING proteins tested (Figure 2.5B). The expression of the fusion proteins in yeast was verified by immunoblotting (Figure 2.5 C,D,E,F).

DSK2a and DSK2b are localized in the cytosol and nucleus and ubiquitously expressed in plants

To analyze the subcellular localization of DSK2a and DSK2b, we made YFP-DSK2 fusion constructs driven by the constitutive 35S promoter and expressed them transiently in *Nicotiana tabacum* (Tobacco). Confocal microscopic analysis of leaf epidermal cells revealed that both DSK2a and DSK2b are localized to the cytosol and nucleus (Figure 2.6A). We also made transgenic plants co-expressing YFP-DSK2 and the peroxisome marker, CFP-PTS1. Confocal images taken from transgenic plants showed that, similar to results from the transient expression, YFP-DSK2 proteins are

not targeted to peroxisomes but instead localize to the cytosol and the nucleus (Figure 2.7A).

We initially searched the Arabidopsis EFP browser for information on the expression of the *DSK2* genes from microarray datasets, but discovered that the probe sets do not distinguish the highly similar *DSK2a* and *DSK2b* genes (Winter et al., 2007). To examine the expression pattern of the two genes, we conducted RT-PCR to analyze their transcript levels in various plant tissues, including 10-day-old seedlings, inflorescence, flowers, stems, rosette and cauline leaves. Although both *DSK2* genes are expressed in all tissues, *DSK2b* is expressed at a much higher level than *DSK2a* in most of them (Figure 2.6B).

The *DSK2* amiRNA lines do not have obvious defects in plant growth and peroxisomal import and function

To evaluate the role of DSK2a and DSK2b in Arabidopsis development and peroxisome functions, we obtained T-DNA insertion mutants of *DSK2a* and *DSK2b* genes, but none of the homozygous mutants showed altered transcript levels of the two genes (Figure 2.7B, C). *DSK2a* and *DSK2b* are tandem duplicates on chromosome 2, thus making the generation of double mutant a difficult task. To this end, we made amiRNA constructs that target two conserved regions of the transcripts (see Methods) and transformed Col-0 plants with the constructs. Transgenic lines with significantly reduced expression of both genes were identified (Figure 2.8A). However, these lines did not show any noticeable growth or developmental abnormalities.

Given that DSK2a and DSK2b interact with two of the RING peroxins, PEX2 and PEX12, we used physiological assays to ascertain if the amiRNA lines displayed any defects in peroxisome metabolism. Peroxisome based β-oxidation of stored lipid during seed germination provides carbon source and energy for the seedling, thus mutants deficient in many peroxisomal proteins are severely compromised in their ability to grow on media lacking exogenous sucrose (Baker et al., 2006). To test the efficacy of β-oxidation, we compared the hypocotyl growth of dark grown seedlings germinated on MS plates with or without 1% sucrose (Figure 2.8B). Peroxisomes also carry out the conversion of the protoauxin IBA (indole-3-butyric acid) to the active auxin IAA, causing root inhibition to plants (Zolman et al., 2000). Thus, we tested the capacity of the amiRNA lines to effectively metabolize IBA by measuring root lengths of the seedling germinated on increasing concentrations of IBA (Figure 2.8C). In both assays, *DSK2* amiRNA lines showed no difference from the wild-type plants, whereas the peroxisome biogenesis factor mutant *pex14* displayed sugar dependence and IBA resistance (Figure 2.8B, C), leading us to conclude that peroxisome metabolism is unaffected in the absence of DSK2 function.

Since PEX2 and PEX12 are important constituents of the peroxisome matrix protein import machinery, we checked if *DSK2* silencing affected peroxisomal protein import. A peroxisome marker protein, consisting of cyan fluorescent protein (CFP) fused to the tripeptide Ser-Lys-Leu, a type 1 peroxisome targeting signal (PTS1), was introduced into the amiRNA plants. Confocal microscopy was used to assess protein import in T2 transgenic plants. No discernible defects were observed in the amiRNA plants in the peroxisomal targeting of CFP-PTS1, peroxisome morphology, or

peroxisome abundance (Figure 2.8D). These data suggest that DSK2s are not directly involved in regulating peroxisome functions or they may be acting redundantly with other ubiquitin receptors. It is also possible that some yet-unknown compensatory mechanisms are invoked to alleviate the effect of *DSK2* silencing in Arabidopsis.

DISCUSSION

In this study, we have shown that like their yeast counterparts, all three Arabidopsis RING peroxins can function as E3 ubiquitin ligases. One notable difference is that in yeast, PEX2 and PEX10 have been reported to be polyubiquitin ligases (Platta et al., 2009), but we only find monoubiquitination activity for the Arabidopsis proteins. Due to the insoluble nature of the full-length protein, we used the RING domain to test for activity. So it is possible that in vivo the full-length proteins do possess polyubiquitination activity and may even act synergistically as multiprotein complexes to exert their functions, as recently shown for the yeast RING peroxins (El Magraoui et al. 2012). Further, in vitro assays have their limitations, thus we cannot rule out the possibility that specific stimuli or accessory proteins exist within the cell that are required to promote catalytic activity. Moreover, some E2-E3 combinations are linked specifically with monoubiquitination, as seen in case of the DNA repair pathway, where Rad6-Rad18 monoubiquitination of the proliferating cell nuclear antigen (PCNA) activates recruitment of DNA polymerases (Hibbert et al., 2011). Recycling of most of the characterized PTS receptors in yeast appears to be dependent on monoubiquitination (Kiel et al., 2005; Kragt et al., 2005; Carvalho et al., 2007; Platta et al., 2007; Hensel et al., 2011). Thus, Arabidopsis RING peroxins may also play a role in

PEX5 recycling by targeting PEX5 for monoubiquitination, although other targets may also exist (Hu et al., 2012). Finally, monoubiquitination plays a role in various cellular processes such as trafficking of ubiquitinated endosomal receptors, modulation of protein activity, transcriptional regulation, and in facilitating protein interactions (Hicke, 2001). It has also been reported that the activity of proteins with ubiquitin binding domains (UBDs) can be regulated by monoubiquitination that results in inhibition of their binding to ubiquitinated substrates (Hoeller et al., 2006). Considering that DSK2s also harbor UBD, i.e, C-terminal UBA, and associate with PEX2 and PEX12, they might undergo monoubiquitination. In this scenario, the monoubiquitination of DSK2s would prevent them from recognizing ubiquitinated substrate proteins like PEX5 and thus increase target protein stability in the cell.

Despite having an incomplete RING domain, PEX12 also exhibits E3 ligase activity. The conserved residues that are changed in $PEX12^{RING}$ are at position 3, 4 and 8 in the C3HC4 RING domain, i.e. Cys->Ser, His->Phe and Cys->Thr (Figure 2.1A). These substitutions make this domain look more like that of the U-box proteins, a new class of E3 ligases derived from RING proteins. U-box E3 ligases lack the conserved Cys/His and instead use charged and polar residues (Ser, Thr, Asp, Glu) to maintain the structural integrity of the protein (Aravind and Koonin, 2000; Ohi et al., 2003). Thus, $PEX12^{RING}$ appears to have features of both the RING and U-box E3 ligases. It is well known that E3s pair with a very narrow set of E2s to carry out substrate specific ubiquitination (Christensen and Klevit, 2009; Ye and Rape, 2009). For example, the tomato pathogen *Pseudomonas syringae* Type III effector AvrPtoB, a U-box protein,

exhibits E3 ligase activity in the presence of UbcH5a and UbcH5c but not UbcH5b (Abramovitch et al., 2006). Several mammalian U-box ligases also exhibit a preference for UbcH5c (Hatakeyama et al., 2001). This suggests that the choice of E2 is critical in determining the specificity of the E3 assays. In agreement with this, PEX12 shows enhanced activity when UbcH5c is present in the E3 assay in our study. Moreover, UbcH5c is one of the three members of the human E2D family that ubiquitinate PEX5 for its recycling (Grou et al., 2008), further supporting our results.

We also report the identification of the tandemly duplicated UBL-UBA proteins DSK2a and DSK2b as specific interactors of $PEX2^{RING}$ and $PEX12^{RING}$. Our expression analysis revealed that DSK2a has much lower expression levels than DSK2b in plants, thus explaining why we did not find DSK2a in our initial Y2H screen. Arabidopsis DSK2s and their homologs in other species have been characterized as molecular adaptors, sometimes called E4, that regulate the relay of ubiquitinated substrate proteins to the proteasome for degradation (Fu et al., 2010). Although the interactions of some of the UBA-UBLs with ubiquitin and proteasomal subunits have been extensively studied, the physiological significance of these interactions remains undefined. In yeast, *dsk2* null mutants are viable and only exhibit subtle phenotypes such as increased cellular polyubiquitination (Biggins et al., 1996; Funakoshi et al., 2002). Arabidopsis has at least eight UBA-UBL family proteins, including four Rad23s, two DSK2s, DDI1 and NUB1 (Farmer et al., 2010; Fu et al., 2010). Several lines of evidence suggest that most of these proteins act in a functionally redundant manner. RNAi lines that target *DSK2*s in Arabidopsis are no different from wild-type plants in appearance (Lin et al., 2011), neither are our amiRNA lines generated in this study.

Mutants of another ubiquitin receptor protein, RPN10 (a component of the proteasome), show elevated levels of UBA-UBL proteins including DSK2s, suggesting that plants invoke compensatory mechanisms to counter the perturbed ubiquitin receptor levels. This may also account for our failure to find any peroxisome related phenotypes in the *DSK2* amiRNA lines.

In summary, we provide evidence that the Arabidopsis RING peroxins PEX2, PEX10, and PEX12 have monoubiquitin ligase activity and that the ubiquitin receptor proteins DSK2a and DSK2b specifically associate with PEX2 and PEX12. We speculate that together with ubiquitin receptor proteins, such as DSK2s, the RING peroxins form a peroxisome-based recycling/degradation system in plants. Identification of the target protein(s) of this machinery would be instrumental to defining the role(s) and cellular consequences of such a surveillance system.

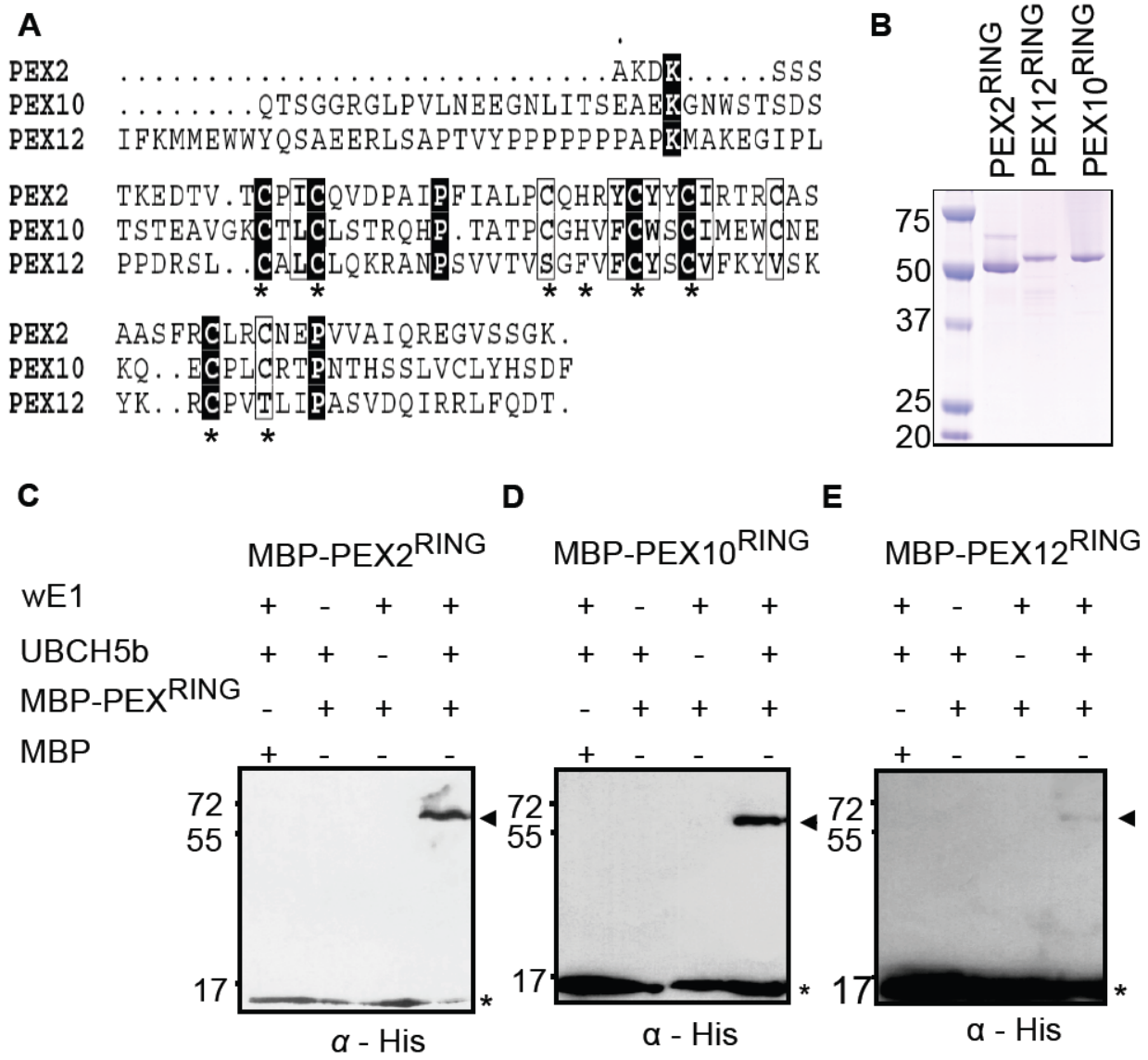

Figure 2.1. Arabidopsis RING peroxins possess E3 ligase activities.

(A) Sequence alignment of the RING domains of Arabidopsis PEX2, PEX10 and PEX12, which were used for generating PEX^{RING} constructs in this study. Positions of zinc binding Cys and His residues are marked by asterisks. Identical residues are shaded in black whereas similar residues are boxed. (B) Purified recombinant MBP-PEX2^{RING}, MBP-PEX10^{RING} and MBP-PEX12^{RING} proteins at expected molecular weight of ~ 51,

Figure 2.1.(Cont'd)

53 and 54 kDa, respectively. Numbers on the left are molecular weight (MW) markers in kDa.

(C-E) In vitro ubiquitination assays to test for E3 ligase activity. Assays were carried out in the presence of wheat E1, human UBCH5b, 6xHis tagged ubiquitin, and MBP fused $PEX2^{RING}$ (C), $PEX10^{RING}$ (D), or $PEX12^{RING}$ (E). Reactions were analyzed with immunoblots using anti-His antibodies. Sizes of molecular weight markers are indicated on the left in kDa. Arrowheads point to the RING-Ub conjugates, and asterisks indicate His-Ub.

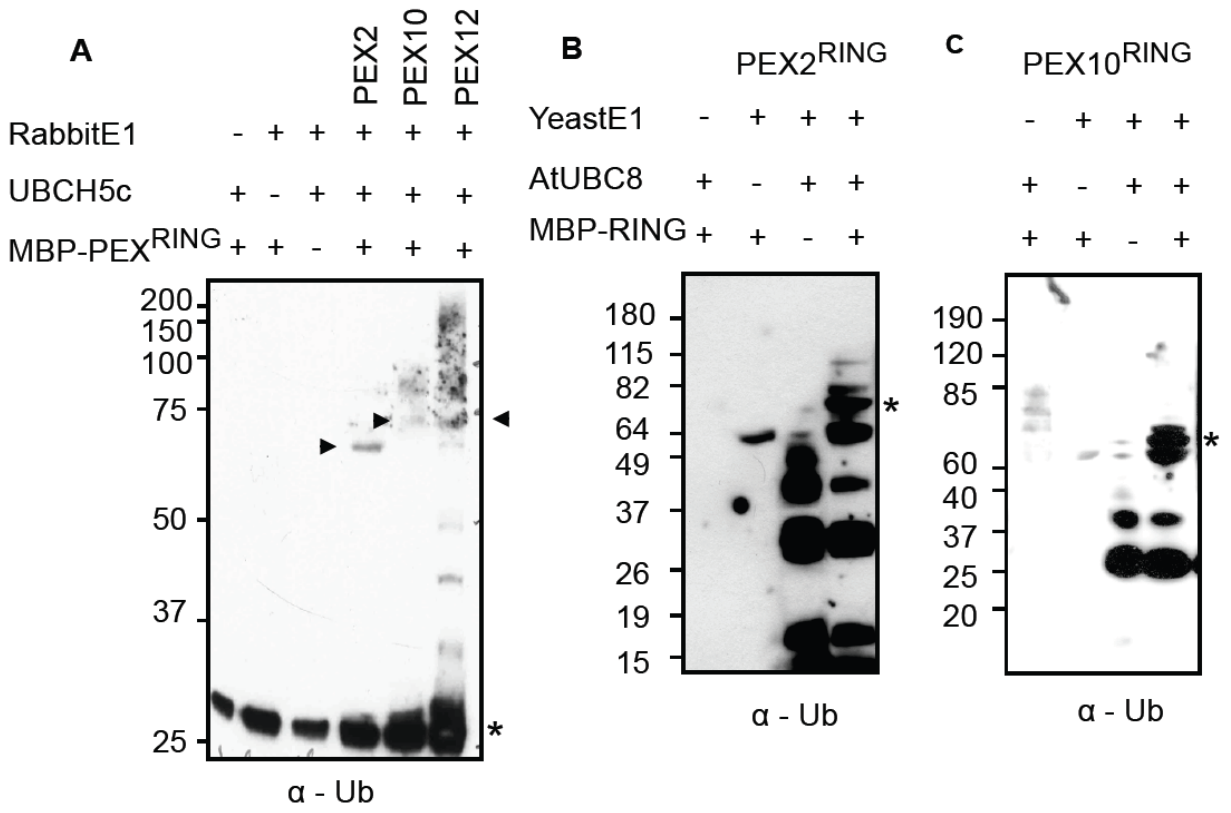

Figure 2.2. The RING domain of the Arabidopsis RING peroxins contains E3 ligase activity in different in vitro systems.

(A) E3 ligase assays using rabbit E1, human UBCH5c (E2), bovine ubiquitin, and MBP-PEX^{RING} proteins. UBCH5c seemed to promote the E3 activity of PEX12^{RING}. MBP-PEX2^{RING} was used in the minus E1 and minus E2 reactions. Arrowheads point to monoubiquitinated MBP-PEX^{RING}. Asterisk indicates Ubs.

(B-C) E3 ligase assays using yeast E1, AtUBC8, bovine ubiquitin, and MBP-PEX^{RING} proteins. Asterisks indicate Ub-conjugated MBP-PEX^{RING} proteins. Low MW bands are mostly E2-Ub conjugates. Numbers to the left of the gels are MW markers in kDa.

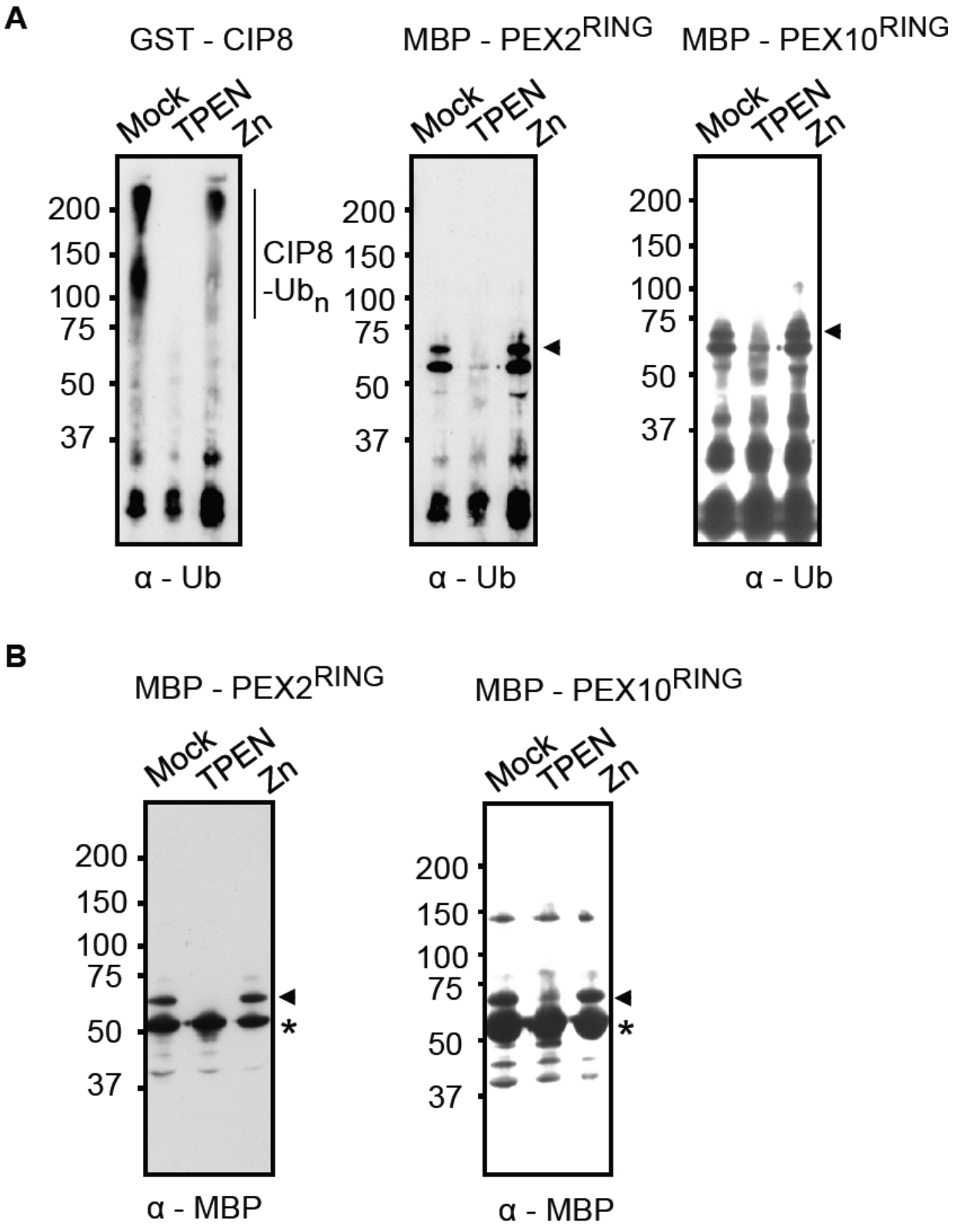

Figure 2.3. Effect of Zn depletion on the E3 ligase activity of PEX2^{RING} and

Figure 2.3. (Cont'd)

PEX10^{RING}. GST-CIP8, MBP-PEX2^{RING} and MBP-PEX10^{RING} were subject to mock (5% ethanol) and 5 mM TPEN treatment, and TPEN followed by 1 mM $ZnCl_2$, which restored the E3 activity in the presence of yeast E1 and Arabidopsis UBC8. Reactions were analyzed with anti-ubiquitin (A) or anti-MBP (B) antibodies. Arrowhead indicates PEX2/10^{RING}-Ub. Asterisks indicate PEX2/10^{RING}. Low MW bands cross-reacted to Ub antibodies are mostly E2-dependent ubiquitination products, as they were not observed in the minus E2 samples (Supplemental Figure 1B and C). Numbers to the left of the gels are MW markers in kDa.

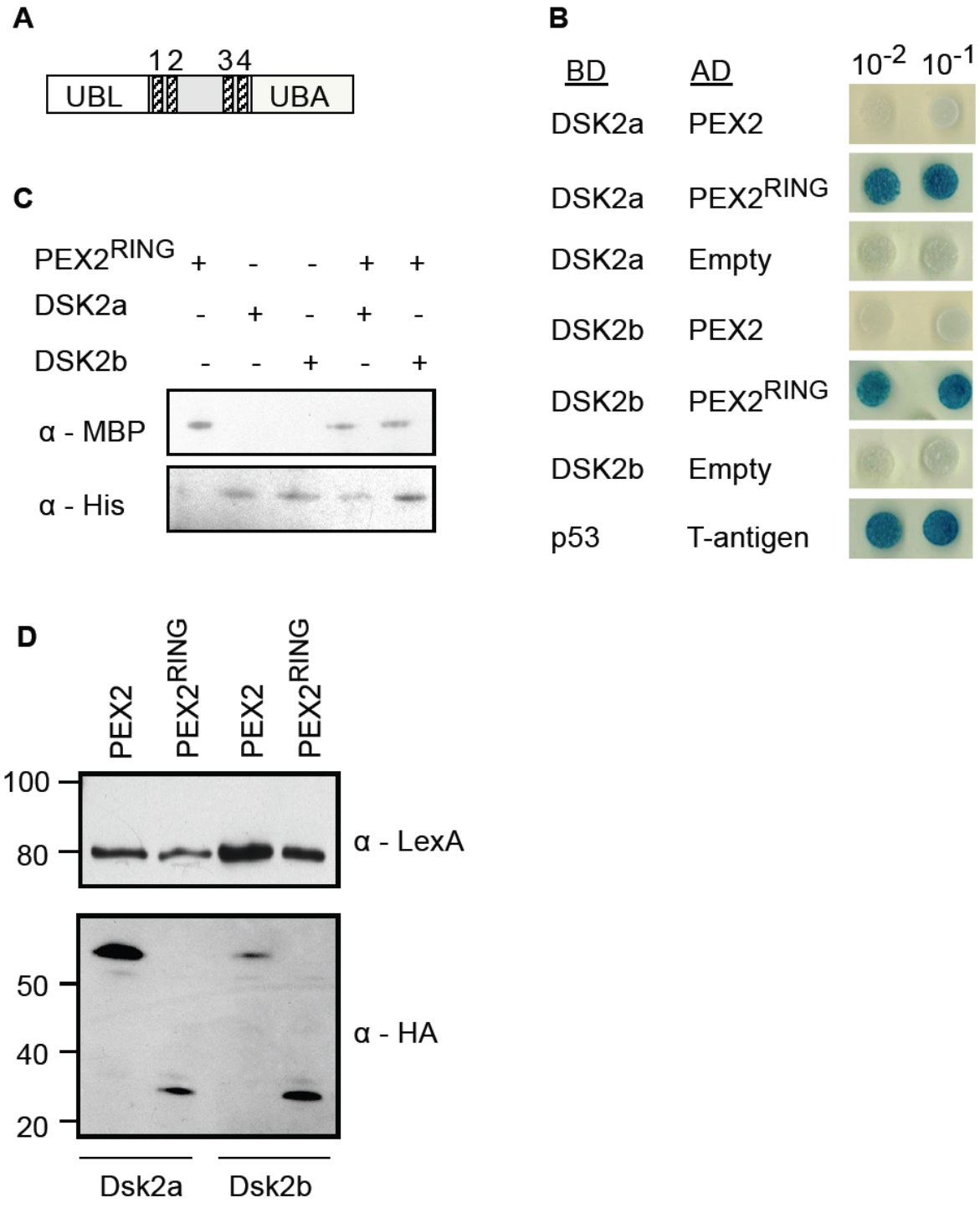

Figure 2.4. DSK2a and DSK2b are PEX2 interacting proteins.

(A) Schematic showing the domain organization of the DSK2 protein. Gridlined boxes

Figure 2.4.(Cont'd)

numbered 1-4 represent the four chaperonin domains. Domains were not drawn to scale.

(B) Y2H analysis to test for interaction between DSK2s and PEX2. Yeast strain EGY48/p8opLacz transformed with AD and BD fusion constructs were plated on selection media containing galactose and X-gal. Blue color indicates interaction and white indicates no interaction. Positive control strain used contained LexA-p53 and pB42AD-T antigen, while transformants containing empty vectors served as negative controls.

(C) In vitro pulldown assays to confirm the interaction between DSK2 and $PEX2^{RING}$. Recombinant 6xHis-DSK2 retains MBP-$PEX2^{RING}$ as detected by MBP antibodies. His antibodies were used to confirm the presence of the bait proteins.

(D) Immunoblot analysis of fusion proteins extracted from yeast strains used for Y2H assays shown in (B). BD- and AD-fusion proteins were detected with anti-LexA (top panel) and anti-HA (bottom panel) antibodies, respectively. Numbers to the left of the gels are MW markers in kDs.

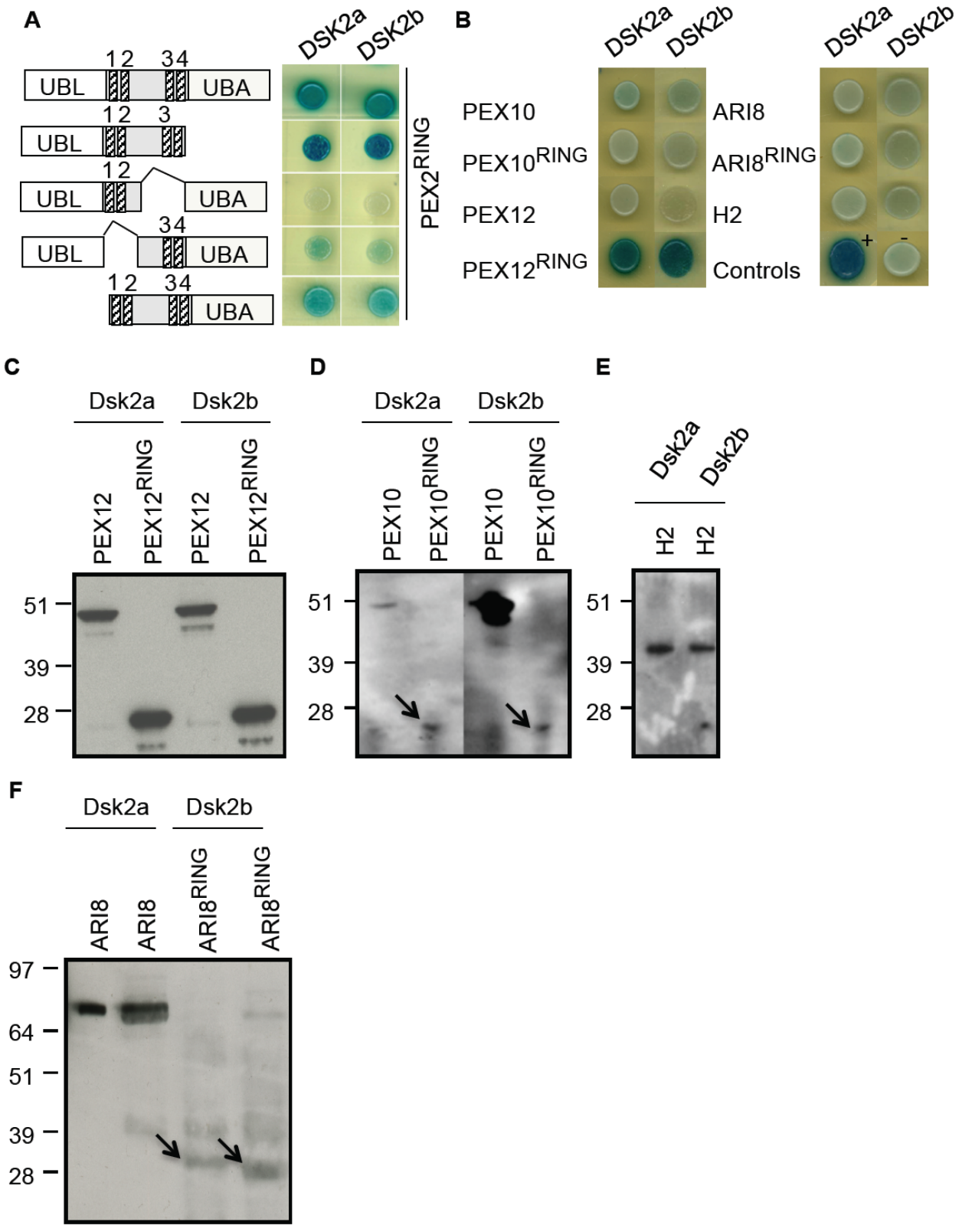

Figure 2.5. Y2H analyses to dissect DSK2 domains responsible for interaction with

Figure 2.5. (Cont'd)

PEX2^{RING} and to test for DSK2's interaction with other Arabidopsis RING proteins.

(A) Y2H assays to assess which domain in DSK2 is responsible for its interaction with PEX2^{RING}. Schematics show the deletion constructs used in the assays.

(B) Y2H assays to test for interaction between DSK2s and other RING proteins. The full length RING peroxins PEX10 and PEX12 and their RING domain constructs (PEX10^{RING} and PEX12^{RING}), and two representative proteins from two other types of RING domains (ARI8 from HCb type and At2g44330 from H2 type) were tested. Positive control strain used contained LexA-p53 and pB42AD-T antigen, while transformants containing empty BD and AD vectors served as the negative control.

(C-F) Immunoblot analysis of fusion proteins extracted from yeast strains used for Y2H assays shown in Figure (B). Anti-HA antibodies were used to detect AD-fusion proteins (RING proteins). Numbers to the left of the gels are MW markers in kDa. Arrows indicate the expressed RING domains.

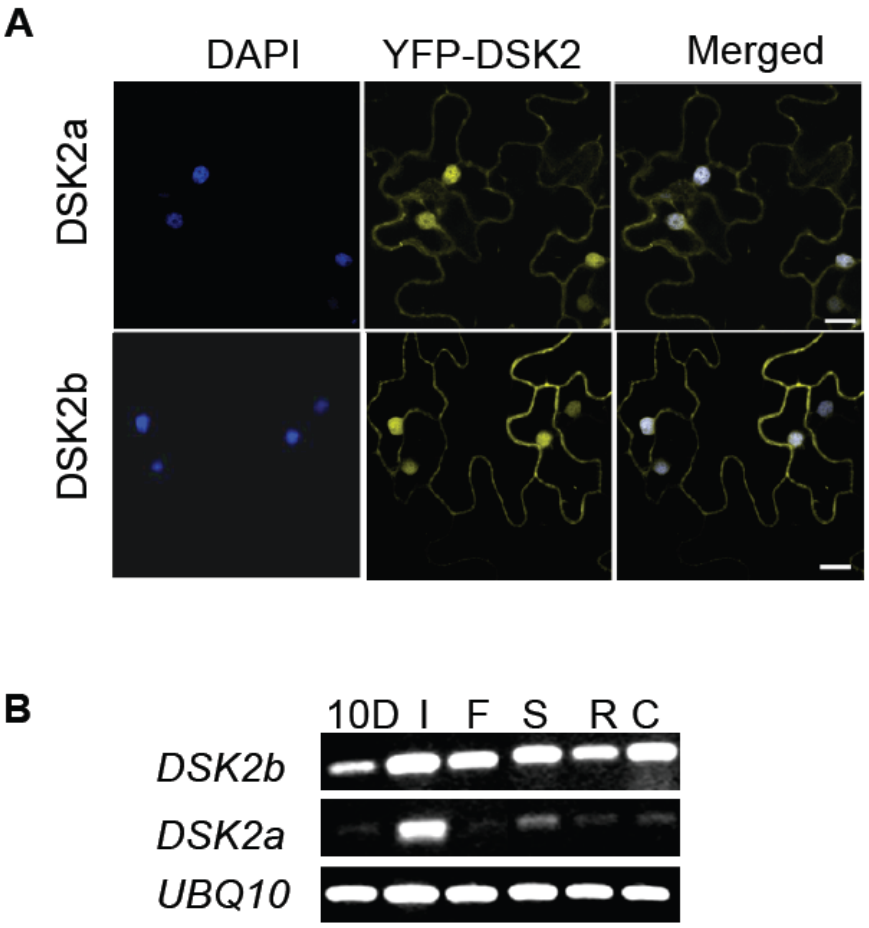

Figure 2.6. Subcellular localization of the DSK2 proteins and expression analysis of the *DSK2* genes.

(A) Confocal images taken from tobacco leaf epidermal cells transiently expressing YFP-DSK2 fusion proteins. DAPI was used for nuclear staining. Merged images show the co-localization of some YFP-DSK2 proteins with the nucleus. Scale bar is 10 μm.

(B) RT-PCR analysis of total RNA extracted from wild-type 10-d seedlings, inflorescence (I), flowers (F), stems (S), rosette leaves (R), and cauline leaves (C). The *UBQ10* transcript was used as a loading control.

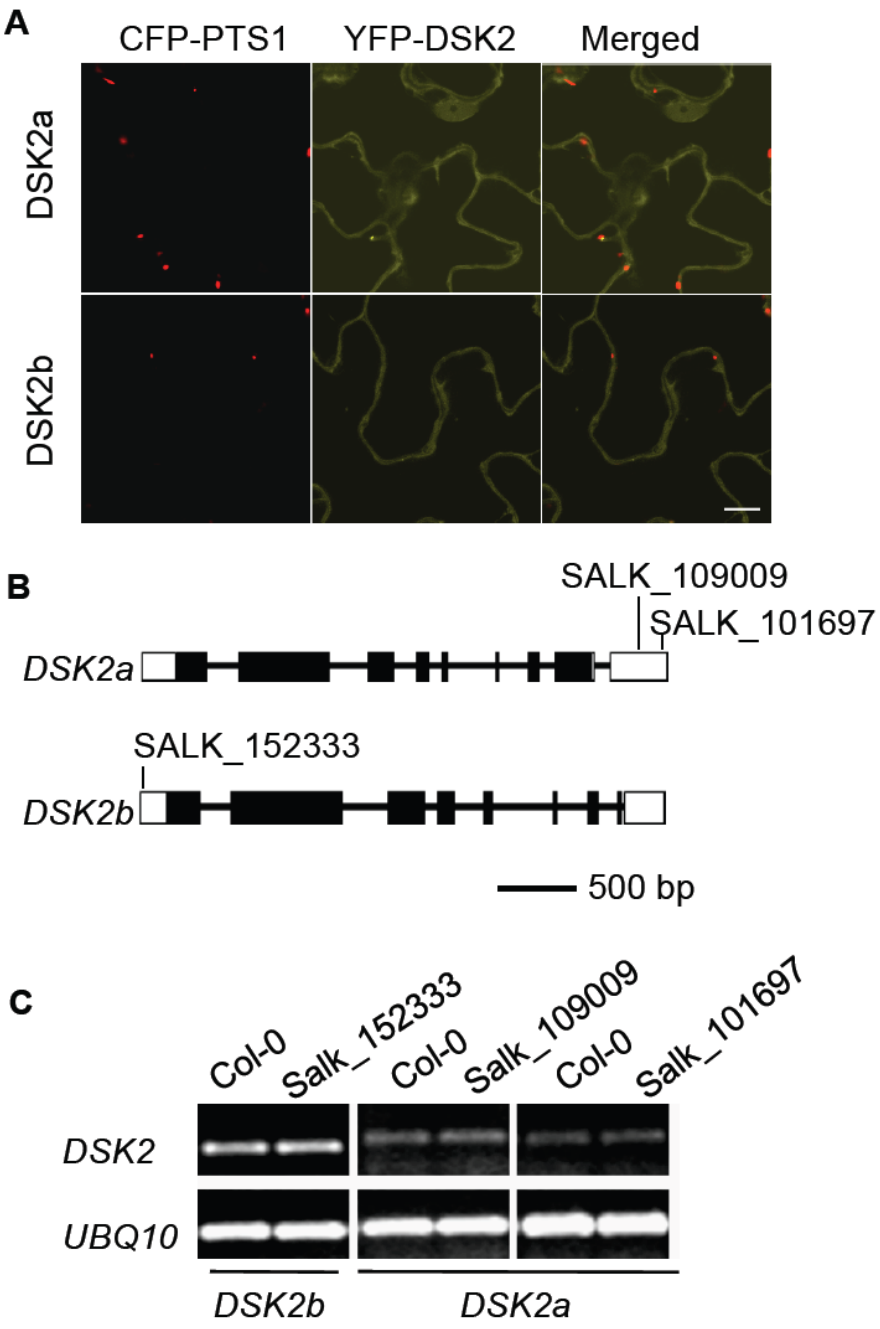

Figure 2.7. Subcellular localization of YFP-DSK2 in transgenic plants and identification of T-DNA insertion mutants of *DSK2* genes.

(A) Confocal images from Arabidopsis transgenic lines co-expressing YFP-DSK2 and the peroxisomal marker CFP-PTS1. Scale bar = 10 µm.

(B) Gene structure of *DSK2a* and *DSK2b*. Positions of the T-DNA insertions are

Figure 2.7. (Cont'd)

indicated. Black boxes are coding regions and white boxes are UTRs.

(C) RT-PCR analysis of homozygous T-DNA insertion lines for *DSK2a* and *DSK2b*. *Ubiquitin 10* (*UBQ10*) served as a loading control.

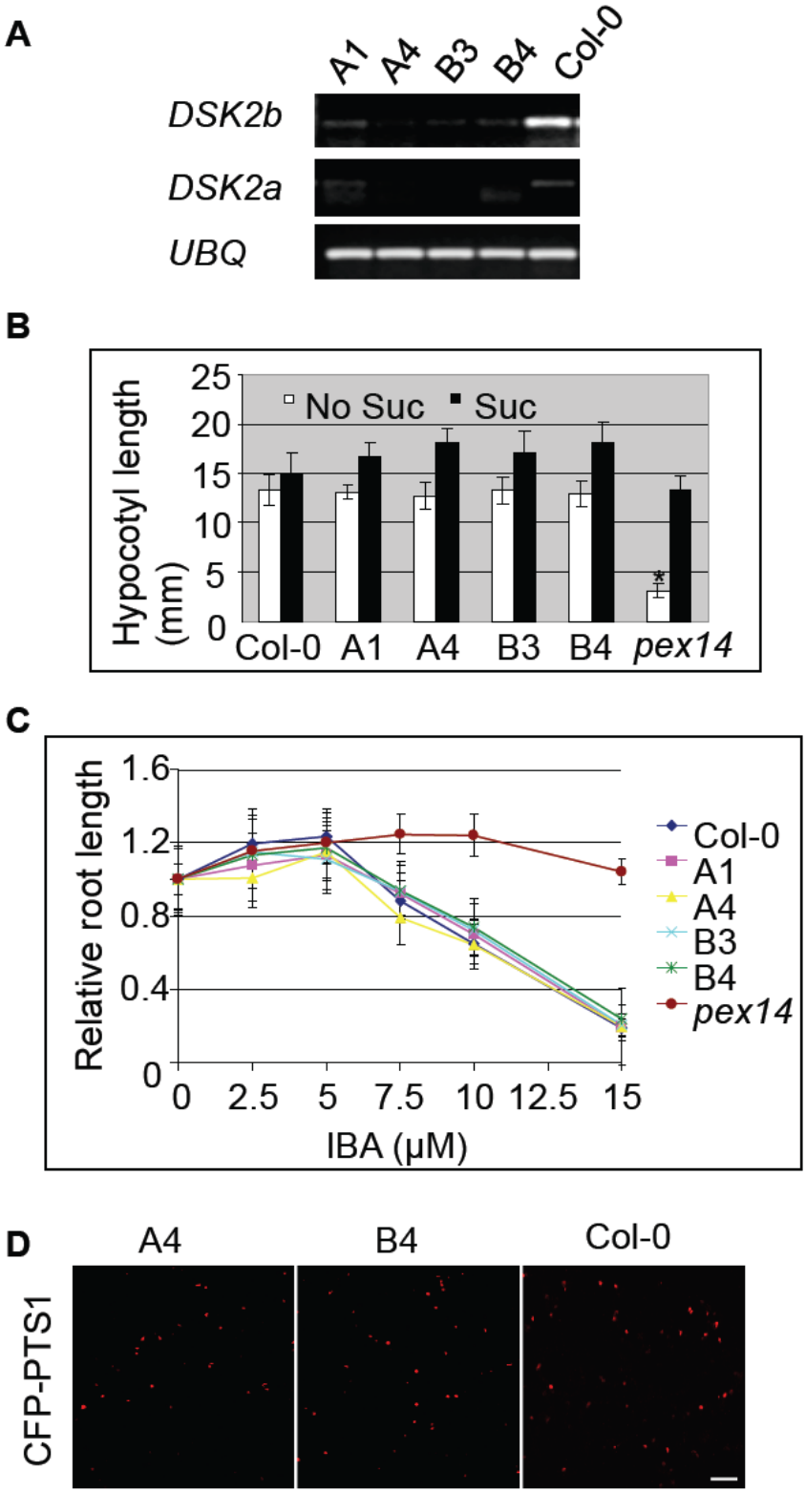

Figure 2.8. Identification and phenotypic analysis of *DSK2* amiRNA lines.

(A) RT-PCR analysis of the *DSK2a* and *DSK2b* transcripts in the amiRNA lines. *UBQ10* was used as the loading control.

(B) Hypocotyl lengths of amiRNA seedlings grown in the dark for 7 days in the presence or absence of 1% sucrose. Error bars indicate standard deviations of n > 25. Student's t-test analyses did not reveal any statistically significant differences compared to the wild type except for *pex14* plants (indicated by an asterisk).

(C) Relative root length (on IBA vs. without IBA) of amiRNA lines grown on increasing concentrations of IBA in light. Error bars indicate standard deviations of n > 25. Student's t-test analyses did not reveal any statistically significant differences for the amiRNA lines compared to the wild type.

(D) Confocal images from leaf epidermal cells of 14-day-old amiRNA lines expressing the peroxisome marker CFP-PTS1. Scale bar is 10 µm.

A1, A4, B3, and B4 are independent *DSK2* amiRNA lines.

Table2.1 Primers used in this research

Primer	Sequence 5'-3'
PEX2RINGF	CAGAATTCCTGCTCAACTCTTCAGCTGTTAAG
PEX2RINGR	GCTCTAGATCATTTGCCACTTGAAACACCTTC
PEX10RINGF	CAGAATTCCAAACTTCAGGAGGGAGAGG
PEX10RINGR	GCTCTAGAGCCTAAAAATCAGAATGATACAAAC
PEX12RINGF	CAGAATTCATGGAATGGTGGTATCAATCC
PEX12RINGR	GCTCTAGAGCCTAAGTGTCCTGAAACAACC
A I miR-s	GATAGTCGTTCTACAGCTGCGTTTCTCTCTTTTGTATTCC
A II miR-a	GAAACGCAGCTGTAGAACGACTATCAAAGAGAATCAATGA
A III miR*s	GAAAAGCAGCTGTAGTACGACTTTCACAGGTCGTGATATG
A IV miR*a	GAAAGTCGTACTACAGCTGCTTTTCTACATATATATTCCT
B I miR-s	GATATCATTTCACGCATACGCTCTCTCTCTTTTGTATTCC
B II miR-a	GAGAGCGTATGCGTGAAATGATATCAAAGAGAATCAATGA
B III miR*s	GAGAACGTATGCGTGTAATGATTTCACAGGTCGTGATATG
B IV miR*a	GAAATCATTACACGCATACGTTCTCTACATATATATTCCT
His190F	ATGGCTAGCGGTGGTGAAGCAGATTC
His190R	GGAATTCCCAATCCACAAGTTCGCG
His200F	CCATGGGGTGGAGAGGGAGAT
His200R	GGATCCCTACTGTCCGATACTCCC
190F	AAAAAAGCAGGCTTCATGGGTGGTGAAGCAGA
190R	AAGAAAGCTGGGTCTTACTGGCCAATACTCCCCAAG
200F	AAAAAAGCAGGCTTCATGGGTGGAGAGGGAGA
200R	AAGAAAGCTGGGTCCTACTGTCCGATACTCCCCAAGAGTC
190RTF	ATGGGTGGTGAAGCAGATTCGAGGC
190RTR	GATCAGCCTGCAAACCATAGCTGAGGAG
200RTF	ATGGGTGGAGAGGGAGATTC
200RTR	GGTGGTGCTGAAGAAGGTGC
UBQ10F	TCAATTCTCTCTACCGTGATCAAGATGCA
UBQ10R	GGTGTCAGAACTCTCCACCTCAAGAGTA
Y190F	GGAATTCATGGGTGGTGAAGCAGATTC
Y190R	CCGCTCGAGCGGTTATCCACAAGTTCGCG
Y200F	GAATTCATGGGTGGAGAGGGAGATTCAAGTC
Y200R	CCATGGCTACTGTCCGATACTCCCCAAGAGTC
Y190R1	CATGCCATGGCATGTTAAGGAGAAGAAGGCACAAAAC
Y190F2	GCGGAATTCCCTCCTGAAGAGCGATTTGCG
Y200R2	CATGCCATGGCATGTTATGCTGAAGAAGGTGCAGAGCC
Y200F2	GCGGAATTCCAATCCAATGTTCCTCCTGAAGAG
y190st34R	GCCCTATCAGTATTTCTCATAAGGCCAGGGAACAAAG
y190st34F	ATGAGAAATACTGATAGGGCTATGAG

Table2.1 (Cont'd)

y190st12R	GTTGTAGAGACATGAGTCCACCAAGGCCTC
y190st12F	ATGTCTCTACAACAATCACTTTTCTCTCAG
y200st34R	CTATCAGTATTCCGACCCAGAGGATTAAATC
y200st34F	CGGAATACTGATAGGGC
y200st12R	GATTGCTGTAGAGTCATATCCGCTCCAAGCATA
y200st12F	ATGACTCTACAGCAATCACTATC
YPEX2F	GAATTCATGACGCCGTCTACGCC
YPEX2R	CTCGAGTCATTTGCCACTTGAAACAC
YPEX10F	GAATTCATGAGGCTTAATGGGGATTCGGGTC
YPEX10RF	GAATTCACTTCGGAAGCTGAAAAGGG
YPEX10R	CCATGGCTAAAAATCAGAATGATACAAACAAACC
YPEX12F	CCCGGGCAGGAAGAAGATGTTGTTTCAGGTGGG
YPEX12RF	GAATTCGTGTACCCTCCACCTCCACCTCC
YPEX12R	CTCGAGCTAAGTGTCCTGAAACAACCTCC
ARI8F	CCATGGCAATGGAAGCTGATGACGATTTCTAC
ARI8R	CTCGAGTCACCGGCCATGTTCACAC
ARI8R2	CTCGAGTCACCGGCCATGTTCACAC
YH2F	GAATTCATGTCGTCCTCCTCTACCCAAAACC
YH2R	CTCGAGTTAGCCGTCTAAACTTTGCTCCAAATCG
LP33	TTGCTGTGTCTGCACAAGATC
RP33	AAGGTTACACACCAAACGCTG
LP09	ACTGACGTTCAGGGTGACATC
RP09	TGAAGGCTTCAACTAGTTCACTTAG
LP97	AGCGTTTGAGTTGATCCTACG
RP97	ATGGTAACGTTAACGCTGCTG

Table 2.2. Vectors used in this study.

Vector	Description	Reference
pRS300	for amiRNA (miR319a backbone)	(Schwab et al., 2006)
pGILDA	For Yeast 2 Hybrid	Clontech
pB42ADm	For Yeast 2 Hybrid	from Clontech (modified MCS)
pCHF3	Binary vector to overexpress amiRNA	derived from PZP212 (Hajdukiewicz et al., 1994)
pMAL-c4X	MBP tag recombinant protein expression	NEB
pET-28a	6X His tag for recombinant protein expression	Novagen
pDONR207	Gateway cloning donor vector	Invitrogen
pEARLEY101	Gateway destination vector to clone YFP -Gene of interest	(Earley et al., 2006)

METHODS

Sequence alignment

PEX2, PEX10 and PEX12 protein sequences were downloaded from The Arabidopsis Information Resource (TAIR) at www.arabidopsis.org. RING domains were identified using SMART (Letunic et al., 2012). Sequence alignment was performed with ClustalW2 (Larkin et al., 2007) and rendered in graphical format using ESPript (Gouet et al., 1999).

Protein expression and purification

The RING domains of PEX2 (aa 248-333), PEX10 (aa 287-381) and PEX12 (aa 296-393) were amplified with the primers listed in Table 2.1. The amplified PCR products were digested with *Eco*RI and *Xba*I and cloned into the pMAL-c4x expression vector (New England Biolabs, USA) to generate constructs encoding MBP-tagged proteins. Full-length coding sequences of DSK2a and DSK2b were cut with *Nhe*I and *Eco*RI (DSK2a) or *Nco*I and *BamH*I (DSK2b) for cloning into the pET28a plasmid (Novagen, USA) to generate 6xHis-tagged proteins. Protein expression constructs were transformed into *E. coli* strain BL21-DE3 (Stratagene, USA). At O.D 0.6, bacterial cultures harboring different constructs were induced with 0.5 mM isopropyl thiob-d-galactoside, incubated at 37°C for three hours and harvested by centrifugation. Cell pellet was resuspended in column buffer (200 mM NaCl, 20 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM PMSF, 1 mM DTT) for the MBP-tagged proteins and lysis buffer (50 mM NaH2PO4 pH 7.5 , 300 mM NaCl,10mM imidazole, 1 mM PMSF) for His-tagged proteins. Cells were disrupted by sonication in an ice-water bath (4°C) , and lysed cells

were centrifuged at 10,000 rpm for 10 min. Cleared lysates of MBP-tagged protein were applied to amylose resin column and purified according to manufacturer's instructions (New England Biolabs, USA, USA).

In vitro ubiquitination assays

For assays presented in Figure 1C, D and E, crude extracts containing recombinant wheat E1, human UbcH5b (E2; approximately 40 ng), purified MBP-PEX2^{RING}, MBP-PEX10^{RING} or MBP-PEX12^{RING} (E3; ~ 1 µg), and purified His-ubiquitin (~2 µg) were used for E3 ubiquitin ligase activity assays as described previously (Xie et al., 2002; Zhang et al., 2007). Reactions were stopped by adding 4X SDS-PAGE buffer and samples were separated on 12% SDS-PAGE gels followed by western blotting analysis using anti-His antibody (Santa Cruz, USA).

Assays presented in Figures 2.2 and 2.3 were performed according to (Stone et al., 2005). For the zinc-chelating assays, RING proteins bound to the amylose beads were incubated in column buffer containing either mock (Ethanol) or 5mM TPEN (N,N,N',N'-Tetrakis(2-pyridylmethyl)ethylenediamine) (Sigma-aldrich, USA) for 16 hours at 4°C on a rotary shaker. Solutions were changed every four hours, after which beads were washed twice with column buffer. TPEN-treated fraction was split into two aliquots, one of which was further incubated with 1 mM $ZnCl_2$ for four hours at 4°C with three solution changes followed by three washes with column buffer. The bead-bound RING proteins thus treated (Mock, TPEN, or TPEN+$ZnCl_2$) were subsequently used in ubiquitination assays. Ubiquitination assays were done using 50 ng of yeast E1 (Boston

Biochem, Cambridge, MA), 250 ng of AtUBC8, 500ng of bead-bound MBP-RING protein and 2 μg bovine ubiquitin (Sigma) incubated in reaction buffer comprising of 50mM Tris-HCl, pH 7.5; 10mM $MgCl_2$; 0.05mM $ZnCl_2$; 1 mM ATP; 0.2Mm DTT; 10 mM phosphocreatine and 0.1 unit of creatine kinase (Sigma-aldrich) for two hours at 30°C. Reactions were stopped by adding 5X SDS-PAGE buffer, split and run on 10% NuPAGE gels (Invitrogen) followed by western blotting analysis using either ubiquitin (Sigma, USA) or MBP (New England Biolabs, USA) antibodies. E3 assays shown in Supplemental Figure 1A were performed in the same way, except that rabbit E1 and human UbcH5c were used instead of yeast E1 and AtUBC8.

Yeast two-hybrid assays

An Arabidopsis cDNA library made from seedlings was screened for proteins interacting with PEX2, using the GAL4 Y2H system with $PEX2^{RING}$ as bait. The Matchmaker LexA system (Clontech, USA) was used to perform further Y2H assays to confirm the interactions. Coding sequences for full-length PEX2, PEX10, PEX12, ARI8 (At1g65430; HCb) and At2g44330 (H2) and deletions comprising only the RING domain, were amplified by PCR and cloned into the pGILDA bait vector to generate LexA DNA binding domain fusions. Full-length coding sequences for DSK2a and DSK2b and deletions thereof were PCR amplified and cloned into a modified pB42AD plasmid to generate prey constructs. Primers used for cloning are listed in Supplemental Table I. To test the interaction between DSK2 and the RING domain proteins, the bait and prey constructs were co-transformed into yeast (*Saccharomyces cerevisae*) strain EGY48 (p8opLacZ),

using transformation protocol described previously (Gietz and Woods, 2002). Yeast strains transformed with empty bait/prey vectors were used as negative controls, and yeast strain transformed with pLexA-53 and pB42AD-T plasmids was used a positive control. Transformants were selected on SD-glucose (BD Biosciences) media supplemented with –Ura/-His/-Trp synthetic dropout solution. Transformants were grown overnight in SD-glu /–Ura/-His/-Trp liquid medium, centrifuged, washed twice with distilled water and plated on SD-galactose/raffinose -Ura/-His/-Trp-inducing media containing 80 µg/mL of X-gal. Plates were incubated at 30°C and imaged after 48 hours. LexA (Invitrogen) and HA-tag antibodies (Aves Labs) were used to detect BD and AD-fusion proteins.

In vitro pulldown assays

Cleared lysates of His-tagged DSK2 proteins were incubated with Ni-NTA resin (Qiagen, USA) for an hour at 4°C. The resin was washed three times with lysis buffer and incubated with lysate from MBP- $PEX2^{RING}$ for two hours at 4°C with gentle agitation. Ni-NTA resin was recovered by low speed centrifugation and further washed with lysis buffer at least three times. Bead-bound proteins were then eluted with lysis buffer supplemented with 250 mM imidazole. Elutes were subjected to immunoblotting using His (Cell signaling, USA) or MBP (New England Biolabs, USA) antibodies to determine interaction.

Subcellular protein localization

The open reading frames of DSK2a and DSK2b were PCR-amplified with Gateway primers containing the attB1 and attB2 sequences. The PCR product was recombined into the Gateway entry vector pDONR207 (Invitrogen) using BP clonase. The pDONR clones were transferred to binary destination vector pEARLEY104 via LR clonase-based recombination, resulting in constructs encoding YFP-DSK2 fusions. *Agrobacterium tumefaciens* strain GV3101 (pMP90) transformed with the constructs of interest was grown overnight at 28°C, washed and resuspended in water to an OD_{600}=0.1. Tobacco plants were infiltrated with the bacterial suspension using a needleless syringe and kept in regular growth conditions for two days.

An inverted Zeiss LSM 510 Meta confocal microscope was used for all fluorescent protein imaging. To label the nuclei, the fluorescent dye DAPI (Sigma) was diluted to 10 µg/mL in phosphate buffer at pH 7.2, and the solution was infiltrated into the Tobacco leaves 1h prior to imaging. To observe subcellular localization, we used the 405 nm diode and 514 nm argon laser to excite DAPI and YFP, respectively. The fluorescent emission from DAPI and YFP was acquired through the 420-480 nm and 520-555 band-pass filters respectively. All images were obtained from a single focal plane. For imaging CFP, we used the 458 nm argon laser for excitation and the 465-510 nm emission filter to detect the CFP fluorescence.

Generation of amiRNA lines

The WMD3-Web MicroRNA Designer (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) tool was used to find the best target amiRNA for DSK2a and DSK2b. Two of the targets, designated amiRNA-a (5'- TAGTCGTTCTACAGCTGCGTT- 3') and amiRNA-b (5'-

TATCATTTCACGCATACGCTC-3'), were selected and amplified from the miR319a backbone using overlapping PCR as described in the amiRNA cloning protocol (http://wmd3.weigelworld.org/downloads/Cloning_of_artificial_microRNAs.pdf). The amiRNA precursors were then digested with *Kpn*I and *Xba*I and cloned into binary vector pCHF3. The constructs were transformed into *Agrobacterium tumefaciens* strain GV3101, which was then transformed into Col-0 and CFP-PTS1 Arabidopsis plants by floral-dip (Clough and Bent, 1998). Transgenic plants were selected on 1/2MS supplemented with 0.5% sucrose and 50 µg/ml kanamycin. T1 antibiotic resistant plants were screened with RT-PCR to identify individual plants in which the expresssion of *DSK2a* and *DSK2b* was reduced. Segregation of the transgenes in subsequent generations was assessed with resistance to kanamycin to identify homozygous lines, which were subsequently used for physiological assays.

RT-PCR

Total RNA was isolated from three-week-old plants using the RNeasy plant mini kit (Qiagen, USA) as per manufacturer's instructions. 1 µg of RNA was reverse-transcribed with the Omniscript RT kit (Qiagen, USA) using oligodT primers. Subsequently, 50 ng of cDNA was used in PCR (Promega, USA) amplification using gene-specific primers listed in Supplemental Table I. PCR conditions used were as follows: 95°C for 2 min, 30 cycles of 95°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec, and a final extension of 72°C for 10 min.

Physiological assays

To test for sucrose dependence, seeds from wild-type and amiRNA lines were surface sterilized and plated on 1/2 MS growth medium solidified with 0.6% phytagar, in the presence or absence of 1% sucrose. Plates were wrapped in foil, stratified for two days at 4°C and imaged after seven days of growth in the dark at 22°C.

For IBA response assays, seeds were sown on 1/2 MS medium supplemented with 0.5% sucrose and various concentrations of IBA (Sigma-Aldrich). Following two days of stratification at 4°C, the plates were placed vertically in growth chamber (Percival) with continuous light, covered with a mesh, and scanned after seven days of growth.

ImageJ (http://rsb.info.nih.gov/ij/) was used to measure both the hypocotyl length of the etiolated seedlings in case of sucrose dependence assay and root length for IBA response assays. A previously identified peroxisome import mutant, *pex14* (SALK_007441) served as the positive control in both assays.

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CHAPTER 3

OCP1 is a novel peroxisomal protein involved in peroxisomal metabolism in *Arabidopsis*

Abstract

Plant peroxisomes are dynamic organelles that play a vital role in plant growth and development. Peroxisomes import matrix proteins through recognition of peroxisome targeting signals (PTS) by cytosolic receptors; however, not much is known about how peroxisomes regulate their proteome through proteolysis. Lately, bioinformatic studies and proteomic experiments have identified several proteases in the peroxisomal matrix. Here, we examined the role of a predicted ovarian tumor-like cysteine protease (OCP1) in Arabidopsis. Subcellular localization studies indicate that OCP1 is targeted to the peroxisome. Both N- and C-terminal GFP fusion proteins of OCP1 localize to the peroxisome, indicating the presence of two functional targeting signals, a C-terminal PTS1 as well as a novel N-terminal PTS2. Loss-of-function lines for *OCP1* were isolated and tested for defects in peroxisomal functions and found to display IBA-resistant root phenotype that suggests a putative function in β-oxidation. *ocp1* mutants were hypersensitive to ABA's suppression of germination, indicative of a positive role of OCP1 in seed germination. Arabidopsis seedling peroxisomes contain glyoxylate cycle enzymes, isocitrate lyase (ICL) and malate synthase (MS), which function in channeling acteyl-CoA derived from peroxisomal β-oxidation towards synthesis of gluconeogenic substrates. However, once photosynthesis begins, peroxisomes undergo developmental remodeling, resulting in the degradation of ICL and MS. Testing the abundance of ICL during germination revealed that ICL and MS are stabilized in the *ocp1* alleles compared to the wild-type control. Taken together, these data suggest that OCP1 is a critical factor in facilitating the degradation of ICL and MS from seedling peroxisomes.

Introduction

Peroxisomes are ubiquitous eukaryotic organelles overseeing an array of biochemical processes that cater to the specific metabolic needs of the organism in question. Presence of peroxisomes is fundamental to the survival of mammals and plants given that lack of peroxisomes severely curtails the lifespan of either organism (Hu et al., 2012; Nagotu et al., 2012). Despite exhibiting significant functional diversity, peroxisomes in all organisms carry out two vital functions: β-oxidation and detoxification of hydrogen peroxide (Islinger et al., 2012).

Interestingly, peroxisomal heterogeneity is not just limited to different organisms; often peroxisomes within an organism also show functional variation in different tissue types or developmental stages (Hayashi and Nishimura, 2006; Islinger et al., 2010). In oilseeds like Arabidopsis, this specialization is exemplified by seed peroxisomes and leaf peroxisomes that contain some mutually exclusive proteins, i.e some glyoxylate cycle enzymes in seed peroxisome and some photorespiration related enzymes in leaf peroxisomes (Nishimura et al., 1996).

It is thus necessary that the relevant peroxisomal protein coding gene is transcribed at the right time, properly targeted and imported by the protein import machinery to ensure that functional specialization occurs. Although transcriptional regulation of peroxisomal protein complement by peroxisome proliferator-activated receptor alpha (PPARα) is well known in mammals (Desvergne and Wahli, 1999), no such regulator has been uncovered in plants thus far. The protein import pathway on the other hand is largely conserved among different organisms and has been studied in some detail. The import of resident matrix proteins is governed by the presence of

peroxisome targeting signals (PTS) within the protein sequence, comprising either of a C-terminal tripeptide denoted as PTS1 (SKL and variants) or an N-terminal nonapeptide referred as PTS2 (R[L/I/Q] X_5HL)(Lanyon-Hogg et al., 2010). Cytosolic receptor proteins, PEX5 and PEX7, bind PTS1- and PTS2-containing cargo proteins respectively. The cargo bound receptors then traffic to the peroxisomal docking complex (PEX13, PEX14), deposit their cargo, undergo translocation through the peroxisome membrane and are subsequently released back into the cytosol. The ubiquitin conjugating enzyme complex (PEX4, PEX22) and RING peroxins (PEX2, PEX10, PEX12) are implicated in the translocation process (Rucktaschel et al., 2011). In mammals and plants, PEX7 is dependent on PEX5 to facilitate docking on the peroxisome membrane and PTS2 peptides are cleaved post-import (Lazarow, 2006). Additionally, in yeast and mammals PEX5 is monoubiquitinated on a conserved Cys residue to facilitate it's recycling (Grou et al., 2009). Since transiently expressed enzymes of seedling peroxisomes were reported to be stabilized in mutants of the Arabidopsis protein import machinery, it was suggested that the import machinery directs the developmentally cued remodeling of seedling peroxisomes to leaf peroxisomes, a process termed Peroxisome Associated Protein Degradation (PexAD)(Lingard et al., 2009).

Apart from import, regulated degradation of a subset of peroxisomal proteins could be an alternative mechanism used to modulate peroxisomal proteome. In spite of the advances made in understanding protein import, our knowledge of peroxisomal protein turnover mechanisms is fairly limited. Proteins are commonly degraded via the action of proteases, through the ubiquitin-proteasome mediated pathway or autophagy

(van der Hoorn, 2008; He and Klionsky, 2009; Vierstra, 2009). Although both pexophagy (autophagic degradation of peroxisomes) and ubiquitin-proteasome mediated degradation of PTS receptor proteins have been documented to occur in yeast and mammals (Manjithaya et al., 2010), neither process has been reported to occur in plants. Proteases found in plant peroxisomes include DEG15, LON2 and PXM16 (Reumann et al., 2007; Eubel et al., 2008; Reumann et al., 2009), of which DEG15 and LON2 have been characterized. DEG15 was demonstrated to be the protease responsible for cleavage of PTS2 peptide, but its absence does not appear to hinder activity of PTS2 protein(s) or have any impact on plant physiology (Helm et al., 2007; Schuhmann et al., 2008). *lon2* mutants show retarded growth phenotype and the LON2 protein is attributed to a possible function as a molecular chaperone. In that capacity, LON2 facilitates continual import of matrix proteins (Lingard and Bartel, 2009). Querying Arabidopsis database with PTS sequences revealed five potential protease candidates (Reumann et al., 2004). One of them, annotated as an ovarian tumor-like (Otubain) cysteine proteases (named OCP1), was chosen for further analysis.

Otubains are an evolutionarily conserved class of putative cysteine proteases found in all kingdoms of life (Reyes-Turcu et al., 2009). Most of the characterized otubains possess linkage-specific ubiquitin isopeptidases activity that is an important determinant of the stability of target proteins. As a direct consequence of their deubiquitinase activity, otubains have been linked with a plethora of biological processes such as regulation of gene expression, DNA repair, innate immune responses and dislocation of misfolded proteins from the ER (Arguello and Hiscott, 2007; Frias-Staheli et al., 2007; Komander and Barford, 2008; Ernst et al., 2009;

Stanisic et al., 2009; Nakada et al., 2010; Chenon et al., 2012). In Arabidopsis, they constitute a 12-member family, only one of which has been characterized till date. Arabidopsis OTLD1 was shown to be a histone deubiquitinase which interacts with a histone demethylase (KDM1C) to repress gene expression (Krichevsky et al., 2011). However, *otld1* plants lacked any observable phenotype, questioning the contribution of OTLD1 in plant physiology.

Research undertaken in this chapter was aimed at characterizing OCP1 to answer the following questions- Is OCP1 a peroxisomal protein? What is its physiological role in plants and peroxisome metabolism? Does the protein have proteolytic activity? What are its possible substrates?

Results

OCP1 is a plant specific peroxisome localized protein

Blast searches using OCP1 as query revealed that while homologs in other plant species possess PTS1 like sequences (Figure 3.1), none of the metazoan homologs contained any PTS sequences. ClustalW alignment of OCP1 proteins from Arabidopsis, rice, poplar and *Physcomitrella* show that while all these proteins contain PTS1 tripeptide and there is high degree of sequence similarity over the region encompassing the otu domain, the N-terminal halves of the proteins are poorly conserved.

Ser-Lys-Leu (SKL) constitutes a major plant PTS1 and in Arabidopsis 29/46 SKL containg proteins have been documented to localize to the peroxisomes. In order to confirm the predicted localization of OCP1, we generated stable transgenic lines co-expressing a peroxisomal marker, DsRed-PTS1, and the GFP-OCP1 fusion protein

driven by the CaMV constitutive promoter. Consistent with the predicted localization, confocal imaging of transgenic lines revealed that GFP-OCP1 colocalized with spots labeled by DsRed-PTS1 (Figure 3.2A). The GFP-tagged OCP1 also appeared to be targeted to the nucleus, which was supported by DAPI staining (Figure 3.2B). Deletion of the C-terminal tripeptide, SKL, abolished the peroxisome targeting, confirming that SKL is necessary to direct OCP1 to peroxisomes (Figure 3.2C). We also examined the localization of GFP-OCP1 under the control of its own promoter (1kb upstream of the start codon) in Arabidopsis plants expressing DsRed-PTS1. Similar to earlier observations, we found that expression of GFP-OCP1 under the native promoter also results in dual-localization of the protein to the peroxisome and the nucleus (Figure 3.3).

Surprisingly, we found that OCP1-GFP also colocalized with the peroxisomal marker protein (Figure 3.4A). Re-examining the N-terminal protein sequence of OCP1 revealed the presence of a PTS2-like sequence (RNAISKRHL; Figure 3.1). Accordingly, we observed that the N-terminal 30 aa sequence of OCP1, when fused to GFP, was sufficient to direct the reporter to peroxisomes (Figure 3.4B). Elimination of this 30 aa peptide caused loss of peroxisomal targeting, thus establishing RNX_6HL as a bona-fide PTS2 (Figure 3.4C).

The *ocp1* mutants exhibit IBA resistant root growth

To study the physiological function of OCP1, we identified two T-DNA insertion mutants of the *OCP1* gene. Using RT-PCR with primers that amplified the full-length conding sequence of *OCP1* we show that *ocp1-1* is a likely null allele and *ocp1-2* is a partial loss-of-function mutant (Figure 3.5B). The homozygous mutant plants showed

no phenotypic differences compared to wild-type plants of the same age throughout the lifecycle.

Considering that OCP1 is targeted to peroxisomes, we subjected the mutants to physiological assays that assess peroxisome dysfunctions in β-oxidation and IBA activation processes. Since β-oxidation of fatty acids in the peroxisome fuels pre-photosynthetic growth in Arabidopsis, many mutants in of peroxisome-targeted genes are reliant on exogenous sucrose for growth in dark conditions (Hayashi et al., 1998). To test whether *ocp1* mutants were compromised in β-oxidation, we grew mutants in the dark in the presence and absence of sucrose and measured their hypocotyl lengths. While the peroxisomal protein import mutant *pex14* showed a clear dependence on sucrose for growth, neither *ocp1-1* nor *ocp1-2* was compromised in growth on media lacking sucrose (Figure 3.6).

Besides fatty acid catabolism, plants also use a single cycle of β-oxidation to convert indole-3-butyric acid (IBA) to indole-3-acetic acid (IAA), a phytohormone that inhibits primary root growth (Zolman et al., 2000). IBA-mediated root inhibition was examined by comparing primary root lengths of *ocp1* alleles grown on increasing concentrations of IBA to those of wild-type plants. Both *ocp1* mutant plants displayed partial resistance to concentrations of IBA that were inhibotry to wild type plants (Figure 3.7); To rule out the possibility that a defect in perception of IAA manifested as IBA resistance, we also tested the mutants in the presence of IAA and found that *ocp1* mutants exhibited wild-type response to IAA. These data suggest that the IBA resistance seen in the *ocp1* alleles is due to a specific lack of OCP1 functions within the peroxisome.

We also transformed mutants with peroxisome marker constructs CFP-PTS1 and PTS2-CFP to observe matrix protein import and/or peroxisome morphology and number (Figure 3.8A). In the mutants, both PTS1 and PTS2 proteins were imported normally to peroxisomes and no abnormalities were seen in peroxisome morphology or number. PTS2 processing was also unaffected as we only detected the mature form of 3-ketoacyl-CoA-thiolase (KAT2) in both mutants. Further analysis did not reveal any alterations in the steady state protein levels of PEX5 or PEX7 either (Figure 3.8B). We conclude that OCP1 by itself does not influence import of peroxisomal proteins and has no effect on peroxisome appearance and abundance in Arabidopsis.

MBP-tagged OCP1 protein does not appear to have deubiquitinase/protease activity

Given that otu domain proteins have deubiquitinase activity, we evaluated the possibility that OCP1 acts as a ubiquitin isopeptidase. We expressed and purified OCP1 as a MBP-fusion protein from *E.coli* to use in enzymatic assays. A mutant version of OCP1, which contains a Ser substitution at the catalytic Cys (C179) residue was also expressed and purified for use as a control (Figure 3.9A). The purified protein was incubated with Lys48-linked tetraubiquitin to determine if recombinant OCP1 could hydrolyze covalently linked ubiquitin moieties. Western blotting with Ub antibody revealed that contrary to expectation, OCP1 did not disassemble tetraubiquitin (Figure 3.9B). Recombinant OCP1 was also tested for deubiquitinating activity using a general synthetic substrate, Z-LRGG-AMC containing carboxy-terminal four aa residues of ubiquitin coupled to a fluorogenic molecule, AMC. The failure to detect cleavage of this

substrate, as evidenced by an absence of increase in fluorescence upon incubation with OCP1, suggested that recombinant OCP1 did not possess ubiquitin isopeptidase activity (Figure 3.9C). Considering its functional annotation as a putative Cys protease, we also subjected OCP1 to universal protease activity assay using casein as a non-specific substrate, but still failed to find enzymatic activity (Figure 3.9D). No change in absorbance was registered in the presence or absence of the recombinant OCP1, while a control protease, RP5, was found to have hydrolyzed casein resulting the release of Tyr residues thus causing a detectable increase in absorbance by Folin's reagent.

ocp1 mutants are hypersensitive to ABA mediated suppression of seed germination

To gain further insights into the role of OCP1 during plant development, we used web-based microarray database to estimate the expression profile of this gene. Normalized expression data obtained from the EFP browser (Winter et al., 2007) revealed that OCP1 was highly expressed in dry seeds and stage 12 flowers (Figure 3.10). We isolated total proteins from these developmental stages, reasoning that an endogenous target protein for OCP1 may be enhanced in the *ocp1-1* mutant compared to the wild-type, enabling its identification by mass spectrometry (MS). However, no noticeable differences were seen between wild-type and mutant protein samples from dry seeds and flowers on SDS-PAGE gels, so samples were not sent for MS analysis (Figure 3.11).

Proceeding with the hypothesis that OCP1 might play a more prominent role in seeds and open flowers, we studied the effect of the *ocp1* mutations on mature flowers,

pollen viability, pollen germination and seed germination. Although we observed no differences in pollen viability or germination, we found that *ocp1* mutants were hypersensitive to ABA, a phytohormone that inhibits seed germination (Figure 3.12).

OCP1 is required for the timely degradation of ICL and MS

The ABA hypersensitive seed germination phenotype displayed by the *ocp1* mutants suggests that OCP1 promotes seed germination, prompting us to examine the abundance of peroxisomal enzymes that are predominantly expressed during germination. Two such enzymes are isocitrate lyase (ICL) and malate synthase (MS) which operate in the glyoxylate cycle and are intimately associated with seed germination (Eastmond et al., 2000). Moreover, ICL and MS are only transiently expressed and appear to be degraded within a span of 5 days after seed germination (Lingard et al., 2009). We analyzed protein extracts from wild-type and *ocp1-1* mutant seedlings at 3, 5, 7 and 9 days after germination with antibodies against ICL and MS. The degradation of ICL is delayed in *ocp1-1* mutant compared with the wild type (Fgure 3.13). Given the apparent stabilization of ICL in *ocp1-1*, a null allele, we also checked ICL levels in the partial loss-of-function *ocp1-2* mutant. ICL was present in 7 days old *ocp1-2* but not in wild-type seedlings, confirming that OCP1 function(s) may be important for its timely degradation. Retarded degradation of ICL and MS has previously been reported only for mutants for protein import components (Zolman et al., 2005; Lingard et al., 2009; Monroe-Augustus et al., 2011). To determine whether OCP1 plays a role in the import of these proteins, we transformed *ocp1-1* and wild-type plants with YFP-ICL constructs and confirmed that the import of ICL is unaffected in the mutant

(Figure 3.14A). However on quantifying the percent co-localization between CFP-PTS1 and YFP-ICL we found that in comparison to wild type plants the *ocp1-1* mutant showed much reduced levels of co-localization with the peroxisomal marker (Figure 3.14B).

Since the major function of the glyoxylate cycle is to provide a usable carbon source to the germinating seed in the absence of photosynthesis, ICL and MS mutants show sucrose dependence when grown in the dark (Eastmond et al., 2000; Cornah et al., 2004). As *ocp1* mutants showed elevated levels of ICL and MS, we also evaluated the effect of *OCP1* overexpression by conducting sucrose dependence assays on 35Spro:YFP-OCP1 transgenic lines (Figure 3.15). We found that hypocotyl growth of dark-grown YFP-OCP1 seeds was compromised in the absence of exogenous sucrose ($P<0.001$). Furthermore, YFP-OCP1 plants were significantly smaller than wild-type plants of the same age (Figure 3.15D).

To address whether OCP1 directly regulates the abundance of ICL and MS, we employed the transient tobacco expression system to co-express YFP-OCP1 and CFP-tagged ICL or MS fusion proteins. YFP-OCP1 and CFP-ICL/MS colocalized, but the expression level of the CFP fusion proteins was extremely low and inconsistent (Figure 3.16A). Nonetheless, results from this experiment would suggest that YFP-OCP1 most likely does not directly degrade ICL/MS. We also attempted a similar experiment with a 35S-HA tagged OCP1 construct, but the protein proved unstable and was barely detectable in western blots (Figure 3.16B). We noted, however that a ~ 22kDa breakdown product appeared to accumulate in HA-OCP1C*S (catalytic mutant) infiltrated samples. This implies that OCP1 undergoes a cleavage step *in planta* and that the N-terminal fragment may be degraded by the enzymatic activity of the C-

terminal fragment. Thus, it is possible that the failure of the catalytically inactive protein to further process the truncated fragment results in the elevated levels of the N-terminal fragment.

Discussion

Plant peroxisomes have been associated with a wide range of metabolic processes largely through the activities of constituent proteins. Experimental proteomic and bioinformatic approaches coupled with in vivo target validation have vastly expanded the number of known peroxisomal proteins, several of which are unique to plants (Hu et al., 2012). Here, we confirmed that OCP1 is a novel component of Arabidopsis peroxisomes and has a conserved PTS1 signal in plant lineages. We found that OCP1 possesses both PTS1 and PTS2 signals, either of which is sufficient for peroxisome targeting. A handful of other matrix proteins, such as the yeast intraperoxisomal organizer (Pex8p) and Arabidopsis LACS7, CSY2 and CSY3, have been reported to harbor both types of PTSs (Fulda et al., 2002; Pracharoenwattana et al., 2005; Zhang et al., 2006). Our bioinformatic analysis identified three additional Arabidopsis proteins that also have N and C-terminal PTS signals (Kaur and Hu, 2011). The presence of redundant targeting signals is somewhat puzzling and seemingly wasteful because PEX7, which is the receptor for PTS2 proteins, cannot associate with peroxisome membranes in the absence of PEX5. For import to occur, proteins destined for the peroxisomal matrix need to be bound by receptor proteins. PTS1 proteins far outnumber PTS2 proteins (98 vs. 19) in Arabidopsis, implying that several proteins would compete to bind PEX5 simultaneously and receptor availability would dictate the rate/efficacy of protein import. Additionally, some emerging data suggests that under

certain environmental conditions PEX5 is (co)-dependent on PEX7 for PTS1 import to occur (Ramon and Bartel, 2010). In this context, two targeting signals would confer a specific advantage ensuring such proteins are efficiently imported into the organelle.

Although OCP1 is annotated as a Cys protease and homologs in other species show deubiquitinase activity, we were unable to detect either ubiquitin hydrolase or even general protease activity in in-vitro assays. It is possible that the large MBP tag interfered with protein folding or enzymatic activity. In parallel, we also expressed OCP1 as a soluble protein using a 6xHis tag, but the yield of the soluble protein was extremely low and purified protein fractions were prone to degradation. It is also likely that the assay conditions needed to be further optimized for buffers, temperature, pH or substrates used. Lastly, expressed HA-OCP1 in tobacco seems to undergo a processing step. It is not known whether OCP1 cleaves itself or this activity is provided in trans by another protease. Synthesis of proteases as inactive precursors with N-terminal autoinhibitory domains is a well-known strategy to keep indiscriminate or premature activity in check (Lopez-Otin and Bond, 2008). The N-terminal domain of OCP1 may be acting in a similar fashion to repress enzyme activity, which may also explain why in vitro enzyme assays did not succeed.

Analysis of *ocp1* mutant plants revealed that they displayed IBA resistance, which indicates that OCP1 influences peroxisome metabolism. Interestingly, this phenotype was more pronounced on higher concentrations of IBA. A range of peroxisomal mutants such as those involved in matrix protein import, peroxisome biogenesis, β-oxidation enzymes, transporters and miscellaneous enzymes show IBA resistance (Hu et al., 2012). While we can discount an involvement in protein import or

biogenesis, the possibility exists that OCP1 modulates the activity of some metabolic enzyme(s) within the peroxisome, manifested as IBA resistance in the mutants. Alternatively, absence of OCP1 could have a deleterious affect on the production or scavenging of peroxisomal H_2O_2 or the pool of available cofactors, or result in build up of toxic metabolite intermediates, any of which could lead to the observed IBA resistance.

ABA promotes seed dormancy. Germination of *ocp1* mutants is hypersensitive to the suppressive effect of ABA on seed germination. ABA is known to induce expression of some peroxisomal genes such as catalase and betaine aldehyde dehydrogenase possibly through ABA-induced ROS generation (Missihoun et al., 2011; Guan and Scandalios, 2000; Guan et al., 2000; Xing et al., 2007, 2008; Mhamdi et al., 2010). Perturbed H_2O_2 levels may explain the lower germination rate exhibited by the ocp1 mutants.

The *ocp1-1* mutant shows delayed degradation of ICL and MS compared to wild-type plants. Several lines of evidence indicate that ICL and MS are sensitive to the levels of peroxisomal H_2O_2 , among them the observations that ICL is inactivated by H_2O_2 and physically associates with catalase (Vandenabeele et al., 2004; Yanik and Donaldson, 2005; Eastmond, 2007; Anand et al., 2009). It has been hypothesized that due to diminished β-oxidation, the peroxisomal ABC transporter, *pxa1* mutant has lower levels of H_2O_2 while catalase mutants have higher levels of H_2O_2. In line with this, catalase mutant shows reduced activities and accelerated degradation of ICL and MS while *pxa1* has persistent levels of ICL and MS (Lingard et al., 2009). Further, it has

been frequently alluded that oxidative damage caused by H_2O_2 triggers peroxisome associated degradation of ICL and MS. Like the peroxisomal ABC transporter mutant, ICL is not mislocalized in *ocp1-1* null allele either. We speculate that the underlying cause of the observed stabilization in *ocp1* alleles might also relate to peroxisomal H_2O_2 metabolism.

Our transient co-expression assays seem to suggest that YFP-OCP1 does not directly affect the stability of ICL/MS. A simple possibility is that, like the MBP fusion protein, the YFP fusion does not have enzymatic activity. However, it must be noted that ICL/MS are normally present in seedling peroxisomes that contain some proteins not found in leaf peroxisomes, two of which are plant specific proteases (Quan et al unpublished data). In view of this, OCP1 could modulate ICL/MS abundance indirectly *via* activating/inactivating these proteases. Some of these discrepancies would have been resolved by using a tagless OCP1 overexpression line, but despite numerous attempts we could not recover such transgenic plants.

YFP-OCP1 overexpressors appear to phenocopy glyoxylate cycle mutants by showing a marked dependence on exogenous sucrose for growth in dark conditions. Unlike *icl/MS* mutants, the YFP-OCP1 overexpression lines show retarded growth compared to wild-type plants of the same age. At this point, we cannot rule out the possibility that the overexpressed YFP-OCP1 proteins function in a dominant negative manner. YFP-OCP1 might still interact with proteins in the peroxisomes and inhibit their functions. We also don’t know whether OCP1 nuclear localization impacts plant growth and development.

In sum, we have identified a novel constituent of Arabidopsis peroxisomes that is conserved across plant species. Analysis of mutants reveals that OCP1 functions are linked to peroxisomal IBA metabolism and have an effect on the timely removal of ICL and MS during germination. We speculate that OCP1 exerts these effects either due to proteolytic activity or possibly by modulating intraperoxisomal H_2O_2 levels. Whether OCP1 acts alone, or in concert with other proteins remains to be elucidated.

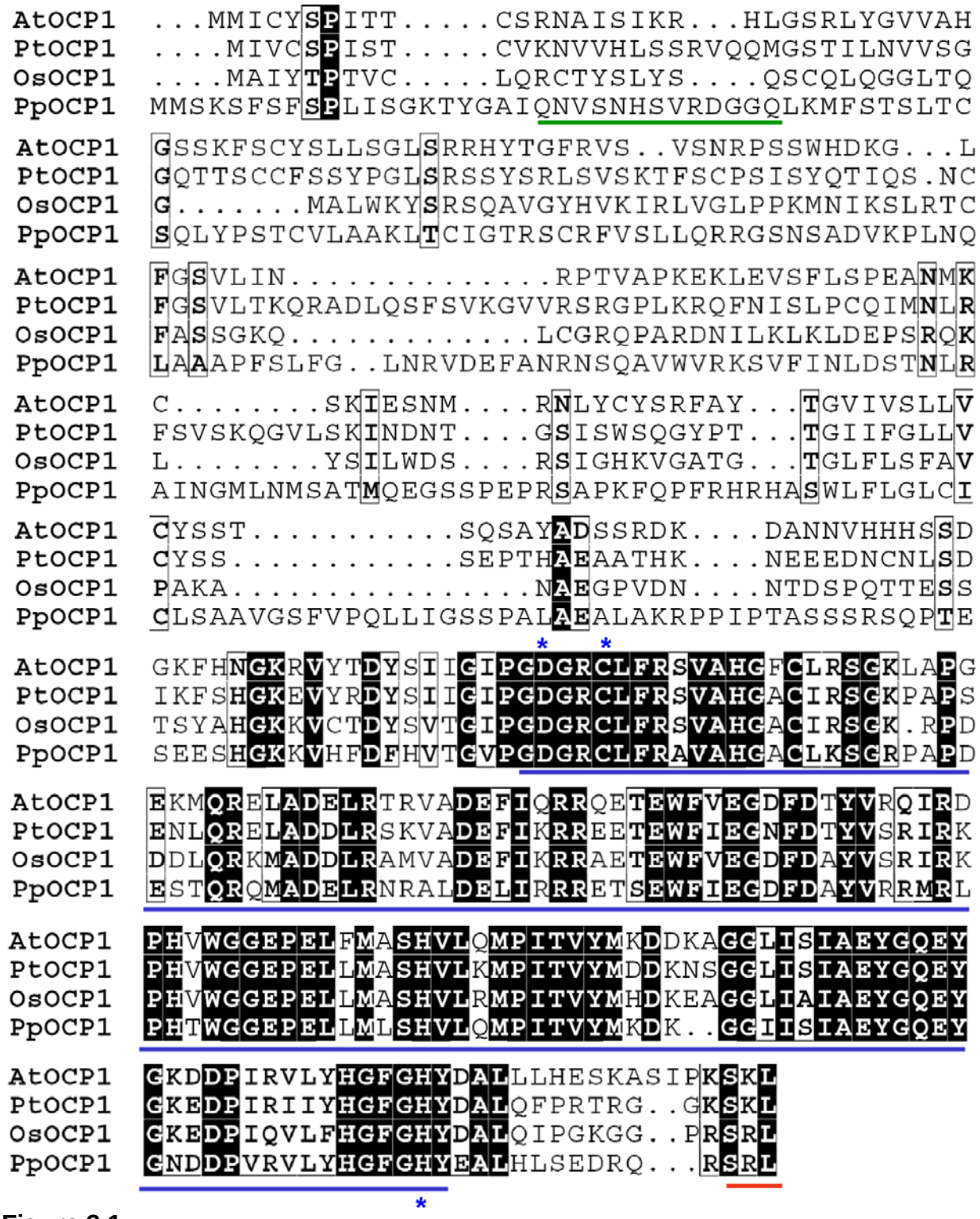

Figure 3.1.

Plant OCP1 homologs have PTS1 sequences and contain a conserved C-terminal OTU domain.

Figure 3.1. (Cont'd)

OCP1 homologs from *Arabidopsis thaliana* (At), *Populus trichocarpa* (Pt), *Oryza sativa* (Os) and *Physcomitrella patens* (Pp) were aligned with ClustalW. Areas shaded in black indicate completely conserved residues and partially conserved residues are outlined in boxes. PTS1 and PTS2 sequences are underlined in red and green respectively. OTU domain is underlined in blue and asterisks mark positions of the putative catalytic residues.

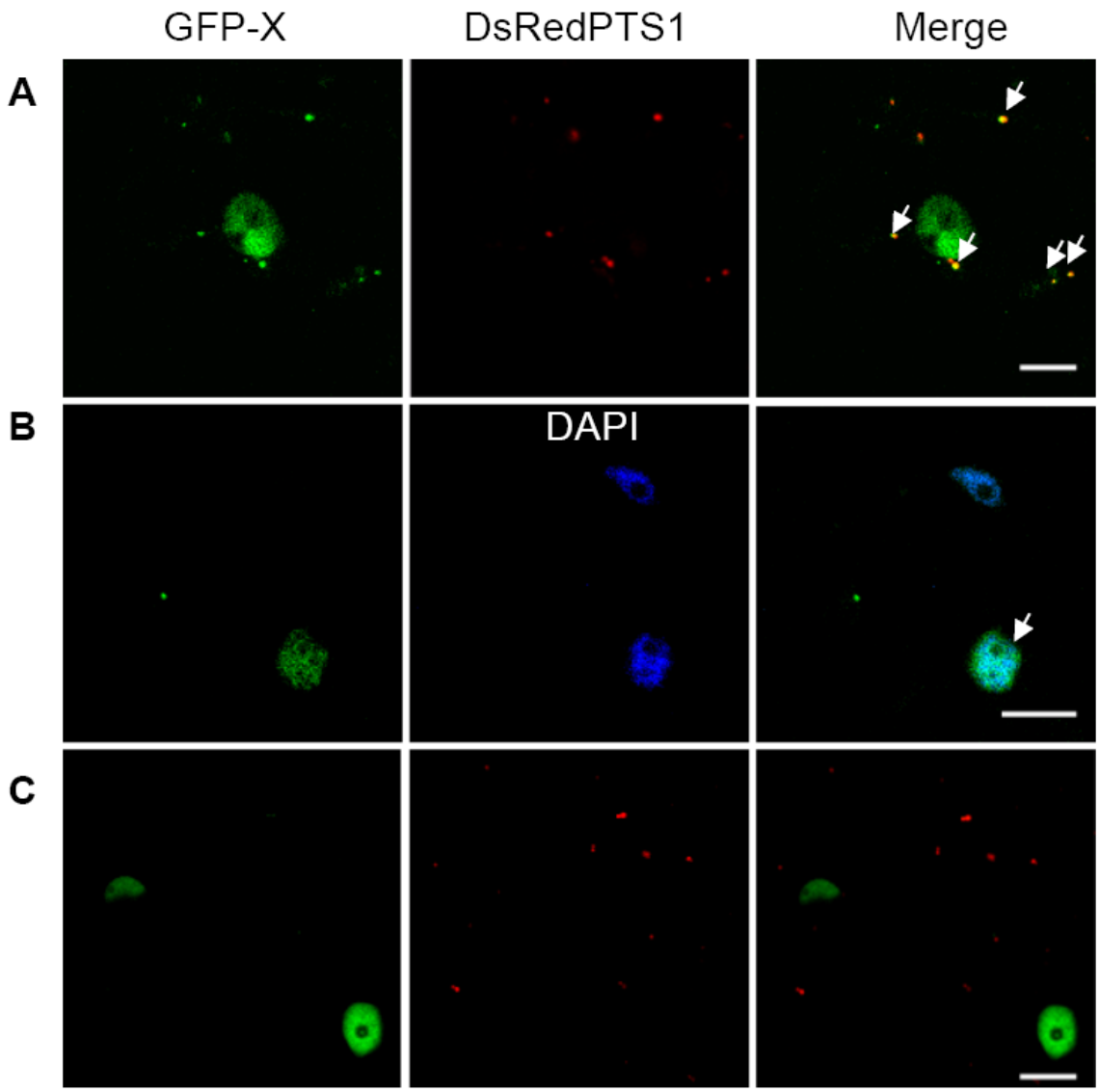

Figure 3.2. GFP-OCP1 localizes to the peroxisomes through a C-terminal PTS1. Confocal images of N-terminal GFP tagged OCP1 (green) coexpressed with (A) peroxisome marker DsRedPTS1(red) or (B) DAPI (blue) in tobacco epidermal cells. Arrowheads indicate colocalized spots. (C) SKL is necessary for peroxisome targeting of GFP-OCP1. Confocal images of N-terminal GFP-OCP1ΔSKL (green) co-expressed with DsRedPTS1 (red) in tobacco epidermal cells. White bar represents scale of 10μm.

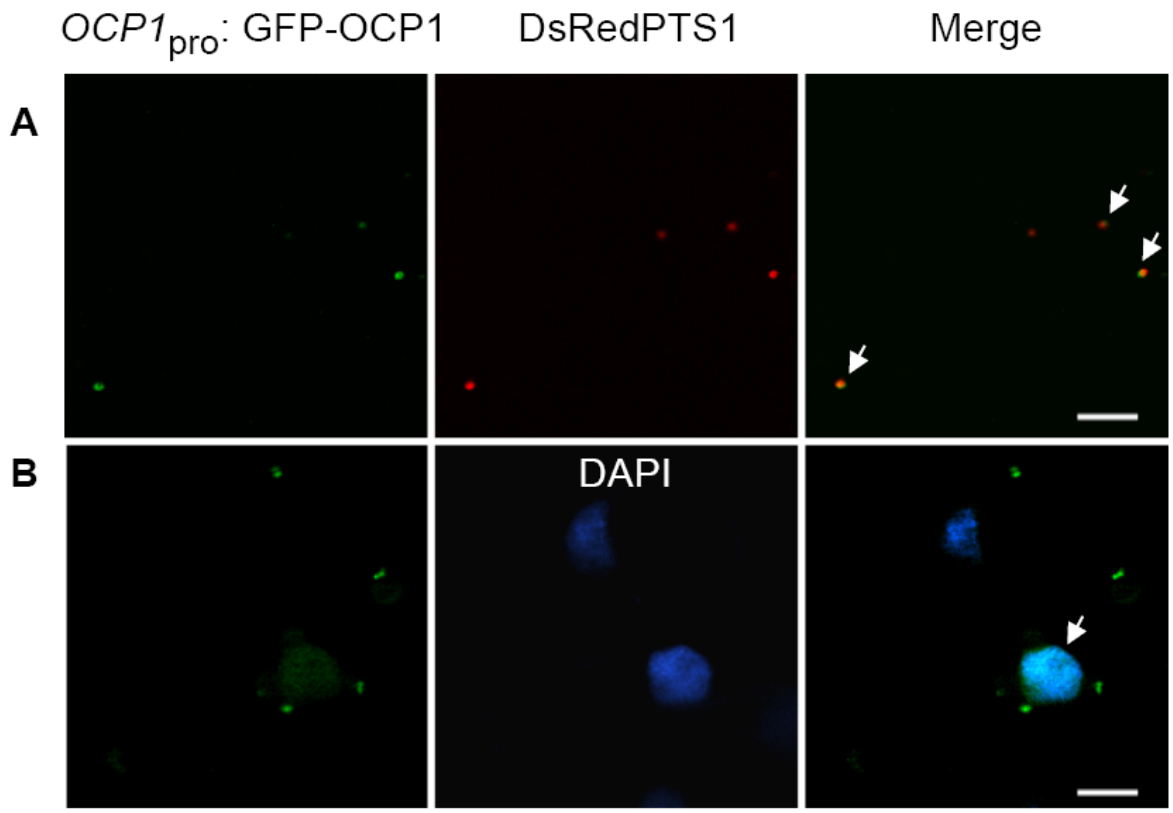

Figure 3.3. Native promoter targets GFP-OCP1 to the peroxisomes and the nucleus.

(A) Confocal images of OCP1 native promoter (*OCP1*$_{pro}$) driven N-terminal GFP tagged OCP1 (green) and peroxisome marker DsRedPTS1 (red) in transgenic Arabidopsis plants.

(B) Confocal images of OCP1 native promoter driven N-terminal GFP tagged OCP1 (green) transgenic Arabidopsis plants stained with DAPI (blue) to visualize nuclei. White bar represents scale of 5 µm.

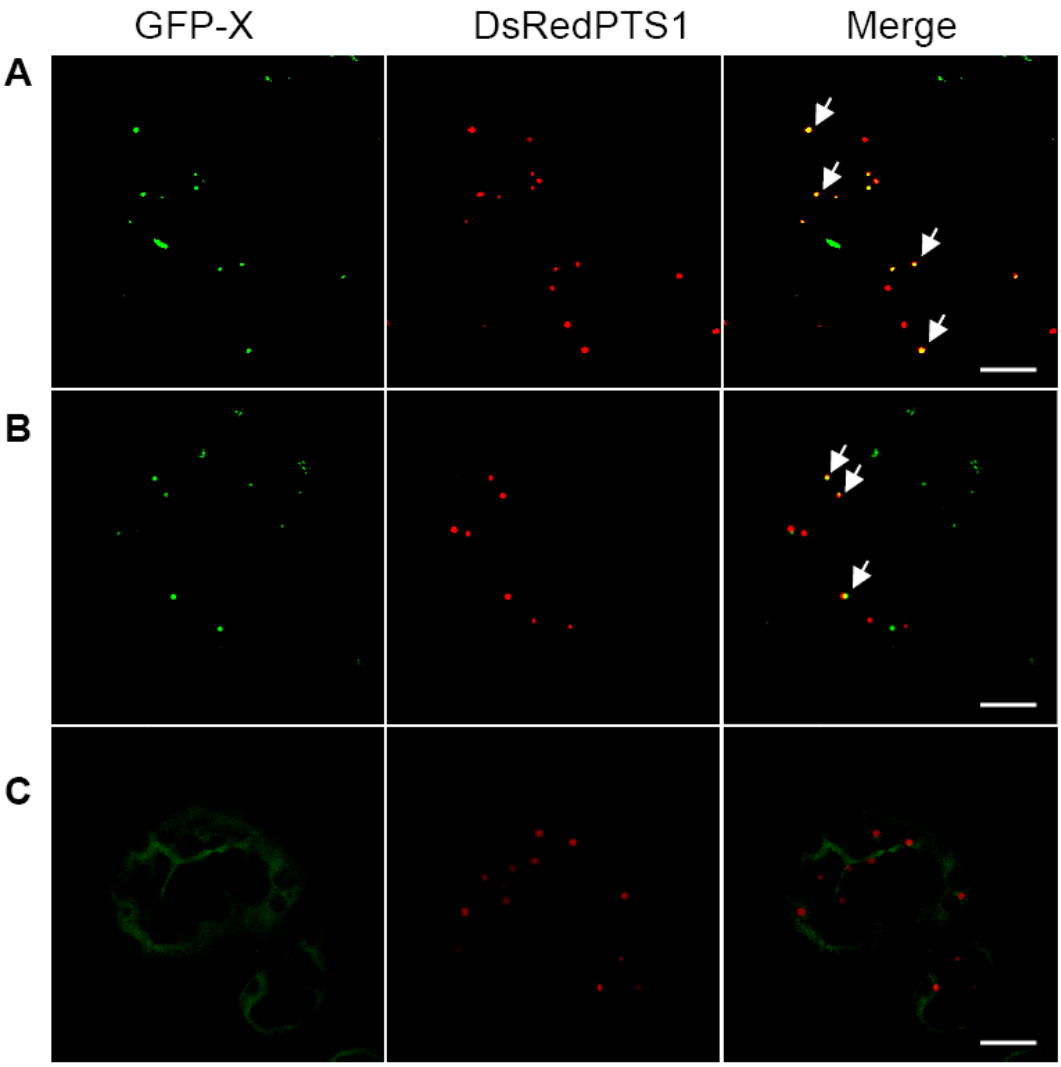

Figure 3.4. OCP1-GFP localizes to the peroxisomes via a novel PTS2.

(A) Confocal images of C-terminal GFP tagged OCP1(green) co-expressed with peroxisome marker DsRedPTS1(red) in tobacco epidermal cells.

(B) Validation of new PTS2 peptide. First 30 amino acids of OCP1 comprising the peptide RNX_6HL were fused to GFP (green) and co-expressed with peroxisome marker DsRedPTS1(red) in tobacco.

(C) Deletion of PTS2 peptide RNX_6HL abolishes peroxisome targeting. White bar represents scale of 10 µm.

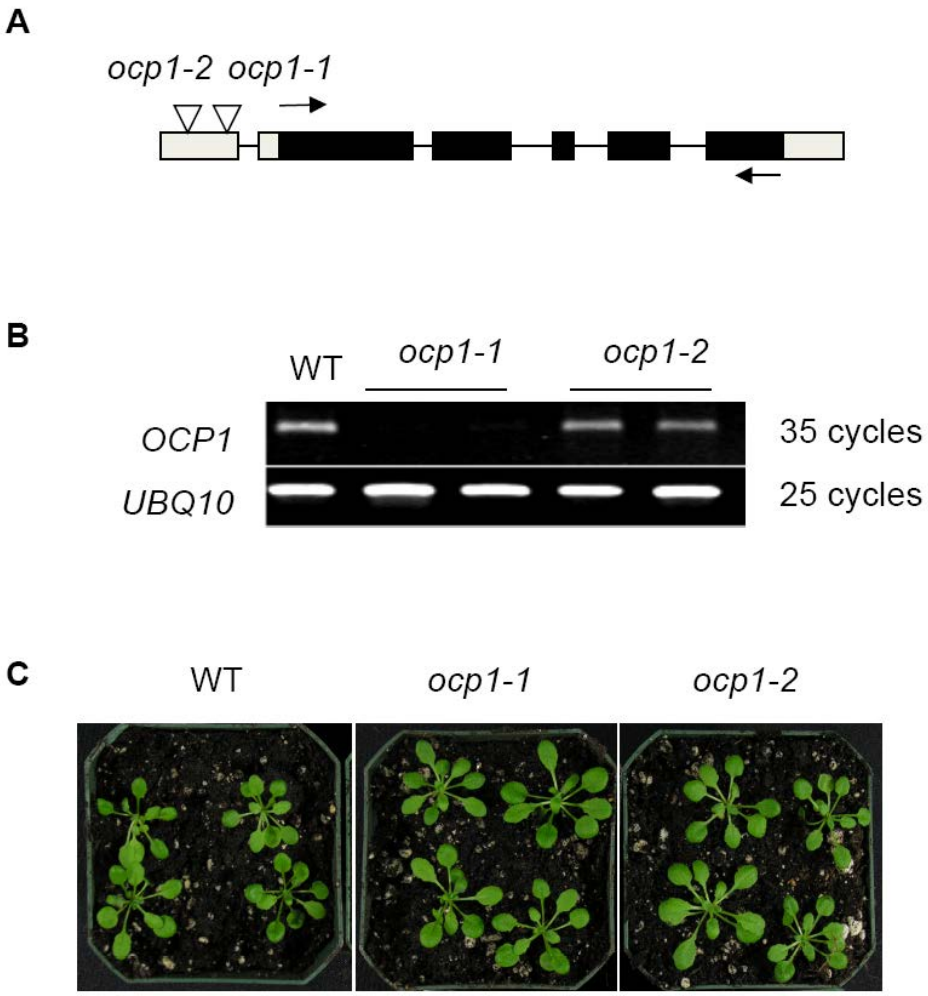

Figure 3.5. Identification of *ocp1* loss-of-function mutants.

(A) Schematic showing insertion sites of T-DNA in the OCP1 gene. Identified mutants were in the 5'UTR (gray box) region. Exons are represented as black boxes and black lines depict introns. Arrowheads show positions of the RT-PCR primers.

(B) RT-PCR showing the expression level of *OCP1* in the two mutants; *ocp1-1* was found to be a null mutant while *ocp1-2* was found to be partial loss-of-function allele. *UBQ10* transcripts was used as a loading control

(C) Plant morphologies of 3 weeks old mutant and wild-type plants (WT). Images were taken under the same magnification.

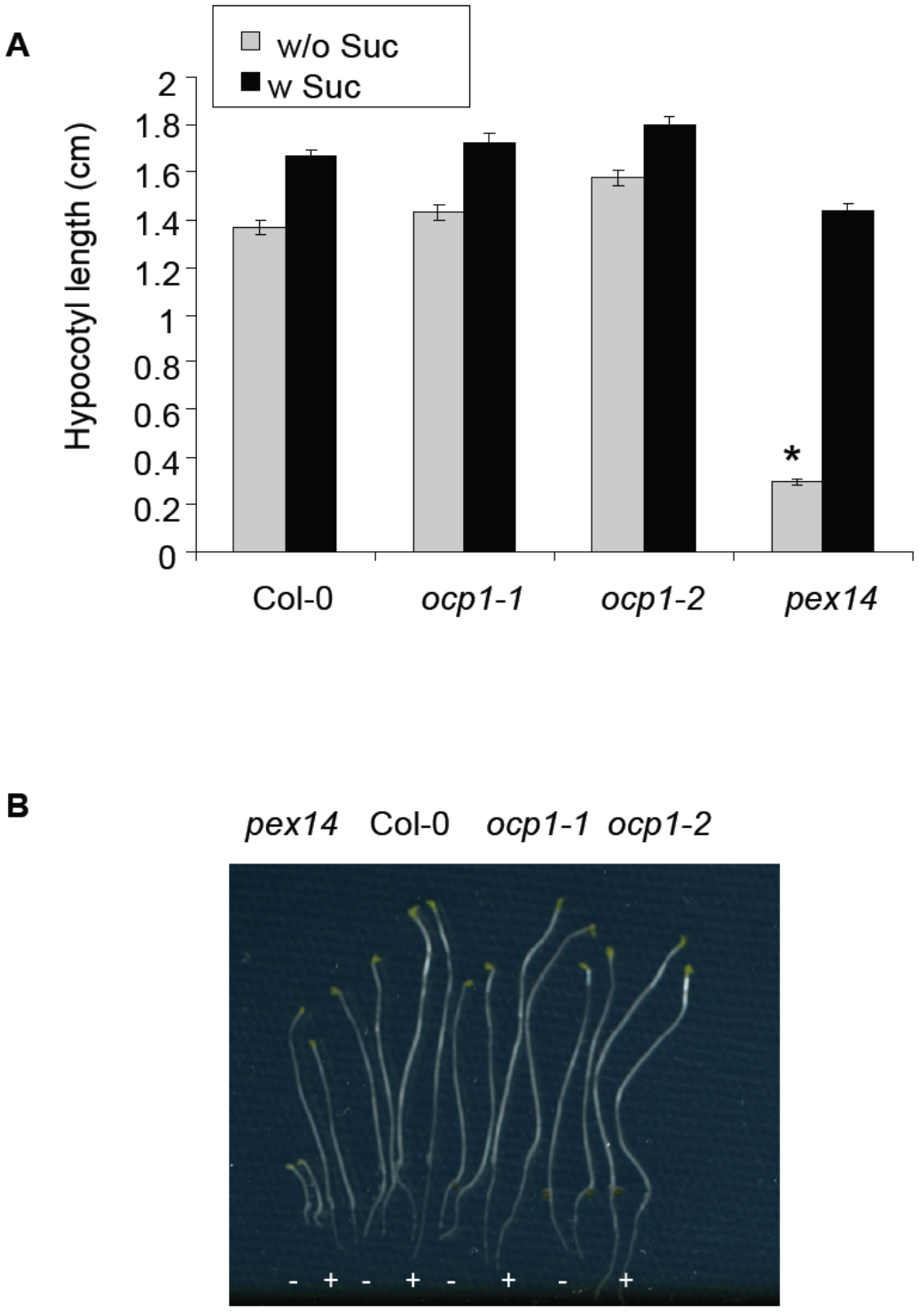

Figure 3.6. *ocp1* mutants are not sucrose dependent.

(A) Hypocotyl lengths of 7 day old etiolated seedlings grown on half-strength MS media in the absence (grey bars) or presence (black bars) of 1% sucrose were quantified.

Figure 3.6. (Cont'd)

pex14, peroxisome import mutant, was used as a positive control. Error bars represent SE (n> 45 for each genotype). * indicates statistically significant differences (Student's *t*-test, p<0.001) in comparisons between mutants and wild-type (Col-0).

(B) Images of seedlings of indicated genotypes grown in the (-) absence or (+) presence of sucrose.

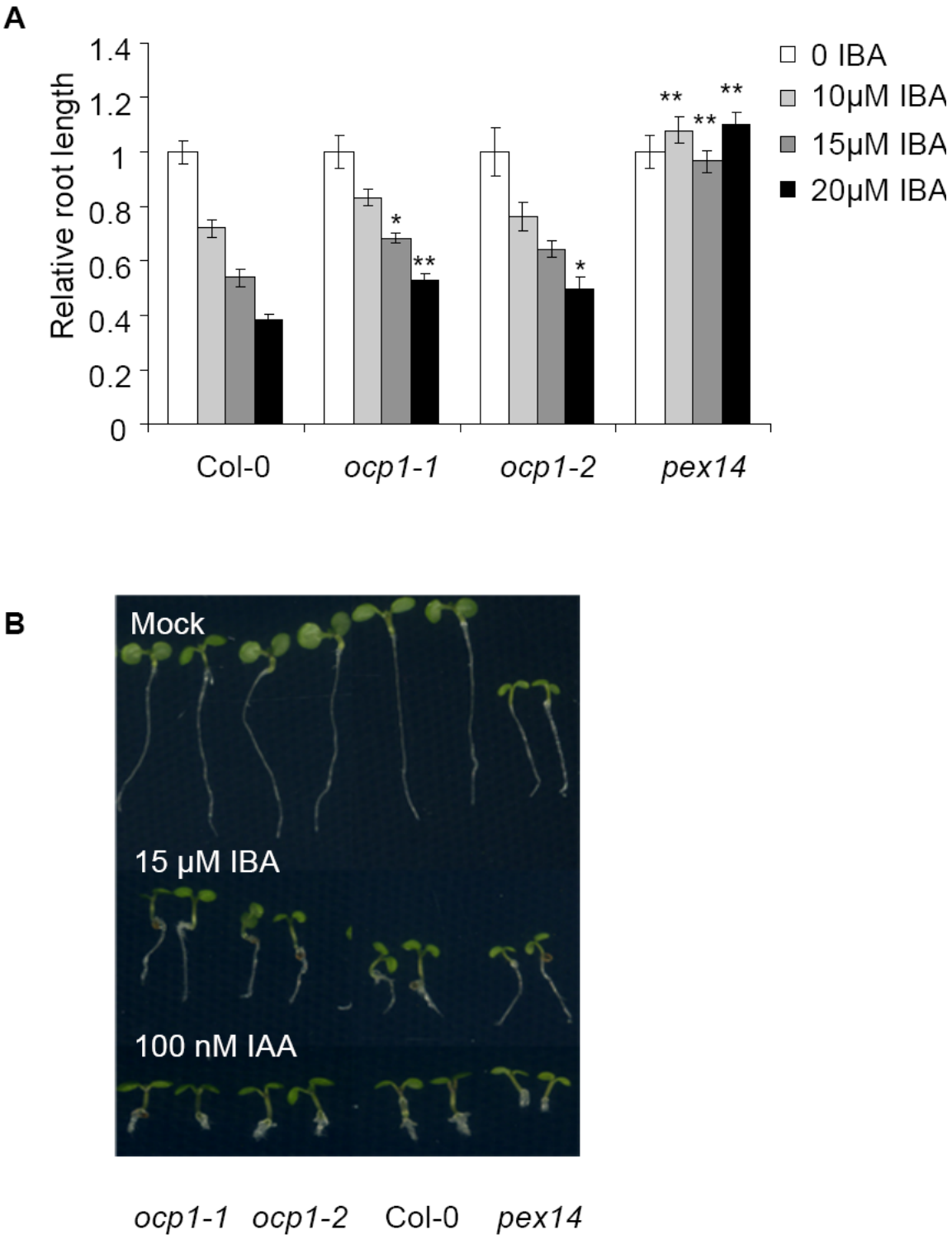

Figure 3.7. *ocp1* mutants are partially resistant to IBA mediated root inhibition.

A) Root lengths of 7 day old seedlings grown on indicated concentration of IBA

Figure 3.7. (Cont'd)

normalized to their respective growth on media without IBA. *pex14* was used as a positive control. Error bars represent SE (n> 60 for each genotype). * and ** indicate statistically significant differences of p< 0.05 and p<0.001 (Student's *t*-test) in comparisons between mutants and wild-type (Col-0).

(B) Two seedlings representing the observed phenotypes on control plates (top panel), 10µm IBA (middle) and 100nm IAA (lower panel).

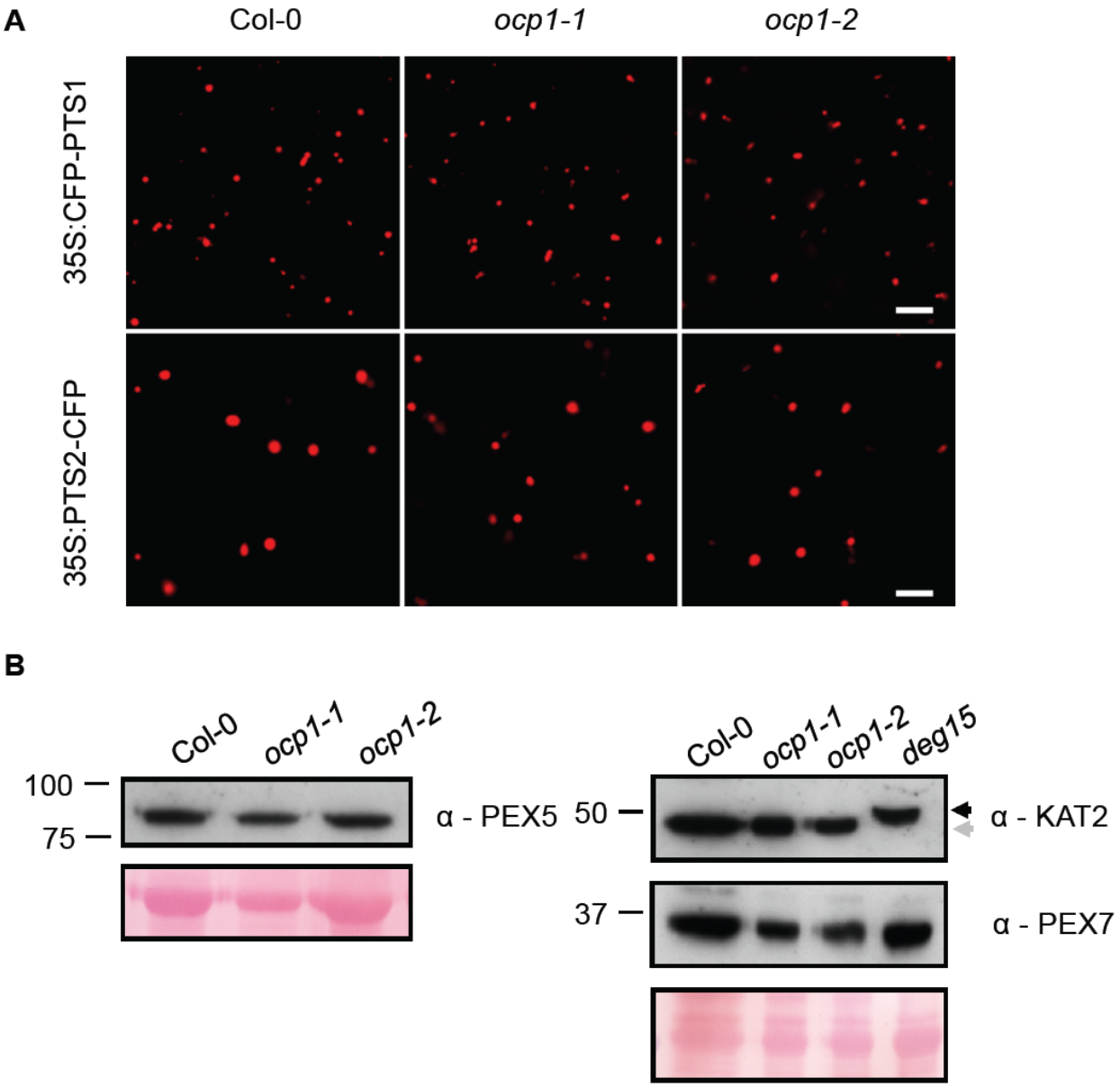

Figure 3.8. Matrix protein import and peroxisome morphology is unchanged in *ocp1* mutants

(A) Confocal images from leaf epidermal cells of wildtype and ocp1 mutant plants expressing CFP modified with a PTS1 (upper panel) or PTS2 signal (lower panel). Scale bar is 5 µm.

(B) Immunoblots using KAT2, PEX7 and PEX5 antibodies to analyze PTS2 processing

Figure 3.8. (Cont'd)

and levels of PTS2 and PTS1 receptor levels in wildtype and *ocp1* mutants respectively. Numbers on the sides indicate molecular weight markers in kDa. Black and grey arrowheads indicate premature (P) and mature (M) bands of KAT2. Expected molecular weights for KAT2P, KAT2M, PEX7 and PEX5 are 48.5, 44.8, 35.4 and 81 kDa respectively. *deg15* mutant is shown as a control for PTS2 processing. Ponceau stained membrane is shown as a loading control.

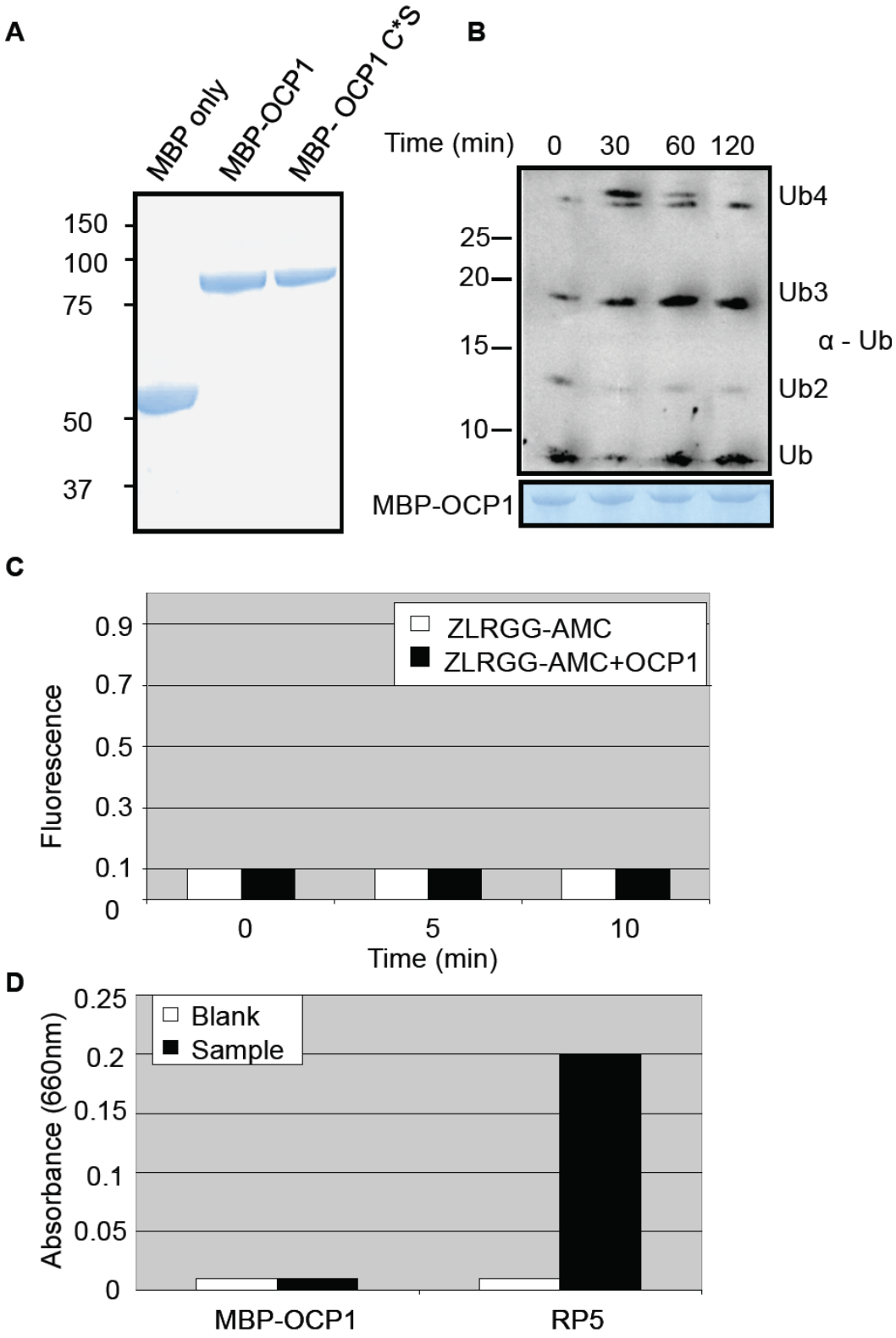

Figure 3.9. MBP-OCP1 does not have DUB or Cys protease activity.

Figure 3.9. (Cont'd)

(A) Purified MBP, MBP-OCP1 and MBP-OCP1 C*S (catalytic mutant) recombinant proteins. Sizes on left are molecular weight markers in kDs.

(B) Lys 48 linked tetraubiquitin was incubated with MBP-OCP1 for the indicated times. Dismantling of Ub chains was assessed by immunoblotting with Ub antibody (upper panel). Blot was stained with NBB to visualize MBP-OCP1 (lower panel).

(C) MBP-OCP1 was incubated with Z-LRGG-AMC at 37°C for 10 min. Release of fluorescence with excitation 340nm and emission 480 nm was monitored for the duration.

(D) MBP-OCP1 was incubated with casein and release of Tyr quantified colorimetrically using Folin's reagent. RP5 is a mitochondrial ubiquitin protease that was used as a control.

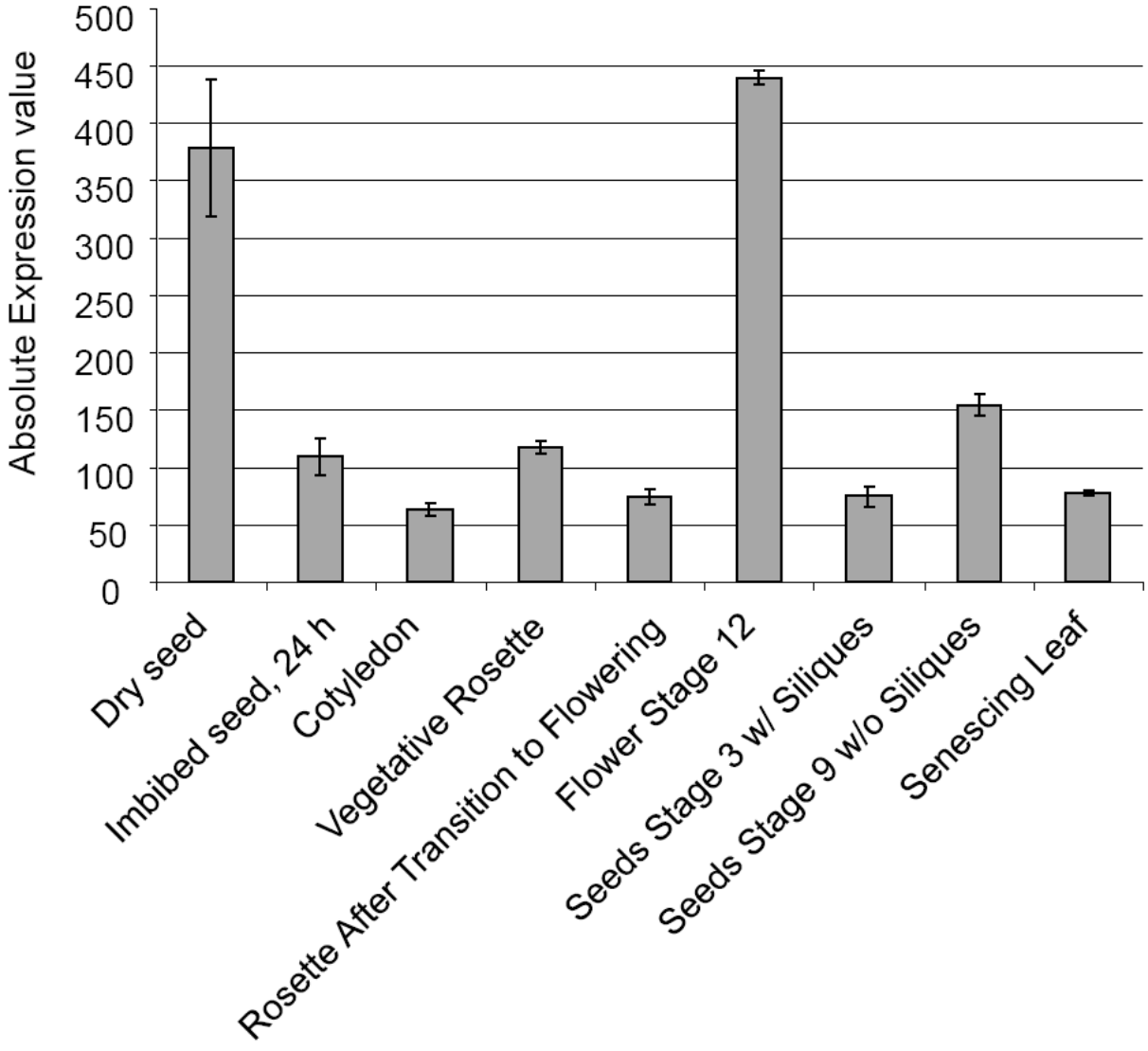

Figure 3.10. *OCP1* is highly expressed in dry seeds and open flowers.

Absolute expression values were retrieved from Arabidopsis EFP browser.

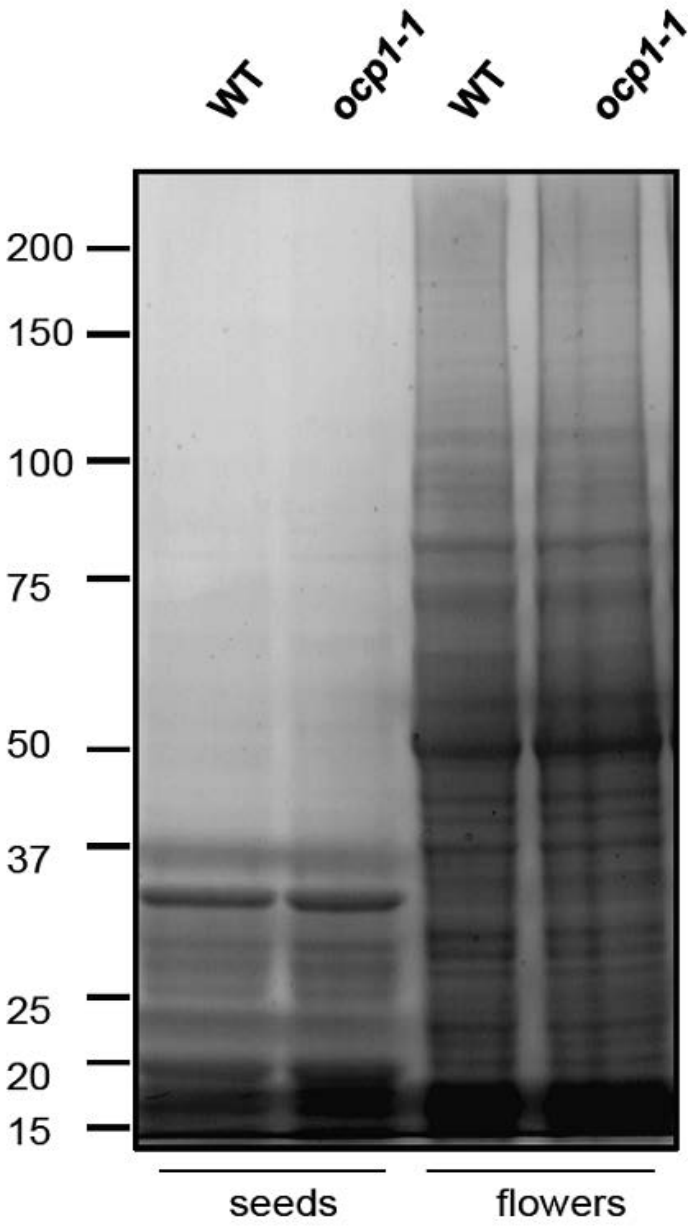

Figure 3.11. Protein profiles of wild-type (WT) and *ocp1-1* null mutant have no obvious differences.

SDS-PAGE of proteins extracted from dry seeds and open flowers of wildtype and *ocp1-1* mutant. Numbers on left represent migration of molecular weight markers in kDs.

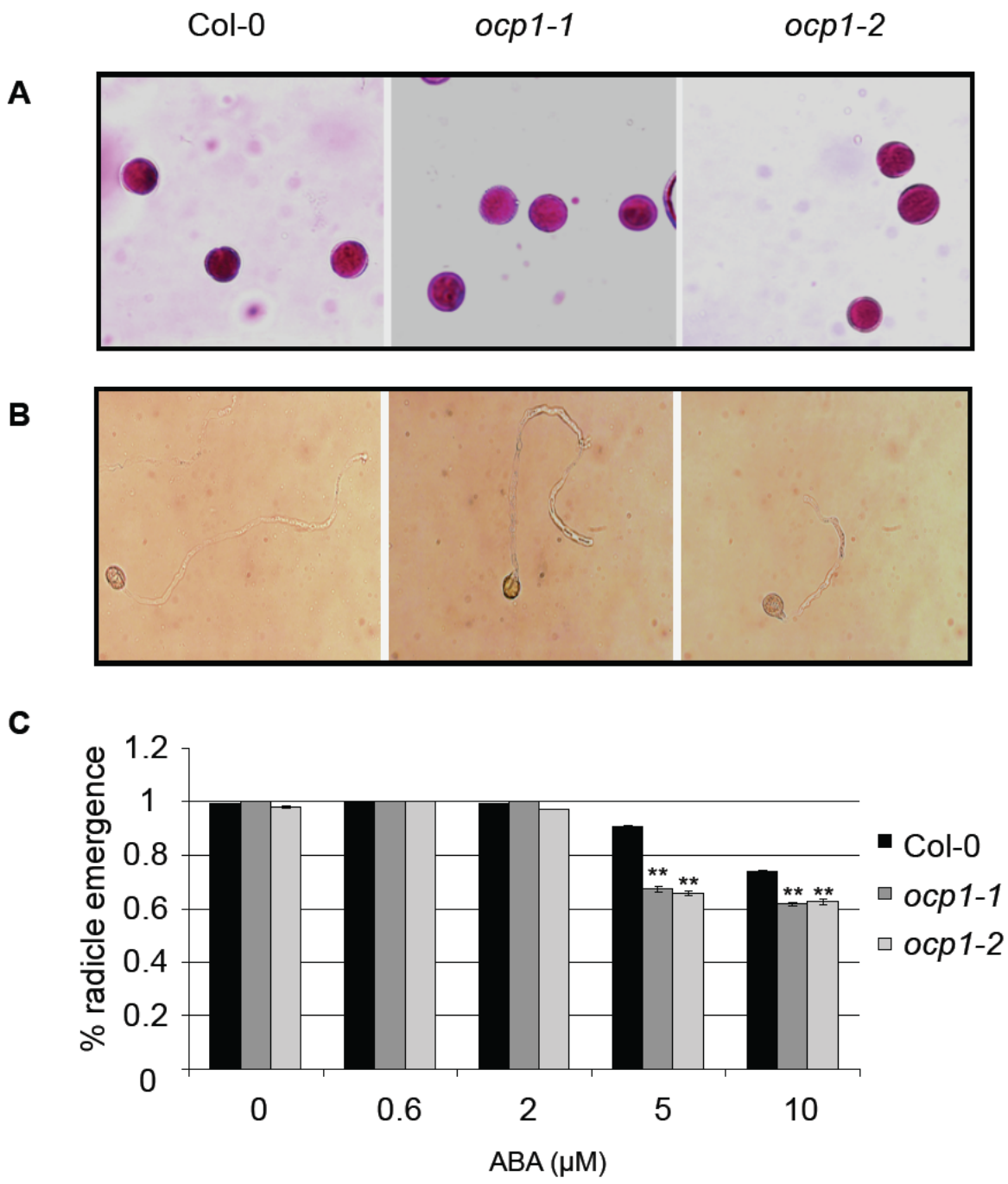

Figure 3.12. *ocp1* mutants produce viable pollen that germinates normally but *ocp1* seeds are hypersensitive to ABA mediated suppression of germination.

(A) Pollen from open flowers of wild-type (Col-0) and *ocp1* mutants was collected and stained with Alexander stain to observe pollen viability. No differences in viability were

Figure 3.12. (Cont'd)

observed.

(B) In-vitro germination assays were conducted on pollen collected from wildtype and *ocp1* mutants. No differences in pollen germination were observed.

(C) Effect of ABA on germination. Quantification of radicle emergence of each genotype on the indicated concentration of ABA. Error bars are SE (n>130). ** denotes stastically significant difference (Student's *t*-test, $p<0.001$) in comparisons between mutants and wild-type (Col-0).

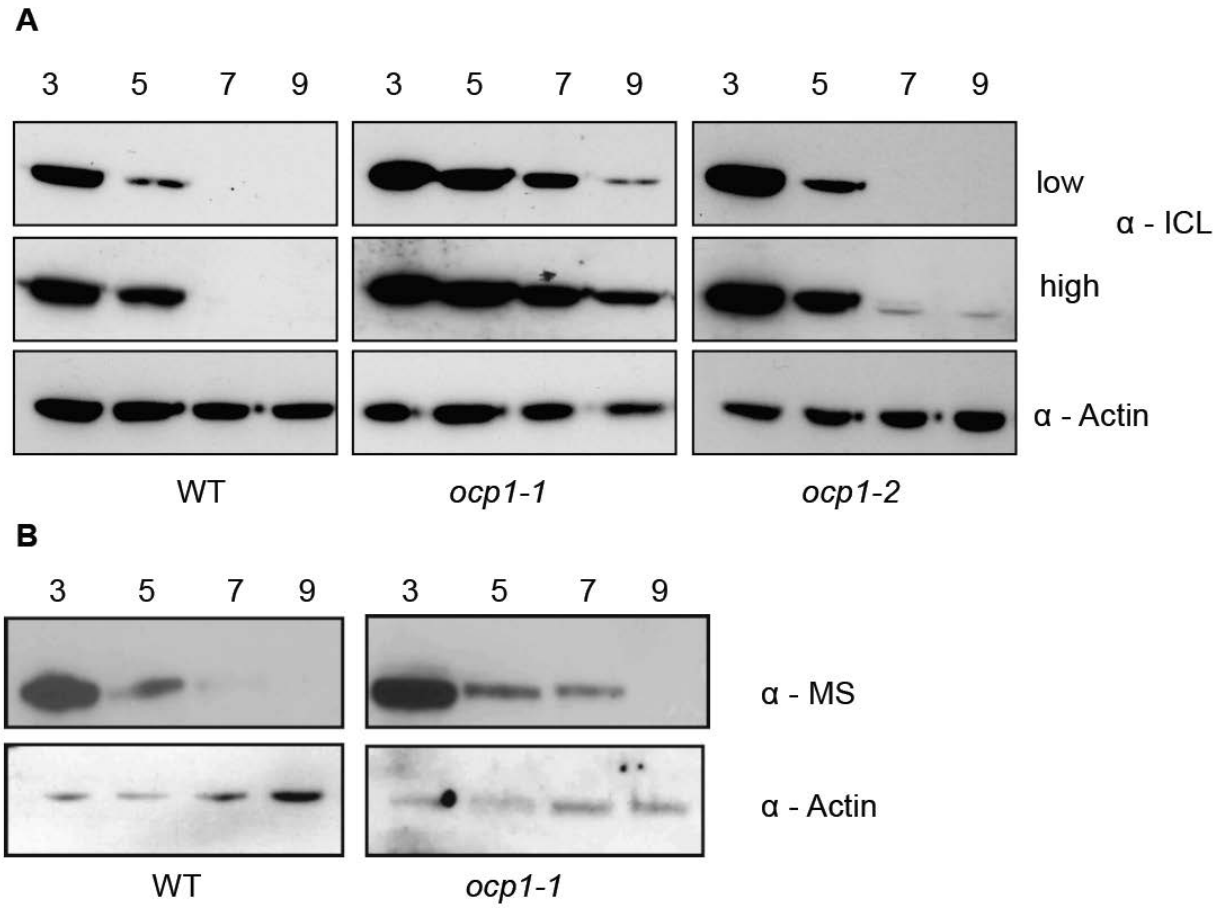

Figure 3.13. *ocp1* has a role in timely degradation of ICL and MS.

(A) Immunoblots of total protein extracted from wildt-ype (WT), *ocp1-1* and *ocp1-2* seedlings probed with α-ICL (top panel). Low and high refer to the time that the blots were exposed for. Numbers on top of the panels refer to the age of the seedlings. Lower panel shows membranes stripped and re-probed with α-actin antibody to show protein loading. Position of molecular weight markers is shown on left in kDs.

(B) Proteins extracted from WT and *ocp1-1* seedlings were probed with α-MS antibody. α-actin blots show protein loading control. Position of molecular weight markers is shown on left in kDs.

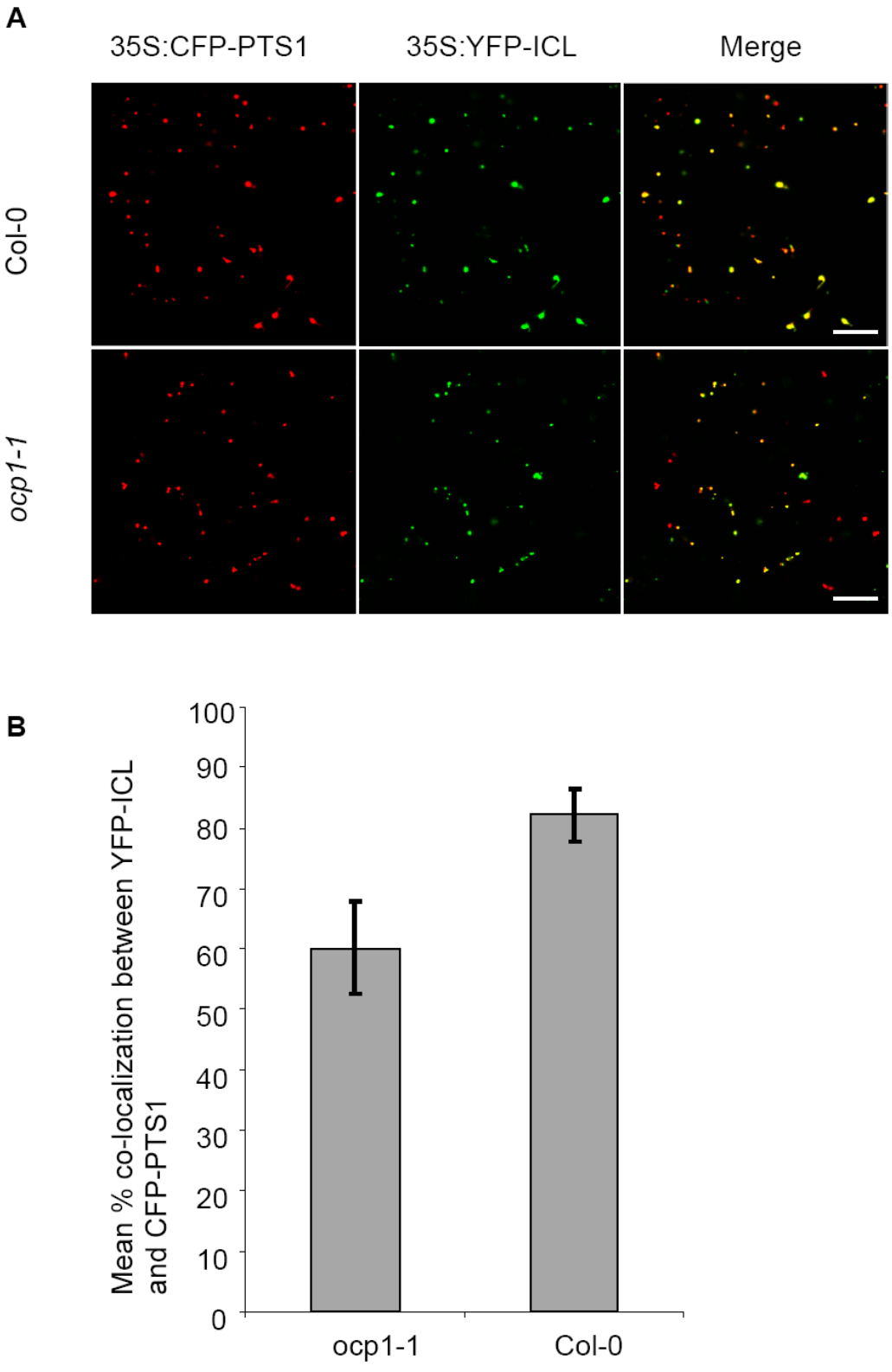

Figure 3.14. ICL is not mislocalized in *ocp1-1*.

Figure 3.14.(Cont'd)

(A) Confocal images from transgenic lines (T1) coexpressing peroxisomal marker (CFP-PTS1) (red) and YFP tagged ICL (green) in the wildtype (top panel) and *ocp1-1* mutant (bottom panel) backgrounds. Scale bar is 10 µm.

(B) Quanitification of overlapping YFP-ICL and CFP-PTS1 in *ocp1-1* and Col-0 genetic backgrounds. An area of epidermal leaf spanning 71.4µM X 71.4 µM was used to quantify percent co-localization. Error bars are SE (n=6).

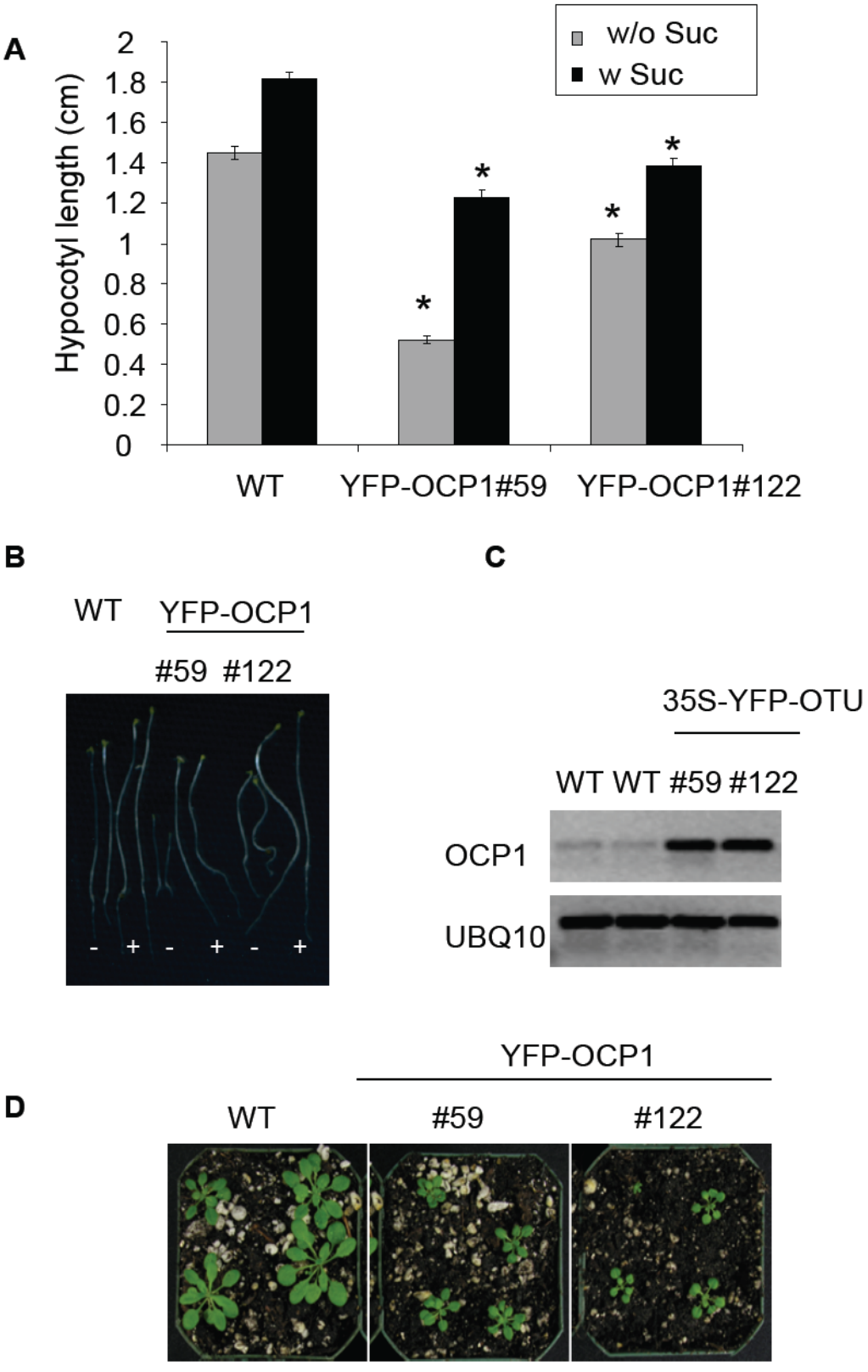

Figure 3.15. 35S:YFP-OCP1 seedlings are sucrose dependent.

Figure 3.15.(Cont'd)

(A) Hypocotyl lengths of 7 day old etiolated wild-type (YFP-PTS1) and YFP-OCP1 seedlings grown in the presence or absence of sucrose. Error bars are SE (n> 25). * indicate statistically

significant differences (Student's *t*-test, p<0.001) in comparisons between transgenic lines and wildtype. 59 and 122 are independent YFP-OCP1 transgenic lines.

(B) Images of WT and YFP-OCP1 seedlings grown in presence (+) or absence (-) of sucrose.

(C) RT-PCR showing levels of OCP1 mRNA in WT and YFP-OCP1 transgenic lines. UBQ10 was used as a loading control.

(D) Plant morphologies of 3 weeks old WT and YFP-OCP1 transgenic lines. Images were taken under the same magnification.

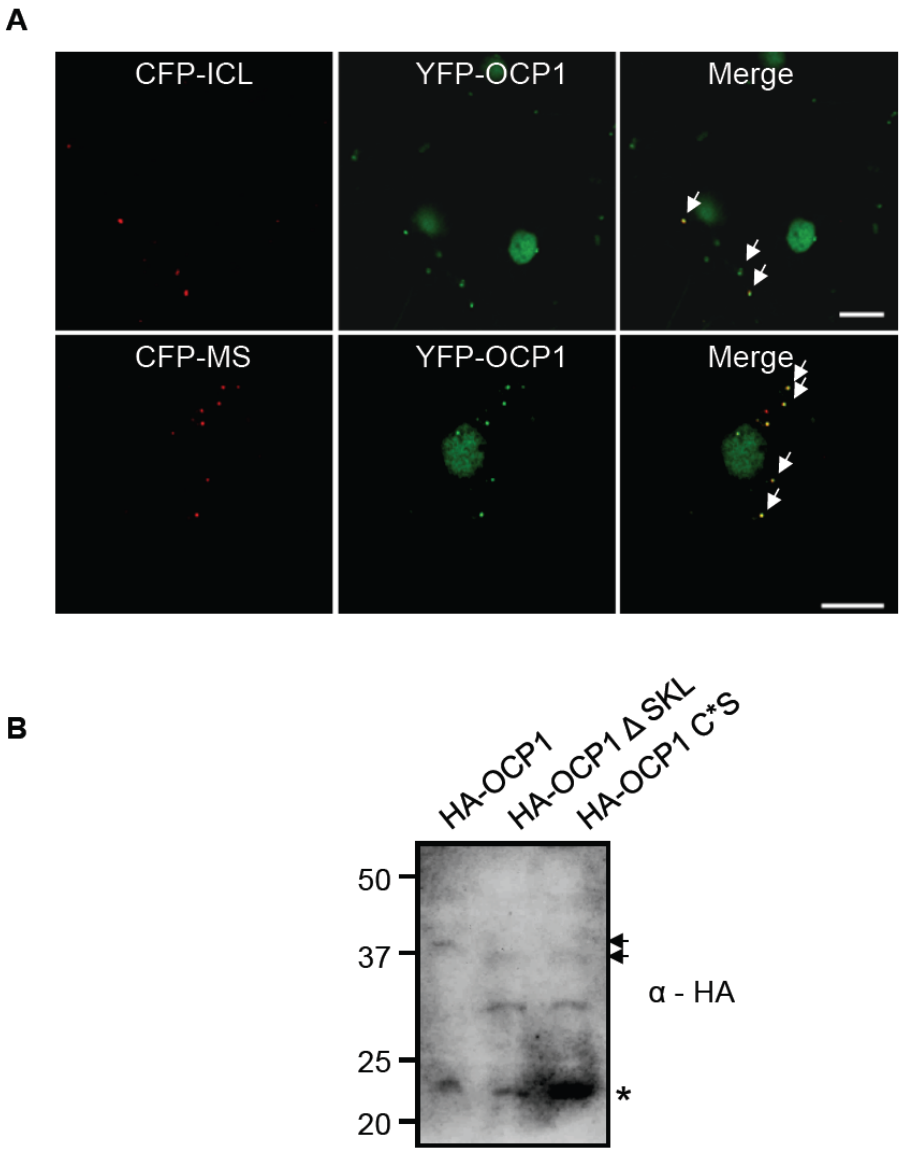

Figure 3.16. OCP1 does not directly degrade ICL/MS in transient assays but is processed itself.

(A) Confocal images from transient co-expression of YFP-OCP1 (green) and CFP-ICL (red) (top panel) or CFP-MS (red) (lower panel) in tobacco epidermal cells. Scale bar is 10 μm.

(B) Transient expression of HA-fusion OCP1 proteins was detected with HA antibody. Arrowheads indicate positions of full-length protein and * denotes a processed form

Table 3.1 Primers used in this research

Primer	Sequence 5'-3'
OCP1G5	GCCGGTACCGGTACCATGATGATTTGTTAC
OCP1G3	TCCGTCGACCTAAAGTTTAGATTTTGG
OCP1G3b	AGAGTCGACGTCGACCTATTTTGGAATCGA
POCP15	CGGAATTCAGAGTCCTTTGTGTTGTTTG
POCP13	CCGAGCTCATCTTTTATAATAAGAG
GOCP15	GTACCGGTACCATGATGATTTGTTAC
GOCP13	CTAGATCTAGAAAGTTTAGATTTTGGAATC
GOCP3B	TCCTCTAGATCTAGAAGCCACAACGCCGTA
OCP1D5	CAACATGTTCTGGCTCTCGCCTC
OCP1D3	GAGGCGAGAGCCAGAACATGTTG
MOCP15	GGCAGATCTTTGTTTC
MOCP13	ACAAAGATCTGCCATC
EOCP15	CCGAATTCATGATGATTT GTTACTC
SOCP13	TCCGTCGACCTAAAGTTTAGATTTTGG
OCPg5	AAAAAAGCAGGCTTCATGATGATTT GTTACTC
OCPg3	AAGAAAGCTGGGTCTAAAGTTTAGATTTTGG
ICL5	AAAAAAGCAGGCTTCATGGCTGCATCTTTCTC
ICL3	AAGAAAGCTGGGTCTTACATTCTTGACTTGGCGAC
MLS5	AAAAAAGCAGGCTTCATGGAGCTCGAGACCTC
MLS3	AAGAAAGCTGGGTCTCAGAGCCTTGAGACATTG
ORT5	ATGATGATTTGTTACTCTCC
ORT3	CTAAAGTTTAGATTTTGGAATCGAAGC
UBQ10F	TCAATTCTCTCTACCGTGATCAAGATGCA
UBQ10R	GGTGTCAGAACTCTCCACCTCAAGAGTA
O76L	CTTAGGAGCAACAGTTGGTC
O76R	TGGGCCCATAAAAGAAGTACC
O84L	GTACTAACGGCAAGCTGTTCG
O84R	AGAATCTGCGTACGCTGATTG
DEG15R	TGAGGAAGCATGCTTTTCATC
DEG15L	ATGGAACCAGTTGATACGCTG

Methods

Sequence alignment

OTU domain sequences were retrieved through iterative BLAST searches from National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/protein). OCP1 homologs were aligned with ClustalW2 and shaded with ESPript software (Gouet et al., 1999; Larkin et al., 2007).

Plant material, Growth conditions and Transformation

Arabidopsis seeds were sowed on ½ LS (Caisson Labs) media containing 0.5% (w/v) Suc solidified with 0.6% (w/v) agar and grown at 22°C under constant illumination (75µE m^{-2} s^{-1}). Homozygous T-DNA insertion mutants, *ocp1-1* (SALK_147876) and *ocp1-2* (SALK_048627) were identified by PCR based screening of genomic DNA, using gene specific and T-DNA primer, respectively. Homozygous mutants were transformed *via* floral dip with *Agrobacterium* containing plasmids encoding for CFP appended with either a C-terminal PTS1 (Ser-Lys-Leu) or an N-terminal PTS2 peptide (1-40 aa of 3-ketoacyl-CoA thiolase 2) to visualize matrix protein import. Transformants were screened on ½ LS agar media containing 0.5% (w/v) Suc and gentamicin (60µg/mL). The transformants were screened for presence of CFP fluorescence microscopically (Axio Imager.M1; Carl Zeiss) and transferred to soil for propagation.

Transgenic plants were generated by floral-dip transformation of either Col-0 or plants expressing peroxisomal marker (DsRed2-PTS1, CFP-PTS1) with *Agrobacterium tumefaciens* harboring constructs of interest (described below). Transgenic plants containing were screened on ½ LS agar media supplemented with 0.5% (w/v) Suc and

kanamycin (50µg/mL). Antibiotic resistant plants were further subject to microscopic observation (Axio Imager.M1; Carl Zeiss) to verify expression of fluorescent fusion proteins. Progeny of T_1 individual plants were re-screened with kanamycin in subsequent generations to identify homozygous lines (T_4) based on observed segregation ratio of the transgene.

Nicotiana tabacum plants were grown under 14h light (50 m^{-2} s^{-1}) at 24°C. *A. tumefaciens* carrying plasmid of interest were grown overnight in a 28°C incubator/shaker, centrifuged and diluted to an A_{600} of 0.1 with distilled water. Cultures were either used alone or in combination with CFP-PTS1 containing *A. tumefaciens* to observe colocalization with peroxisomes. Bacterial suspension was infiltrated into the abaxial surface of a fully expanded Tobacco leaf using 1ml needleless syringe. Plants were maintained under the same growth conditions for a period of 2 days and then subject to confocal microscopy.

Cloning, plasmid construction and transgenic plants

To clone 35S-GFP-OCP1, the coding region of *OCP1* was amplified from cDNA reverse transcribed from Col-0 seedling mRNA using OCP1G5 and OCP1G3. The amplified fragment was digested and cloned into KpnI and SalI sites of pCHF3GFP binary vector. OCP1ΔSKL was amplified with OCP1G5 and OCP1G3b and cloned into KpnI and SalI sites of pCHF3GFP binary vector to generate 35S-GFP-OCP1ΔSKL construct. The pCHF3-GFP-OCP1 construct was digested with EcorI and SacI to remove the 35S promoter and the resulting promoterless plasmid purified from agarose gel. The native

OCP1 promoter, 1kb region upstream of *OCP1* start codon was PCR amplified from Col-0 genomic DNA with pOCP15 and POCP13 and ligated into the EcorI and SacI sites of GFP-OCP1 promoterless plasmid to generate the pOCP1-GFP-OCP1 construct. 35S-OCP1-GFP construct was generated by amplifying the coding region of *OCP1* using GOCP15 and GOCP13. The resulting PCR fragment was cloned into KpnI and XbaI sites at amino terminus of GFP in binary vector pCHF3.

A 90-nucleotide fragment encoding the 1^{st} 30 aa of OCP1 was amplified using GOCP15 and GOCP3B cloned into KpnI and XbaI sites at amino terminus of GFP in binary vector pCHF3 to generate the PTS2 (OCP1)-GFP construct. Primers with inbuilt overlapping nucleotides OCP1D5 and OCP1D3 were used in combination with GOCP15 and GOCP13 to amplify *OCP1* lacking the PTS2 region. This product was cloned into KpnI and XbaI sites at amino terminus of GFP in binary vector pCHF3 to generate the OCP1ΔPTS2-GFP clone. Template for catalytic mutant was generated by using mutagenic primers and nested PCR. To generate MBP-tagged proteins, OCP1 or OCP1C*S ORF was cloned into the EcoRI and SalI sites of the pMAL-c4x expression vector (New England Biolabs, USA).

Clones for YFP-OCP1, HA-OCP1, HA-OCP1ΔSKL, HA-OCP1C*S, YFP-ICL, CFP-ICL, CFP-MS were generated by Gateway® recombination cloning technology as per manufacture's instructions. ICL and MS were amplified from cDNA generated from RNA extracted from 3-day old Arabidopsis seedlings using Phusion High-Fidelity DNA polymerase (New England Biolabs). Entry clones were created via BP-recombination between PCR amplicons and the pDONR207 vector. Entry plasmids were mobilized into different destination vectors using site-specific recombination catalyzed by LR

clonase. Listed OCP1 templates in BP plasmids were cloned into pEARLEY201 to create HA-fusion proteins. OCP1 and ICL were cloned in pDest-35S-6xHis-YFP and pEarleyGate101 to generate YFP-OCP1 and YFP-ICL respectively (Earley et al., 2006; Reumann et al., 2009). pGWB445 was the destination vector for expression of CFP-ICL and CFP-MS respectively (Nakagawa et al., 2007). All primers used in this study are listed in Table 3.1.

Confocal Microscopy

An inverted Zeiss LSM 510 Meta confocal microscope was used to capture fluorescent protein images. Small part of plant leaf were cut, mounted in water and observed with 63x oil objective using the following parameters depending on the fluorescent protein being imaged. We used the 488 nm Argon and 543nm HeNe lasers for excitation of GFP and DsRed2 and 505-530 nm and 560-615nm emission filters to acquire GFP and DsRed2 fluorescence images. Transgenic seedlings (GFP-OCP1, GFP-OCP1ΔSKL) were immersed in minipetri-plates containing 10 µg/mL DAPI solution (diluted in phosphate buffer pH 7.2) 1h prior to imaging to enable visualization of nuclei. We used the 405 nm diode and 488 nm Argon laser to excite DAPI and GFP, respectively. Fluorescence emission was detected with the 420-480 nm and 505-530nm bandpass filters, respectively. CFP and YFP were excited using the Argon laser at 458 nm and 514nm respectively and fluorescence was captured using 465-510 and 520-555 filters. All images were obtained from a single focal plane using sequential scanning to avoid spectral bleed-through.

Physiological Assays

Sucrose dependence assays were carried out by plating surface sterilized seeds of indicated genotypes on ½ MS (Caisson Labs), 0.6% (w/v) phytagar plates containing no exogenous Suc or supplemented with 1% (w/v) Suc. Plates were covered, kept at 4°C for two days, followed by seven days of growth in dark in a growth chamber (Percival) maintained at 22°C . Plates were scanned and hypocotyl lengths were measured with ImageJ software (http://rsb.info.nih.gov/ij/).

For IBA root inhibition assays, seeds were sown on ½ MS (Caisson Labs) growth medium solidified with 0.6% phytagar was supplemented with 0.5%(w/v) Suc and various concentrations of IBA (Sigma-Aldrich ; 0,10,15,20 µM) or 120nM IAA. Plates were stratified for 2 days and shifted to a growth chamber (Percival) with continuous light at 22°C, where plates were arranged vertically, covered with mesh and scanned after seven days. Primary root lengths were measured with ImageJ.

The effect of ABA on germination was determined according to (Fujii et al., 2007). Seeds were sown on ½ LS (Caisson Labs) media plates containing 0.5% Suc, 0.6% phytagar and ABA (Sigma-Aldrich) at 0, 0.6, 2, 5 and 10 µM. Plates were incubated in dark at 4°C for 4 days followed by 6 days growth under continuous light at 22°C. Radicle emergence was scored to assess germination efficiency.

Pollen viability and germination assays

Pollen viability assay was performed using the simplified Alexander staining method described in (Peterson et al., 2010). Briefly, anthers were removed from open flowers of

Col-0, *ocp1-1* and *ocp1-2* plants and fixed in Carnoys fixative (6 ethanol: 3 chloroform: 1 glacial acetic acid) for at least two hours. Post-fixation, anthers were placed on a glass slide and a few drops of Alexanders stain (10ml 95% ethanol, 25ml glycerol, 1ml Malachite green (1% solution in 95% ethanol), 5ml Acid fuchsin (1% solution in water), 0.5ml Orange G (1% solution in water), 4ml glacial acetic acid, 54.5ml distilled water) were added. The slide was then heated over a burner in the fume hood until the stain started boiling. A few drops of 25% glycerol were added and coverslip placed in the slide followed by light tapping to release pollen from stained anthers. Slides were imaged with a Zeiss Axio Imager.M1 microscope (Carl Zeiss).

In-vitro pollen germination was performed according to (Hicks et al., 2004). For each genotype (Col-0, *ocp1-1*, *ocp1-2*) flowering plants were kept at room temperature for 2h to synchronize pollen germination. Anthers were picked from open flowers and brushed on glass slides containing 30µl of pollen germination media (18% Sucrose, 0.01% Boric acid, 1mM $CaCl_2$, 1mM Ca $(NO3)_2$, 1mM $MgSO_4$). The slides were then inverted (hanging drop technique) and kept in dark and high humidity conditions (tip box containing prewet kimwipes) overnight. Tubes of germinated pollen grains were visualized with a Zeiss Axio Imager.M1 microscope (Carl Zeiss).

Immunoblot analysis

3, 5, 7 and 9 day old seedlings (twenty each) were collected from Col-0, *ocp1-1* and *ocp1-2* plants and ground in liquid N_2 in 100µl of extraction buffer (50 mM Tris-HCL pH 8., 2% SDS, 2.5% glycerol, 0.1 mM EDTA pH 8.0, and 1X protease inhibitor cocktail (Roche)). Samples were centrifuged to remove cell debris and heated for 10 min at

70°C and centrifuged at 13,000g for 10 min. 5 µl of 4X SDS-PAGE sample buffer was added to 15 µl of the supernatant and samples were briefly boiled, separated on 10% SDS-PAGE and transferred to PVDF membrane. Membranes were blocked with 5% non-fat milk in 1X TBST buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween 20) for 1h at room temperature. Membranes were incubated overnight with either ICL (rabbit, 1:5,000) , MS (rabbit, 1:5000), PEX5 (1:100), PEX7 (1:800), KAT2 (1:3000) or Actin (mouse monoclonal, 1: 5,000; Sigma-aldrich) antibodies. Blots were washed with 1XTBST four times and incubated with HRP conjugated goat anti-rabbit (1:20,000; Thermo Scientific) or anti-mouse (1:10,000) secondary antibodies for 1 h at room temperature. Blots were subject to 3 washes with 1XTBST and developed using SuperSignal® West Dura Extended Duration Substrate (Pierce Biotechnology).

Protein expression and Purification

MBP-OCP1 or MBP-OCP1C*S were expressed in *Escherichia coli* Rosetta-gami™ 2(DE3) Competent Cells (Novagen) at 18°C for 22 hours following induction with 100µm IPTG (isopropyl *β*-D-thiogalactoside) at an A_{600} of 0.6. Cell cultures were harvested by centrifugation and resuspended in 25ml lysis buffer (50 mM Tris-HCl, pH 8.0, 25 mM EDTA, 10 mM β-mercaptoethanol) and subjected to six cycles of sonication, with each cycle comprising 30 sec of sonication followed by 1 min rest. Cell lysate was clarified with centrifugation (15,000g at 4°C for 15 min) and incubated with amylose resin (New England Biolabs) for 1h at 4°C with gentle agitation. The resin was washed

three time with ice cold column buffer (200 mM NaCl, 20 mM Tris-HCl pH 7.4, 1 mM EDTA,1 mM DTT). Protein was eluted with column buffer supplemented with 10mM maltose. Eluted protein was concentrated using amicon ultra centrifugation filter (EMD Millipore). Protein purity was evaluated by running a 10% SDS-PAGE and staining with simply blue safe stain (Invitrogen).

Proteins from Col-0 and *ocp1-1* seeds and flowers were extracted by grinding tissue in liquid N_2 using a mortar and pestle. Pulverized tissue was transferred to a micro centrifuge tube and 400µl extraction buffer (4% SDS, 2% β-mercaptoethanol, 20% glycerol, 100 mM Tris–HCl (pH 8.0)) was added to it, followed by vortexing and boiling for 3 min. Samples were centrifuged at 13,000g for 10 min at room temperature. Supernatant were transferred to a 2ml microcentrifuge tube and proteins were precipitated by adding 4 volumes of ice-cold acetone. Tubes were kept at -20°C for 1h and then centrifuged 13,000 g for 10 min at 4°C. Supernatant was decanted and pellet resuspended in 100µl of 2X SDS-PAGE gel loading buffer.

Ubiquitin chain hydrolysis assay

300ng of purified MBP-OCP1 was incubated with 500ng of K48 linked tetraubiquitin (Boston Biochem) in reaction buffer (50 mM Tris pH 7.5, 5mM $MgCl_2$, 5mM DTT) For 0, 30, 60 and120 minutes in 37°C waterbath. Reaction was terminated by adding SDS-PAGE buffer. Samples were run on 12% SDS-PAGE and transferred to nitrocellulose membrane (Whatman). Membrane was autoclaved for 10' prior to blocking in 5% non-fat dry milk in TBST (50mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20).

Ubiquitin levels were assessed by probing the blot with ubiquitin antibody (polyclonal rabbit, 1:100, Sigma-aldrich). HRP-conjugated secondary antibody (goat anti-rabbit IgG 1:10,000, Millipore) and SuperSignal® West Dura Extended Duration Substrate (Pierce Biotechnology) were used to develop the blot.

Z-LRGG-AMC cleavage assay

Fluorogenic substrate Z-LRGG-AMC (Enzo Life Sciences) was used to assay in-vitro deubiquitinase activity. Reactions were initiated by adding 500ng of purified MBP-OCP1 to 100μM of Z-LRGG-AMC in reaction buffer (50 mM Tris pH 7.5, 5mM $MgCl_2$, 5mM DTT) in a quartz cuvette placed in a temperature controlled (37°C) spectrofluorometer (Molecular Devices). The release of AMC fluorescence was monitored over time and detected with 480nm with 340nm excitation using a SpectraMax M2 spectrofluorometer (Molecular Devices). A no protein control was also run in parallel to monitor for non-catalytic AMC release.

Universal protease activity assay

Protease activity assay was performed using casein as a substrate according to instructions on colorimteric protease assays posted on the Sigma website (http://www.sigmaaldrich.com/life-science/learning-center/life-science-video/universal-protease.html). Briefly, 1μg of MBP-OCP1 was incubated with 0.65% solution of casein (5ml) at 37°C for 10 min. A second set (Blank) omitting the protease was run in parallel. Both sets were treated with TCA (5ml) to precipitate the protein. 1μg of MBP-OCP1 was

added to the Blank set after the addition of TCA. Following a 30 min incubation at 37°C solutions from both sets were filtered. Folin's reagent (1ml) and 500 mM Sodium Carbonate Solution (5ml) was added to the filtrate (2ml) and incubated at 37°C for 30 minutes. Absorbance at 660 nm was recorded and value of the Blank sample subtracted from the Absorbance of the protease samples and plotted on a tyrosine standard curve to determine specific activity.

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CHAPTER 4

Conclusions and future perspectives

Overview

Our knowledge of peroxisomal contributions to plant development and physiology has grown tremendously in the last decade. Many aspects of peroxisome biology that include targeting and import of matrix proteins, division and proliferation mechanisms have been extensively studied and have led to the identification of numerous factors involved in these processes (Hu et al., 2012). Data from published peroxisomal proteins coupled with those found through proteomics experiments on purified peroxisomes reveal that Arabidopsis peroxisomes have at least 163 proteins (Kaur and Hu, 2011). However, despite the fact that peroxisomes house so many proteins, we do not know how plant peroxisomes regulate protein abundance or degrade obsolete, damaged or superfluous proteins. The work presented in this thesis was undertaken to identify peroxisomal proteins that mediate degradation.

Arabidopsis RING Peroxins are E3 ubiquitin ligases that interact with two homologous ubiquitin receptor proteins

When this project was initiated, very little was known about the occurrence of ubiquitination or related degradation processes in peroxisomes. Ubiquitination of PEX5 in yeast species was just being established. Although parallels had been drawn between ERAD and components of the peroxisome import machinery, supporting experimental evidence was lacking. Given the reported ubiquitination of PEX5 in yeast and the conservation of protein import apparatus across kingdoms, it seemed logical to presume that similar events occur in plant peroxisomes too. A major impediment to studying this process in plants had been the lethal phenotype associated with mutants

of peroxisome biogenesis genes (Lin et al., 1999; Hu et al., 2002; Schumann et al., 2003; Sparkes et al., 2003; Fan et al., 2005). I used in-vitro assays to determine if RING peroxins have E3 ligase activity. I demonstrated that RING domains of AtPEX2, AtPEX10 and AtPEX12 possess monoubiquitin ligase activity. Additionally, I show that AtPEX2 and AtPEX10 activity is dependent on the presence of Zn ions. Hitherto, E3 ligase activity of RING peroxins had not been shown to occur in any organism apart from the yeast *S.cerevisiae*, we thus conclude that this property of RING peroxins is evolutionarily conserved. Collectively, these findings suggest that plant peroxisomes are functionally equipped with proteins that have the potential to form a membrane-associated quality control system.

In an effort to understand the function of AtPEX2, a yeast two-hybrid screen was conducted. A potential AtPEX2 interactor was found to be an UBA-UBL family protein (DSK2b), whose homologs in other species had been associated with ubiquitin related degradation processes. Till date the functions of UBL-UBA proteins have never been associated with peroxisomes in any capacity. Research undertaken in Chapter 2 was conceived with the idea that DSK2b was a part of the peroxisome associated degradation machinery and investigating its functional role would enable us to dissect the molecular mechanisms underlying this phenomenon in plants. I showed that both DSK2b and its paralog DSK2a interacted specifically with the RING domain of AtPEX2. Moreover, they also interact with another RING peroxin, AtPEX12. Since DSK2s are tandem duplicates, I generated amiRNA lines to study their role in plants and peroxisomes. However, amiRNA lines did not display any gross defects in plant physiology or appreciable differences in peroxisome metabolism or protein import.

Many outstanding questions still remain to be answered, among them- what exactly is the biological relevance of AtPEX2^{RING} interaction with DSK2s? What are the targets for the plant peroxisome-associated degradation system? As there are several UBA-UBL proteins in Arabidopsis, creating higher order mutants may be necessary to determine the roles essayed by DSK2s. We speculate that in concert with RING peroxins, DSK2s may be involved in degradation of AtPEX5 or possibly damaged or obsolete peroxisomal matrix proteins. Comparing levels of AtPEX5 in the *DSK2* amiRNA lines or higher order UBA-UBL mutants would address this possibility.

Data from other model systems has established that PEX5 ubiquitination at the peroxisomal membrane is a major determinant for its export (Platta et al., 2007; Grou et al., 2009). In yeasts, Pex5p is also polyubiquitinated and degraded and it is evident that the mode of PEX5 ubiquitination is an outcome of the action of distinct (sub)sets of proteins including RING peroxins, Pex4p and cytosolic Ubc4p (Kragt et al., 2005; Platta et al., 2007; Williams et al., 2007). Whether, and if, PEX5 is ubiquitinated at all in plants is not yet known. Outlined below are some approaches that may prove fruitful in resolving this question.

PEX5 ubiquitination has been reported to occur on a conserved Cys residue that makes it difficult to detect due to its thiol sensitive nature (Carvalho et al., 2007; Williams et al., 2007). Reports from mammals indicate that substituting Cys to Lys has no impact on receptor function or ubiquitination but enhances the stability of the tagged protein because of the covalent bond formed between Lys and ubiquitin (Grou et al., 2009). Recent studies from mammals also indicate that stable expression of PEX5 with a mutation in its Cys^{11} residue traps the receptor in the peroxisome membrane and has

a deleterious effect on matrix protein import (Grou et al., 2009; Okumoto et al., 2011). This phenotype could be achieved by expressing a C-terminally truncated mutant PEX5 too (Okumoto et al., 2011). A similar strategy could thus be implemented in plants by overexpressing an epitope tagged AtPEX5 carrying Cys-Lys mutation that would facilitate the detection of ubiquitinated AtPEX5 without jeopardizing peroxisome protein import. In parallel, AtPEX5 with Cys-Ala mutation could be expressed under an inducible promoter so as to circumvent the possible dominant negative effects imposed by an export incompetent AtPEX5.

A second line of investigation might benefit by exploiting chemical genetic approaches to identify small molecules that target peroxisome proteins (Stockwell, 2000). The success of this approach has already been demonstrated in a mini-screen conducted in plants to identify molecules affecting peroxisomal protein import (Brown et al., 2011). Moreover, it has been shown that treatment with small molecules rescued the functions of a dysfunctional mammalian PEX1 protein (Zhang et al., 2010). A more comprehensive screen may be advantageous in identifying new chemical(s) that disrupt peroxisome import processes and would be valuable in deciphering the molecular mechanisms underpinning peroxisome protein import processes. Using small molecules to probe the import process would also overcome the challenges posed by the embryo lethal phenotypes of most plant peroxisome import mutants. These screens can be carried out on either peroxisomal reporter line (YFP-PTS1) or a transgenic line expressing CFP tagged AtPEX5 under the control of its own promoter (Tian et al., 2004). Comparing the effects of the chemicals on peroxisome targeting in both these lines would help in differentiating inhibitors or agonists that are specific to AtPEX5 or

other aspects of import or even peroxisome biogenesis. Novel compounds identified in such screens would also have ramifications as therapeutic drugs to alleviate symptoms of patients suffering from peroxisome biogenesis disorders.

Using synthetic biology, the entire ubiquitin cascade has been faithfully reconstituted in bacteria, eliminating the need for in-vitro assays and separate purifications steps for each of the enzymes and targets involved in the reaction (Keren-Kaplan et al., 2012). It may be worthwhile to examine if co-expressing RING peroxins, AtPEX5 and AtPEX4 (the putative peroxisomal E2 enzyme) results in AtPEX5 ubiquitination.

Finally, considering the lethal phenotype of the RING peroxin T-DNA insertion mutants, TILLING (Targeting Induced Local Lesions in Genomes) lines (Till et al., 2003) had previously been screened in the lab in an attempt to find allelic series of mutations in the genes for Arabidopsis RING proteins. However, no lesions were found that conferred any identifiable partial loss-of-function phenotype. Perhaps screening the tetraploid TILLING populations with higher mutation density may be pursued in the future. A systemic analysis of mutations carried by Zellweger syndrome patients in recent study provides an overview of all known lesions in associated peroxisomal gene loci (Ebberink et al., 2012). Although sequence similarities between human and Arabidopsis RING peroxin genes are low, several missense mutations occur on conserved residues. Moreover, all these mutations result in peroxisome defects; at least in a few cases the homozygous patients survived. Expressing Arabidopsis RING peroxin gene variants with corresponding missense mutations in heterozygous mutant lines might help in recovering alleles that display peroxisome phenotypes.

OCP1 is a novel peroxisomal protein that likely regulates the timely removal of the glyoxylate cycle enzymes ICL and MS

My work presented in Chapter 3 shows that OCP1 is a plant specific peroxisomal protein that is targeted to the peroxisomal matrix via two targeting signals, one of them a novel PTS2. I was able to demonstrate that elimination of either sorting signal resulted in loss of peroxisomal localization. Functional characterization of *ocp1* mutants revealed that they are resistant to IBA-mediated root inhibition and exhibit compromised germination efficiency in the presence of ABA. Further, germinating *ocp1* mutant seedlings were found to retain ICL longer than corresponding wild-type seedlings, suggesting that OCP1 had a functional role in governing the degradation of this transiently expressed peroxisomal enzyme. Several questions arise from these findings – some of which, along with directions for future research are tackled in the following paragraphs.

Although RNX_6HL was established as a novel PTS2 peptide, it was observed that C-terminal GFP tagged OCP1 peptide only showed partial co-localization with the DsRed-PTS1 peroxisomal marker. One drawback of transient assays is that they cannot always completely mimic endogenous systems and this may be why we see incomplete co-localization. Import of PTS2 proteins has been documented to be less efficient vis-à-vis PTS1 import (Wiemer et al., 1996; Wang et al., 2008), and could also be a contributing factor in the partial labeling observed. Co-expression of the PTS2-(OCP1)-GFP construct with a PTS2-DsRed marker could be examined to determine if this is the case. A final possibility is that OCP1-GFP spots that do not co-localize with DsRedPTS1 spots

mark OCP1-GFP containing vesicles. Previous work has shown that a mitochondrial membrane protein (MAPL) is enriched in vesicles that bud off from mitochondria and fuse with the peroxisomes (Neuspiel et al., 2008; Braschi et al., 2010). To test this possibility, the FAST technique could be used to co-express OCP1-GFP and MAPL in Arabidopsis, which could be later, checked for co-localization (Li et al., 2009). Further, since PTS2 peptides are difficult to predict, we can now extend our bioinformatics searches to include OCP1's PTS2 sequence to find other potentially (unidentified) peroxisomal proteins.

My assays for enzymatic activity of OCP1 were not successful in detecting either deubiquitinase (DUB) or protease activity. Does OCP1 have proteolytic activity? First, perhaps recombinant expression in a eukaryotic system such as *Pichia pastoris* could be attempted to resolve possible issue with protein folding in *E.coli*. Secondly, DUBs exhibit distinct substrate preferences, some enzymes that hydrolyze Lys48 linked ubiquitin chains lack activity against Lys63 linked chains and vice versa (Mason et al., 2004). Similarly, while some DUBs are active against Z-LRGG-AMC, others prefer Ub-AMC (Dang et al., 1998). Perhaps testing OCP1 activity against a more diverse array of substrates would have been more successful. Yet another possibility is that OCP1 needs a specific partner that facilitates its activation. Activity based probe profiling is a new method to assay proteases *in-vivo* by using biotin coupled small molecules that react with active site residues (van der Hoorn et al., 2011; Richau et al., 2012). This method has been successfully applied even in transient systems and could therefore be worthwhile to pursue in the future (Gu et al., 2012). In pathogenic bacteria, proteins in the YopJ superfamily of effectors had been functionally assigned as SUMO proteases,

and shown to cause commensurate decrease in SUMOylated proteins upon overexpression (Mukherjee et al., 2007; Lewis et al., 2011). However, YopJ proteins were later found to be acetyltransferases that post-translationally modified Ser and Thr residues (Mukherjee et al., 2006). Similarly, it is also possible that OCP1 proteins do not have DUB activity but exert their functions through some other biochemical activities.

Despite numerous attempts, stable lines overexpressing untagged OCP1 could not be identified. If OCP1 undergoes processing and has an N-terminal domain that acts in an inhibitory capacity, it may be interesting to generate plants expressing just the N-terminal region modified with a PTS1 signal or just the protease domain. Expressing an inhibitory domain may render the endogenous OCP1 protein inactive and would be expected to mirror the mutant phenotypes. Protease domain expression may act in a dominant negative manner but may not be conducive for plant viability.

The apparent stabilization of ICL in *ocp1* mutants could be a byproduct of an alteration in peroxisomal H_2O_2 levels. Testing this possibility would entail expressing a genetically encoded H_2O_2 sensor protein, HyPer, in the *ocp1* mutants and monitoring for changes in its' fluorescence ratios (Belousov et al., 2006). The feasibility of such an approach in determining organellar and specifically plant intra-peroxisomal H_2O_2 has already been demonstrated and should prove relatively simple to analyze (Costa et al., 2010; Malinouski et al., 2011).

An alternative possibility is that OCP1 modulates the activity of other peroxisomal proteases that in turn are responsible for degradation of ICL. For example, the papain family proteases undergo autocatalytic cleavage that releases the inhibitory domain of

the enzyme, thus allowing activity. Similarly if some as of yet unidentified peroxisomal protease(s) hydrolyze ICL, their inhibitory domains could conceivably be substrates for OCP1 activity. Mutants for several of the known and predicted peroxisomal proteases have previously been analyzed, but none showed altered abundance of ICL. Proteomics experiments on peroxisomes isolated from seedling peroxisomes have found two novel proteases that could be possible candidates for degrading ICL. Mutants in these two proteases are available in the lab and can be easily tested. Further, these mutants could be crossed to *ocp1* alleles to generate double and triple mutants, which would be useful in studying the relevance of proteases and their contributions to peroxisome metabolism and plant physiology as a whole. It is possible that proteases have overlapping functions; therefore detailed analysis of higher order mutants could help to clarify this possibility.

Given that other mutants reported to have persistent levels of ICL and/or MS are in genes encoding proteins that function in peroxisome matrix protein import, it might be rational to consider that an inability to import the protease responsible for degrading ICL and MS resulted in the observed enzyme stability. Inconsistent with this possibility is the *pex4 pex22* mutant that does not display obvious defects in PTS1 or PTS2 import (Zolman et al., 2005). It is also hard to reconcile this phenotype with the proposal that import of ICL and MS into the peroxisomes is prerequisite for their timely eradication (Lingard et al., 2009). However, if we assess these phenotypes in context of PEX5 functions, a possible explanation comes to mind. Mutants in *pex14*, *pex5* and *pex6* all show stabilized levels of ICL and/or MS but are deficient in importing these enzymes into their peroxisomes (Lingard et al., 2009; Monroe-Augustus et al., 2011). In the

absence of *pex14*, PEX5 would be unable to dock at the peroxisomal membrane, hence import would be affected. Compromising PEX5 would obviously cause deficiencies in peroxisome matrix protein import. Lower levels of PEX5 in the *pex6* mutant have been linked to the mutants' mislocalization of proteins that is rescued upon overexpressing PEX5 (Zolman and Bartel, 2004). The *pex4* mutant on the contrary has elevated amounts of PEX5 that is speculated to be stalled on the peroxisomal membrane (Ratzel et al., 2011). This has been interpreted as emanating from faulty PEX5 recycling or a block in retrotranslocation of matrix proteins for degradation (Ratzel et al., 2011). I speculate that although *pex4 pex22* mutants import matrix proteins, due to the reduced population of free PEX5, they do so less efficiently or with slower kinetics. Hence, the translated pool of cytosolic ICL/MS may be contributing to the observed stabilization in this mutant. In support of this argument is the observation that a PTS2 enzyme, KAT2, also appears to be stabilized in the *pex4 pex22* mutant (Lingard et al., 2009). On the other hand, proteases in general are inherently unstable and may breakdown in the cytosol especially if, as I propose, import is not abolished but delayed in the *pex4 pex22* mutant. These possibilities can be addressed by (i) testing the localization of peroxisomal proteases in the peroxin mutants, (ii) overexpressing candidate proteases in *pex4 pex22* mutant to see if they rescue the stabilized ICL phenotype, and (iii) assessing levels of a range of PTS1 and PTS2 proteins in the cytosolic and peroxisomal fractions of *pex4 pex22* and wild-type plants.

I do not dismiss the importance of environmental and developmental cues in determining the degradation of ICL and MS in Arabidopsis. Studies done on *Brassica napus* using transcriptional run on assays suggest that ICL is transcriptionally regulated

and actively transcribed post-embryogeny and 2 days after imbibition (Comai et al., 1989). Likewise in Arabidopsis, *ICL* mRNA can be detected only for a limited number of days past germination (Lingard et al., 2009). However, to distinguish between active transcription and a stored mRNA pool present in the seed, it may be more informative to conduct a time course analysis on germinating Arabidopsis seedlings. Treatment with transcriptional inhibitors such as α-amanatin and performing qRT-PCR may help distinguish between these two possibilities. Further, it would be informative to subject *ocp1* mutants to similar analysis.

In some plants like spinach, ICL (and MS) are present in peroxisomes isolated from leaves and do not seem to undergo proteolysis (Babujee et al., 2010). Therefore, it would be interesting to examine if peroxisomal OCP1 (or other peroxisomal protease) homologs exist in spinach.

Summary

Prior to this body of work our knowledge of proteolysis in plant peroxisomes was cursory. I provide evidence that RING domains of three peroxisomal membrane proteins AtPEX2, AtPEX10 and AtPEX12 have E3 ligase activity. Along with the identified interactor proteins, RING peroxins have the potential to function as a peroxisome quality control system. My work on OCP1 hints at a possible post-translational regulation of ICL and MS in Arabidopsis seedlings. Further analysis of OCP1 and other peroxisomal proteases through generation of higher order mutants would be instrumental to clarifying the interplay of proteases within the peroxisomes and elucidate the mechanisms involved in turnover of proteins in the peroxisomes. Future studies based

on my work will be important in clarifying the role of peroxisome associated proteolysis on plant physiological processes such as seed germination and lipid mobilization and may also shed light on the etiology of mammalian peroxisomal disorders.

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