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The *Hansenula polymorpha PDD7* gene is essential for macropexophagy and microautophagy

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Abstract

Hansenula polymorpha PDD genes are involved in the selective degradation of peroxisomes via macropexophagy. We have isolated various novel *pdd* mutants by a gene-tagging method. Here we describe the isolation and characterisation of *PDD7*, which encodes a protein with high sequence similarity (40% identity) to *Saccharomyces cerevisiae* Apg1p/Aut3p, previously described to be involved in random autophagy and the cytoplasm-to-vacuole targeting pathway. Our data indicate that HpPdd7p is essential for two processes that degrade peroxisomes, namely the highly selective process of macropexophagy and microautophagy, which occurs in *H. polymorpha* upon nitrogen starvation.

Keywords: Autophagy; Methylotrophic yeast; Peroxisome; Pexophagy; Selective peroxisome degradation

1. Introduction

Yeast cells adapt to changing environmental conditions by regulating the levels of proteins and organelles in response to metabolic needs. This phenomenon continuously occurs during peroxisome homeostasis in methylotrophic yeasts. In these organisms, numerous peroxisomes proliferate when methanol is used as sole carbon and energy source. Under these conditions, peroxisomes harbour the key enzymes required for methylotrophic growth. However, when methanol-grown cells are shifted to glucose or ethanol, the organelles are rapidly degraded by a highly selective process [1–4] referred to as pexophagy [5]. Morphologically, pexophagy occurs via distinct pathways, depending on the yeast species and the carbon source used to induce the degradation process. When glucose or ethanol is supplemented to methanol-grown *Hansenula polymorpha* cells, peroxisomes are specifically and individually sequestered by multiple membrane layers and subsequently transferred to the vacuole for degradation [1,2], a process that has been designated macropexophagy. In contrast, in the related yeast *Pichia pastoris*, two mechanisms for selective peroxisome degradation have been observed. When methanol-grown *P. pastoris* cells are shifted to ethanol, peroxisomes are degraded by macropexophagy, similarly as described for *H. polymorpha* [4]. However, when the cells are shifted to glucose-excess conditions, peroxisome clusters are engulfed by the vacuole and degraded, a process designated micropexophagy [3,4,6].

Recently we have reported that in *H. polymorpha*, when methanol-grown cells were subjected to nitrogen starvation, peroxisomes and other cytoplasmic material were degraded via a process called microautophagy [7]. In this case, peroxisome clusters are taken up by the vacuolar membrane and degraded. Also in *Saccharomyces cerevisiae*, specific peroxisome degradation has been observed when cells were shifted from peroxisome-inducing conditions (growth on oleate), to conditions where these organelles have become redundant for growth [8,9]. It remained unresolved whether in this case the peroxisome degradation occurs via macro- or micropexophagy. Hutchins et al. [9] also studied the effect of mutations in *APG/AUT* genes, required for non-specific autophagy of cytosol and organelles, on peroxisome degradation and observed that this process required the function of many of these genes. Recently, it was observed in *S. cerevisiae* that two morphologically comparable processes, autophagy (Apg/Aut) and

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cytoplasm-to-vacuole transport (Cvt), require overlapping sets of genes. This suggests that many pathways to the vacuole utilise in part the same machinery (reviewed in [10,11]).

To gain insight in the molecular mechanisms of selective peroxisome degradation in *H. polymorpha*, we have isolated mutants affected in macropexophagy (designated *pdd* mutants) by chemical mutagenesis [12] and gene-tagging [13]. Here we describe the isolation of the *pdd7* mutant and the characterisation of the corresponding gene. *PDD7* encodes the homologue of *S. cerevisiae* Apg1p, a serine-threonine kinase essential for the Apg/Aut and Cvt pathways [14,15]. *HpPDD7* appeared to be essential for both macropexophagy and microautophagy, two morphologically distinct processes.

2. Materials and methods

2.1. Micro-organisms and growth conditions

All *H. polymorpha* strains used in this study are derivatives of NCYC495 [16]. *H. polymorpha* strains were grown in batch cultures on mineral medium [17] using glucose (0.5% w/v), ethanol (0.5% v/v) or methanol (0.5% v/v) as carbon sources or on rich medium (YPD) containing 1% (w/v) yeast extract, 1% (w/v) peptone and 1% (w/v) glucose. Transformants were selected on YND plates containing 0.67% yeast nitrogen base without amino acids (Difco), 1% glucose and 1.5% agar. Leucine was added as required (final concentration 30 mg l^{-1}).

Escherichia coli DH5α [18] was used for plasmid constructions and was cultured on LB medium supplemented with the appropriate antibiotics.

2.2. DNA procedures

Standard DNA techniques were carried out essentially according to [18]. Transformation of *H. polymorpha* was performed as described previously [19]. Southern blot analysis was performed using the ECL direct nucleic acid labelling and detection system (Amersham Corp., Arlington Heights, IL, USA). DNA sequencing reactions were performed at BaseClear (Leiden, The Netherlands) on a LiCor automated DNA sequencer using dye primer chemistry (LiCor Inc., Lincoln, NE, USA). Oligonucleotide primers were obtained from Invitrogen Life Technologies (Merelbeke, Belgium). The TBLASTN algorithm [20] was used to screen databases at the National Centre for Biotechnology Information (Bethesda, MD, USA). Protein sequences were aligned using the CLUSTAL_X programme [21].

2.3. Biochemical methods

Macropexophagy experiments with methanol-grown *H. polymorpha* cells were performed essentially as described [12]. To analyse peroxisome degradation in *H. polymorpha* cells under nitrogen starvation conditions, we followed the procedures described in [7]. Total cell extracts of *H. polymorpha* cells were prepared using the trichloroacetic acid (TCA) method [22]. Alcohol oxidase (AO) activity was assayed as described before [23] using crude *H. polymorpha* cell extracts [24]. Determination of protein concentrations, SDS–PAGE and Western blotting were performed by established procedures.

2.4. Mutagenesis and screening of pdd mutants

Mutagenesis of *H. polymorpha* cells was performed by Random Integration of Linear DNA Fragments (RALF) [13]. *H. polymorpha* mutants affected in selective peroxisome degradation (*pdd* mutants) were selected using the AO activity plate assay as described before [12].

2.5. Isolation of PDD7

After singling out the *pdd7-F31* mutant, the integrated pREMI-Z plasmid [13] and its flanking genomic regions were rescued in *E. coli* by digesting the genomic DNA with *Eco*RI followed by self-ligation. Subsequently, the pREMI-Z flanking regions were sequenced using vector-based primers [13]. Based on the retrieved sequences, two oligonucleotide primers (PDD7-1: 5′-CTC AAG ACT TCA CCT CCT GC-3′ and PDD7-2: 5′-GCA ATA GAC ATA GCT TTG GC-3′) were designed that should produce a 950-bp fragment by PCR when *H. polymorpha* DNA containing the authentic *PDD7* gene is used as template (Fig. 1). These primers were utilised to screen pools containing 1000 clones of a *H. polymorpha* gene library in pYT3 [25] for clones containing *PDD7*. After four successive rounds of screening, a single plasmid was isolated that produced the expected PCR fragment. It had an insert of 6.5 kb, and was designated pYT3-PDD7-1. Subsequently, a subclone, designated pBS-PDD7-1, containing the entire *PDD7* gene was sequenced. The nucleotide sequence of *PDD7* was submitted to GenBank and was assigned the accession number AY053423.

2.6. Construction of a HpPDD7 deletion strain

A Δ*pdd7* strain was constructed by replacing the region of *PDD7* comprising nucleotides 589 through 2083 (see Fig. 1) by an auxotrophic marker. To this end, pBS-PDD7-1 was digested with *Mlu*I, blunted by Klenow treatment and partially digested with *Asp*718. Subsequently, the 5.3-kb vector fragment was ligated to a 2.0-kb *Cla*I (blunted)–*Asp*718 fragment containing the *H. polymorpha URA3* gene [26]. From the resulting plasmid, designated pBS-PDD7-URA, a 3.0-kb *Sal*I–*Nde*I fragment was used to transform *H. polymorpha* NCYC495 (*leu1.1 ura3*) cells. Uracil prototrophic transformants were analysed using the

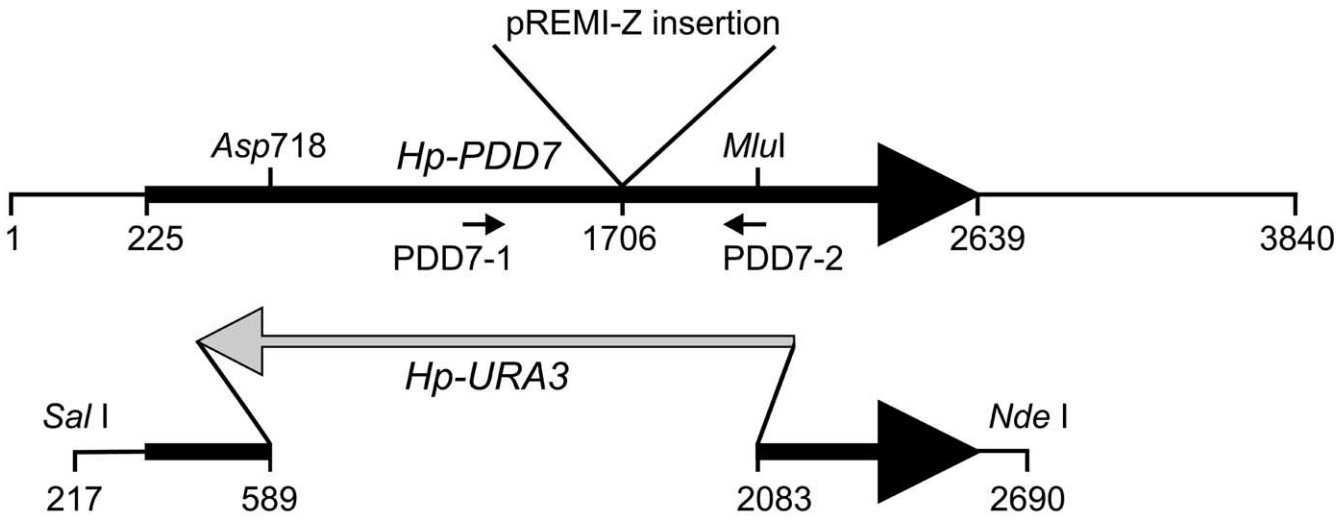

Fig. 1. Schematic representation of the genomic region comprising *HpPDD7* and disruption of *PDD7* by homologous recombination using a *Sal*I–*Nde*I fragment of pBS-PDD7-URA (see Section 2.6). The integration site of pREMI-Z in the *pdd7-F31* mutant, and the location of the primers used for isolation of the *PDD7* gene are also indicated. Only relevant restriction sites are shown. The indicated numbers correspond to nucleotide positions in GenBank accession number AY053423.

AO activity plate assay [12]. Proper integration of the disruption cassette was confirmed by Southern blot analysis (data not shown).

2.7. Electron microscopy

Cells were fixed and prepared for electron microscopy as described previously [24]. Immunolabelling was performed on ultra-thin sections of Unicryl-embedded cells, using specific antibodies against *H. polymorpha* AO and gold-conjugated goat anti-rabbit antibodies [24].

3. Results

3.1. Isolation of H. polymorpha PDD7

H. polymorpha pdd mutants fail to degrade peroxisomes after a shift of cells from methanol- to glucose-containing media. This process can readily be monitored by the fate of AO, one of the key peroxisomal matrix enzymes required for growth on methanol. To screen for novel *pdd* mutants, a series of 450 mutants obtained by gene-tagging using linearised pREMI-Z plasmid [13] was screened by an AO activity plate assay [12]. Subsequently, putative *pdd* mutants were biochemically characterised by specific AO activity measurements and Western blotting of crude extracts, prepared from these cells. This resulted in the isolation of three mutants that were impaired in glucose-induced peroxisome degradation, including mutant *pdd7-F31* (data not shown). To further confirm the Pdd phenotype of this mutant, methanol-grown *pdd7-F31* cells were shifted to glucose and analysed by electron microscopy. Four hours after the shift of mutant cells to glucose, peroxisomes were still present, whereas in WT controls, abundant specific peroxisome degradation had occurred within the same time interval (data not shown).

Subsequently, the integrated pREMI-Z plasmid including the flanking genomic regions was isolated from *pdd7-F31*. Initial sequence analysis indicated that the plasmid had integrated in a gene that was highly similar to *S. cerevisiae APG1* [14,15], which is essential for nitrogen limitation-induced autophagy. Subsequently, using a PCR screening approach and a *H. polymorpha* genomic library, we isolated a plasmid containing the complete gene that was disrupted by pREMI-Z in *pdd7-F31*. Sequence analysis of a 3.8-kb fragment revealed one large open reading frame, which was designated *PDD7*. The sequence of *PDD7* was submitted to GenBank (no. AY053423). *H. polymorpha* Pdd7p has a predicted M_r of 90 kDa and is 40% identical to *S. cerevisiae* Apg1p (Fig. 2). Highest sequence similarity (59% identity) was observed in the region comprising the serine-threonine kinase domain (amino acids 22–322 in Sc-Apg1p). The integration site of pREMI-Z in *pdd7-F31* was identified to be between nucleotides 1706 and 1707 (corresponding to codons 494 and 495) (Fig. 1).

3.2. Δpdd7 is affected in macropexophagy

We constructed a Δ*pdd7* strain by replacing the region of *PDD7* encoding amino acids 122–620 by the *H. polymorpha URA3* gene (Fig. 1). Like the *pdd7-F31* strain, the Δ*pdd7* strain showed a Pdd phenotype that could be functionally complemented by a plasmid containing the entire *PDD7* gene (data not shown).

To determine whether the Δ*pdd7* strain was disturbed in macropexophagy, we exposed methanol-grown *H. polymorpha* WT and Δ*pdd7* cells to excess glucose or ethanol conditions. AO activity assays and Western blots were

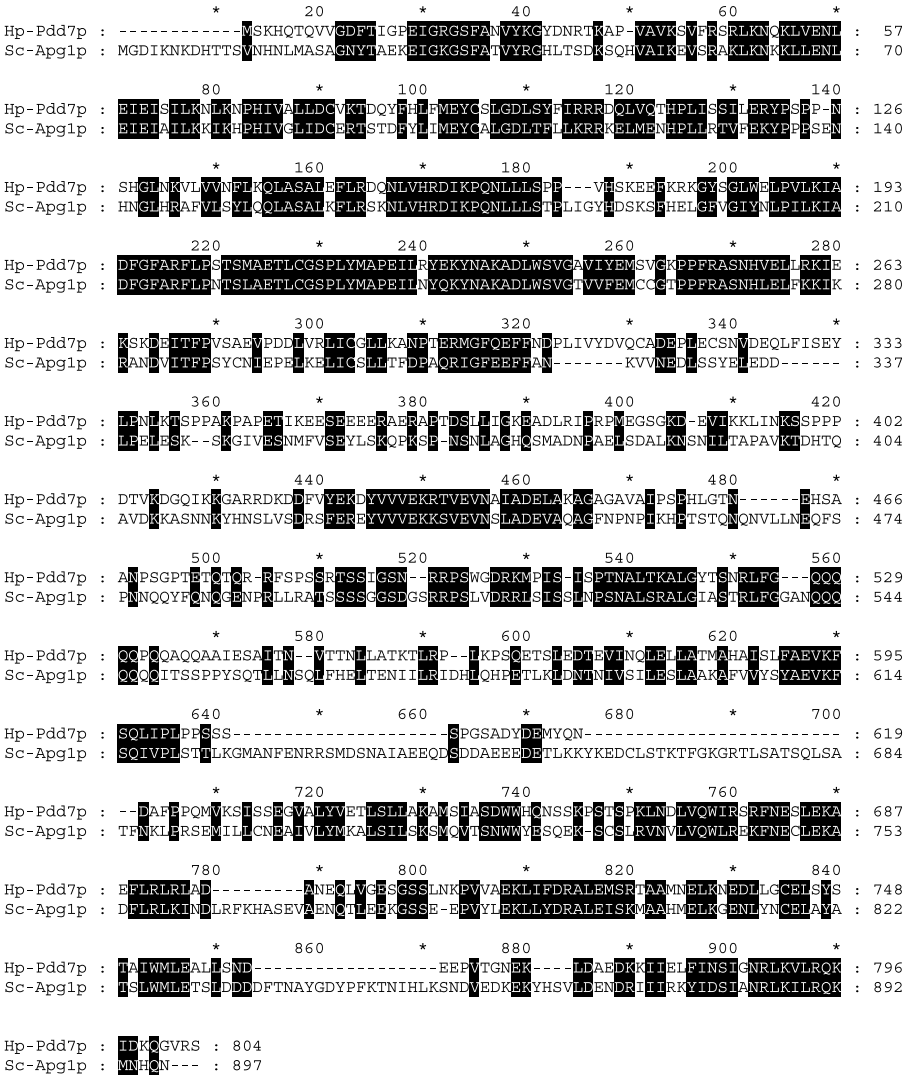

Fig. 2. Sequence alignment of the deduced primary sequence of *H. polymorpha* (Hp) Pdd7p with that of *S. cerevisiae* (Sc) Apg1p (accession number P53104). The one-letter code is shown. Gaps were introduced to maximise the similarity. Identical and conserved residues are shaded. The main similarity between the proteins resides in the N-terminus of the proteins, which contains the serine-threonine kinase domain (residues 22–322 in Sc-Apg1p). Also the ATP-binding region (residues 30–38 and 54 in Sc-Apg1p) and the active-site aspartate (residue 172 in Sc-Apg1p) are conserved in HpPdd7p.

performed on crude cell extracts of samples taken at various time points after the shift of cells (Fig. 3). As expected, in WT cells specific AO activities and AO protein levels had decreased gradually 4 h after the shift to either glucose or ethanol (cf. [12]). In contrast, in Δ*pdd7* cells neither the specific AO activities nor the AO protein levels had been significantly reduced after 4 h of cultivation on glucose- or ethanol-containing media. From this we concluded that Δ*pdd7* cells are impaired in the degradation of peroxisomal AO, a major peroxisomal matrix protein.

Electron microscopical analysis of these cells demonstrated that the degradation defect is associated with the inability of Δ*pdd7* cells to degrade peroxisomes by macropexophagy (Fig. 4A–D). In WT cells, sequestration of peroxisomes, the first event in degradation, was readily observed within 30 min after the addition of glucose (Fig. 4A), and after 4 h of incubation most peroxisomes had disappeared. Immunocytochemistry, using α-AO, resulted in the specific labelling of both peroxisomes and autophagic vacuoles, suggesting that peroxisomes were subject to degradation in the vacuole (Fig. 4C). In contrast, in Δ*pdd7* cells, 4 h after the addition of glucose peroxisomes were still present in a non-sequestered form (Fig. 4B). Furthermore, immunolabelling demonstrated that AO protein was confined to peroxisomes (Fig. 4D). α-AO-specific labelling was invariably absent on vacuolar profiles. From this we conclude that *PDD7* is required in an early step of macropexophagy.

3.3. Δpdd7 is also disturbed in microautophagy

In *H. polymorpha*, microautophagy is induced by nitrogen starvation conditions [7]. We investigated whether the Δ*pdd7* strain was also disturbed in this process by exposing

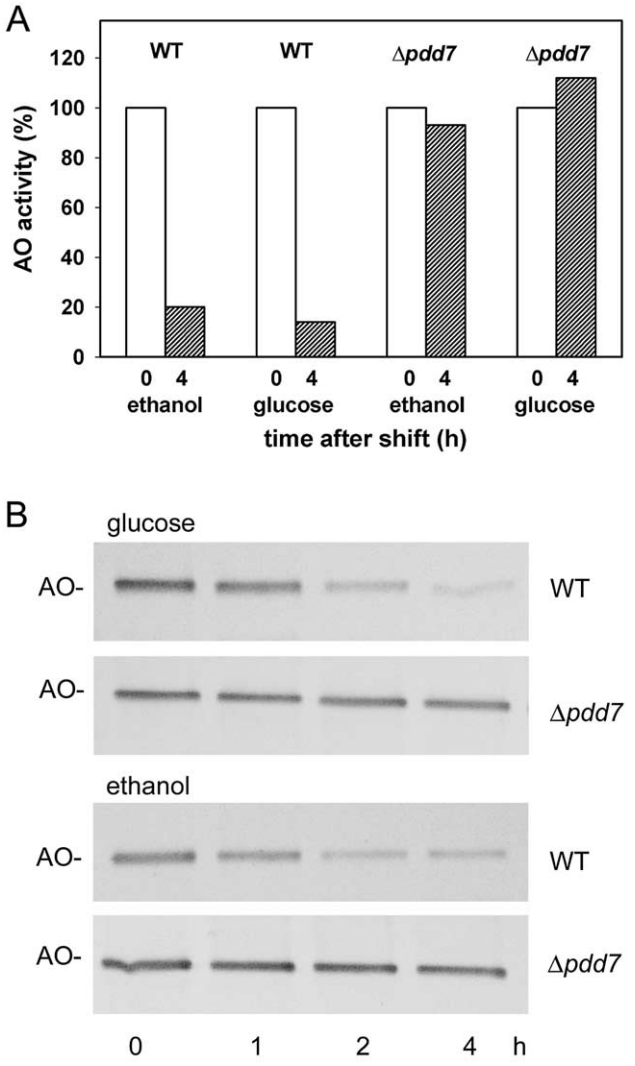

Fig. 3. Biochemical analysis of *H. polymorpha* WT and Δ*pdd7* cells under conditions that induce macropexophagy. Cells were grown in methanol-containing media to the mid-exponential growth phase. Subsequently ethanol or glucose was supplemented to the cultures to induce selective peroxisome degradation. A: The specific activity of the peroxisomal marker protein AO was measured in crude extracts of cells, harvested at $t=0$ or $t=4$ h after the shift to ethanol or glucose. Specific AO activities are expressed as percentage of the value at $t=0$, which was set to 100%. Data are corrected for growth of cells on ethanol or glucose. B: Equal volumes of identically grown cells were taken 0, 1, 2 and 4 h after the shift to glucose or ethanol, TCA-precipitated and prepared for Western blots. The blots were decorated with α-AO.

methanol-grown Δ*pdd7* cells to nitrogen (N) starvation conditions. Specific AO activity measurements on crude cell extracts of samples taken 2 h after the shift demonstrated that during N-starvation the specific AO activity in *H. polymorpha* WT cells used as control had been reduced to 39% of the value of cells grown in the presence of nitrogen source (Fig. 5A). In crude cell extracts prepared of N-limited Δ*pdd7* cells, AO activities remained similar to those observed in cells grown in the presence of nitrogen source (Fig. 5A). Western blots were prepared from the crude cell extracts (Fig. 5B). As expected, in WT cells AO protein levels had decreased gradually 4 h after the shift to N-limited conditions. In contrast, in Δ*pdd7* cells the AO protein levels stayed at the same level after 4 h of cultivation on N-limited media. In control experiments, when WT and Δ*pdd7* cells were grown in the presence of nitrogen source, no decrease of AO levels was observed (data not shown). Essentially the same observations were made for porin, a mitochondrial marker. In WT cells grown under N-starvation conditions, porin levels decreased over time, whereas in Δ*pdd7* cells the porin levels did not decrease during the same time interval. Thus, under conditions which induce microautophagy, neither peroxisomal nor mitochondrial proteins are degraded in *H. polymorpha* Δ*pdd7* cells.

Δ*pdd7* cells undergoing N-starvation were also analysed by electron microscopy, using immunocytochemistry and antibodies against AO (Fig. 4E,F). This analysis confirmed that the absence of AO degradation in Δ*pdd7* cells was caused by the inability to degrade peroxisomes by microautophagy. In WT cells, 2 h after the shift to N-limited conditions, α-AO labelled both peroxisomes and autophagic vacuoles, suggesting degradation of peroxisomes by microautophagy (Fig. 4E). In contrast, in Δ*pdd7* cells, 2 h after the shift to N-limited conditions, α-AO label was confined to peroxisomes, no labelling was observed in the vacuole (Fig. 4F). From this we conclude that Δ*pdd7* cells are disturbed in microautophagy.

4. Discussion

Here we describe the isolation of the *H. polymorpha PDD7* gene. *PDD7* encodes a 90-kDa protein with high sequence similarity to *S. cerevisiae* Apg1p/Aut3p, which appeared to be essential for nitrogen limitation-induced general macroautophagy [27,28], for selective peroxisome degradation [9], and for the cytoplasm-to-vacuole targeting (Cvt) pathway [29].

In *S. cerevisiae*, Apg1p has been identified as the core protein of the so-called Apg1p–Cvt9p complex, which regulates the switch between the Cvt pathway and the process of macroautophagy [29]. This complex consists of Apg1p, which interacts with Cvt9p and Vac8p, two components that are involved exclusively in the Cvt pathway, and Apg17p, a protein that is solely required for autophagy [29–32]. The interaction of Apg1p with Vac8p is mediated by Apg13p, a protein necessary for both processes. Vac8p is thought to facilitate phosphorylation of Apg13p during N-rich conditions, while Apg17p has been proposed to play a role in the Apg1p–Apg13p interaction. In addition, Cvt9p plays a role in the selective Cvt and pexophagy pathways, but not in general autophagy. During N-starvation conditions, Apg1p and Apg13p are partially dephosphorylated which results in a stronger interaction between the two proteins [29]. This leads to the transformation of Cvt vesicles into autophagosomes. Apg1p has

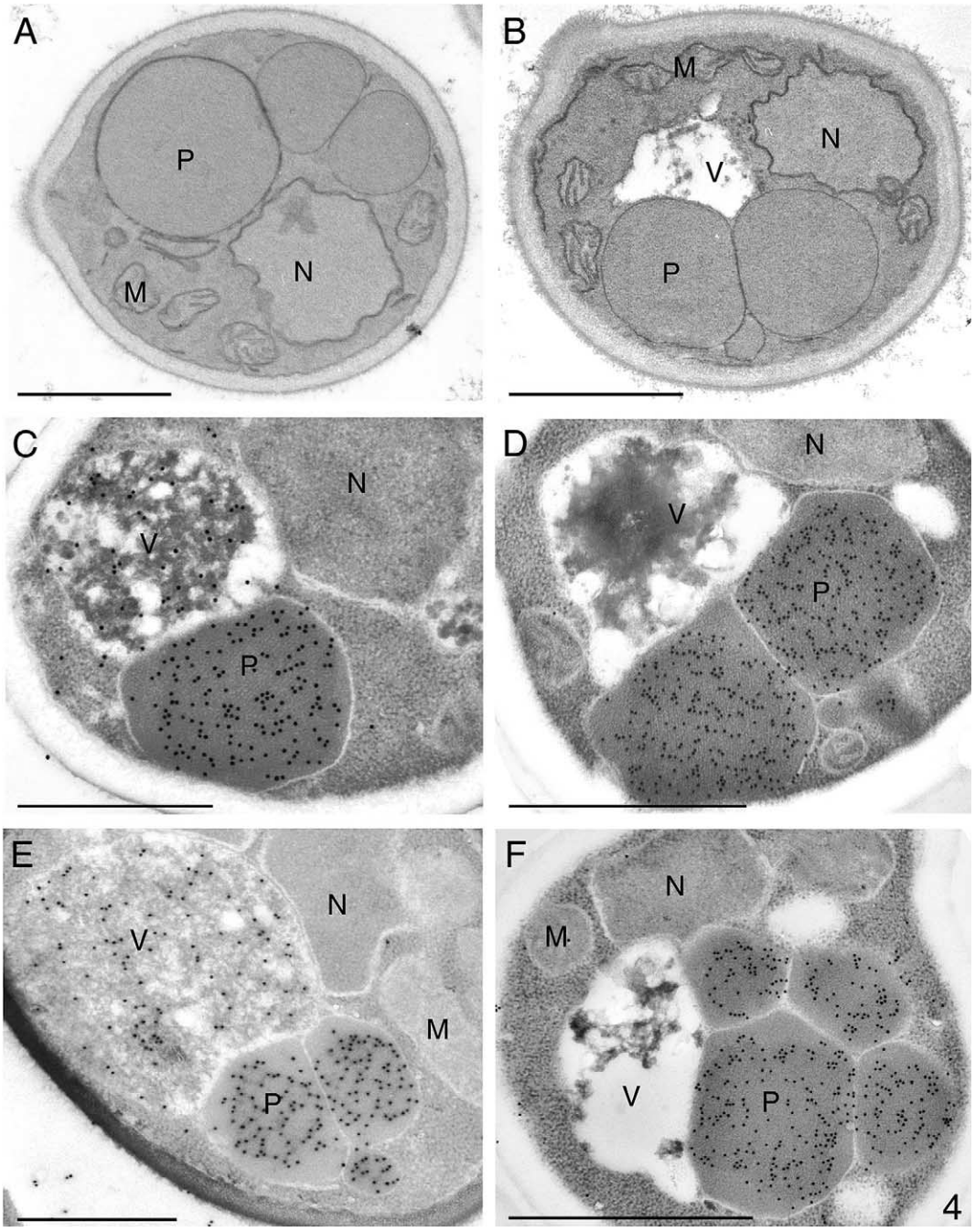

Fig. 4. Ultrastructural analysis of *H. polymorpha* WT and Δ*pdd7* cells during macropexophagy (A–D)-and microautophagy (E–F)-inducing growth conditions. Cells were grown in methanol-containing media to the mid-exponential growth phase. Glucose was administered to induce macropexophagy. To induce microautophagy, methanol-grown cells were shifted to methanol medium without N-source. Samples were taken at various time points after the shift and prepared for electron microscopy. A, B: Morphology, C–F: immunocytochemistry using α-AO. A: The sequestration of peroxisomes in WT *H. polymorpha* cells is already observed 30 min after the shift to glucose. B: In Δ*pdd7* cells, after the shift to glucose, peroxisomes were never observed to be sequestered after 4 h, but remained normally present in the cytosol. C: In WT cells 1 h after the shift to glucose AO protein is present in the vacuole. D: In Δ*pdd7* cells, taken 2 h after the shift to glucose, α-AO-dependent vacuolar labelling is never observed, but remains confined to peroxisomes. E: In WT cells, taken 2 h after the shift to N-limited growth conditions, α-AO-dependent labelling is observed in peroxisomes as well as in the vacuole. F: In Δ*pdd7* cells, 2 h after the shift to N-starvation conditions, AO labelling is confined to peroxisomes, and is never observed in the vacuole. Abbreviations: M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. The bar represents 1 μm.

also been demonstrated to be essential for selective peroxisome degradation [9]. It may be assumed that the Apg1p–Cvt9p complex also signals the onset of this process.

The localisation of Pdd7p in *H. polymorpha* is unknown. Studies in baker's yeast using a strain overproducing Apg1p–GFP have shown a cytosolic location of the fusion protein [15]. However, under physiological conditions Apg1p may be at least in part membrane-bound [32].

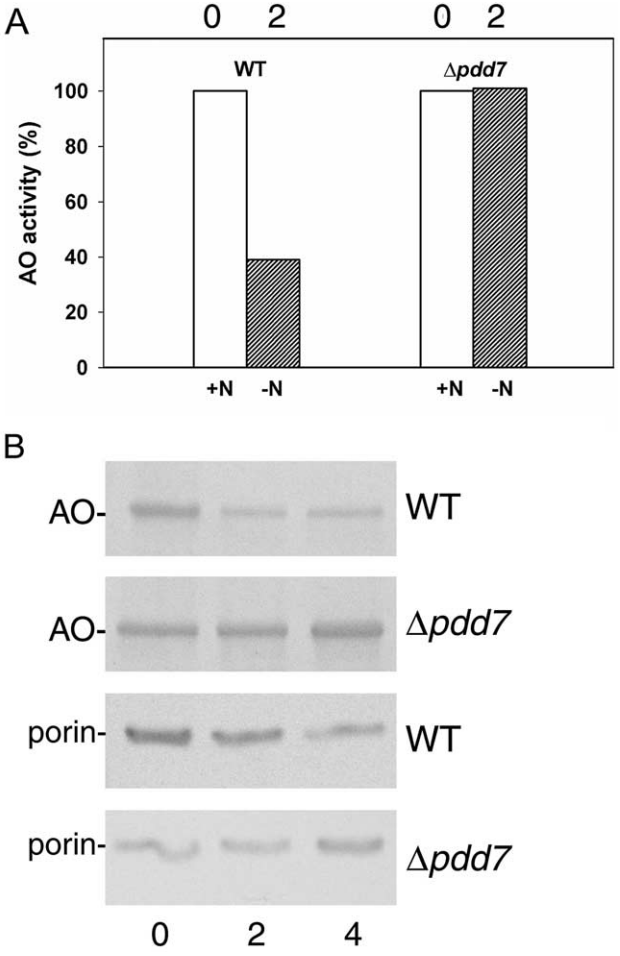

Fig. 5. Biochemical analysis of *H. polymorpha* WT and Δ*pdd7* cells under conditions that induce microautophagy. Cells were grown in methanol-containing media in the presence of ammonium sulphate. At the mid-exponential growth phase, the cultures were harvested by centrifugation and resuspended in pre-warmed methanol-containing media lacking ammonium sulphate (−N). Control cells were suspended in pre-warmed media supplemented with ammonium sulphate (+N). A: The specific activities of the peroxisomal marker enzyme AO were measured in crude extracts of samples taken 2 h after the shift to N-limited conditions. Specific activities are expressed as percentage of the value obtained with extracts of cells grown in the presence of ammonium sulphate, which was arbitrarily set to 100%. B: Equal amounts of protein of identically grown cells were taken 0, 2 and 4 h after the shift to N-limited conditions, TCA-precipitated and prepared for Western blots. The blots were decorated with antibodies against a peroxisomal (α-AO) and a mitochondrial marker (α-porin).

Recently, the Apg1p–Cvt9p signalling complex has been shown to be located on a novel structure, which functions in autophagosome formation [33]. This structure was described as a peri-vacuolar membrane compartment, which probably serves in membrane donation for autophagosome formation [34].

The high similarity between *H. polymorpha* Pdd7p and Apg1p suggests that they are functional homologues. Our morphological data indicate that *H. polymorpha* Δ*pdd7* mutant cells are affected in an initial stage of macropexophagy – as the sequestering event was never observed – implying that these mutants are either affected in signalling or in the initiation of the sequestration process. Such a scenario fits well with the signalling role described for Apg1p in general autophagy and the Cvt pathway [29,32]. In this respect, Δ*pdd7* mutant cells resemble *H. polymorpha* Δ*pdd1* mutant cells, which are mutated in the gene encoding the homologue of *S. cerevisiae* Vps34p [35]. Both *pdd1* and *pdd7* mutant cells are disturbed in the initial stages of macropexophagy and are unable to degrade peroxisomes by microautophagy ([7,35], this study). In contrast, unlike Δ*pdd1* mutant cells [35], Δ*pdd7* mutant cells are not disturbed in the transport of soluble vacuolar proteases to their target organelle, since vacuolar carboxypeptidase Y is not secreted by Δ*pdd7* cells (our unpublished data).

The *HpPDD7* gene is another example of the general notion that various transport pathways to the vacuole (i.e. microautophagy, the Cvt pathway and macropexophagy) require in part the same sets of genes [9–11]. Nevertheless, the process of macropexophagy is morphologically distinct from microautophagy [1,2,7]. Also, many mutants affected in glucose-induced micropexophagy in *P. pastoris* are not affected in macropexophagy [4]. Furthermore, *H. polymorpha pdd2* mutant cells, which are affected in macropexophagy, are not affected in nitrogen starvation-induced microautophagy [7]. This lends support to the notion that although the machineries initiating the different transport processes to the vacuole may utilise the same genes, including *HpPDD1* and *HpPDD7*, the actual transport mechanism during macropexophagy is apparently controlled by a unique set of genes. Clearly, much additional research is required to unravel the differences between macropexophagy and microautophagy at the molecular level.

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