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Arabidopsis homologues of the autophagy protein Atg8 are a novel family of microtubule binding proteins

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Abstract Autophagy is the non-selective transport of proteins and superfluous organelles destined for degradation to the vacuole in fungae, or the lysosome in animal cells. Some of the genes encoding components of the autophagy pathway are conserved in plants, and here we show that *Arabidopsis* homologues of yeast Atg8 (Apg8/Aut7) and Atg4 (Apg4/Aut2) partially complement the yeast deletion strains. The yeast double mutant, a deletion strain with respect to both Atg8 and Atg4, could not be complemented by *Arabidopsis* Atg8, indicating that *Arabidopsis* Atg8 requires Atg4 for its function. Moreover, *Arabidopsis* Atg8 and *Arabidopsis* Atg4 interact directly in a two-hybrid assay. Interestingly, Atg8 shows significant homology with the microtubule binding light chain 3 of MAP1A and B, and here we show that *Arabidopsis* Atg8 binds microtubules. Our results demonstrate that a principle component of the autophagic pathway in plants is similar to that in yeast and we suggest that microtubule binding plays a role in this process.

Keywords: Autophagy; Atg8; Microtubule binding; *Arabidopsis*

1. Introduction

Autophagy mediates the non-selective transport of cytosolic material and superfluous organelles to the vacuole or its equivalent in animal cells, the lysosome, for degradation by a large group of resident proteases. Autophagy is well characterised in yeast [1,2] and homologues of some of the genes involved are present in *Arabidopsis* [3,4]. So far only the *Arabidopsis* equivalents of the yeast autophagy genes, Atg7 (old nomenclature Apg7 [5]) and Atg9 (Apg9) which are both single copy in *Arabidopsis*, have been shown to be involved in *Arabidopsis* [3,4]. An essential protein in the yeast autophagic pathway is Atg8 (Apg8/Aut7). After a series of post-translational modifications, Atg8 promotes the formation of autophagosomes and their delivery to the vacuole for subsequent degradation [2,6]. The processed Atg8 localises to autophagosome membranes in yeast and mammalian cells [7–9]. One of the proteins involved in the post-translational modification of Atg8 in the yeast autophagy pathway is Atg4 (Apg4/Aut2). Atg4 is a cysteine protease that cleaves off the C-terminal amino acid of Atg8 to reveal a C-terminal glycine [10]. This C-terminal glycine is conserved in the *Arabidopsis* homologues of this protein [3].

Microtubules are filamentous polymers of α and β tubulin dimers that are involved in many key processes in eukaryotic cells, including cell division and intracellular transport. Microtubule organisation and function are regulated by microtubule associated proteins (MAPs). All MAPs found in plants appear to have relatives in animal cells but not all animal MAPs are present in plants [11]. MAPs 1A and B, for example, do not appear in the *Arabidopsis* genome, however, light chain 3 (LC3) which regulates the binding of MAP1 to microtubules [12] is conserved in separate open reading frames [13,14]. Interestingly, LC3 is an orthologue of the yeast Atg8 protein. LC3/Atg8 have been found to localise to autophagosome membranes in both yeast and mammalian cells [7,8]. In addition, Atg8 is also homologous to $GABA_A$-receptor-associated protein (GABARAP) and to GATE-16, a Golgi membrane transport modulator [15]. Like MAP1A and 1B LC3, GABARAB also binds to microtubules [16].

Here, we show that *Arabidopsis* homologues to Atg8 and Atg4 are functional homologues of their yeast counterparts. In addition, we show that the *Arabidopsis* Atg8 proteins bind microtubules, suggesting that the microtubule cytoskeleton may be involved in autophagy in plants.

2. Materials and methods

2.1. Yeast complementation

pYES2 (Invitrogen, Paisley, UK) was cut with *Hin*dIII and *Bam*H1, blunt ended, treated with calf intestine alkaline phosphatase (Promega, Southampton, UK) and a GATEWAY cloning cassette introduced into the vector, resulting in pYES2-GW. Complementation of yeast mutants was carried out by introducing pYES2-GW plasmids containing AtAtg genes into yeast deletion strains. Analysis of the complementations was carried out as previously described [17].

2.2. Yeast two-hybrid interaction analysis

The coding sequences of AtAtg8a, 8d and Atg4b were incorporated into the activating domain (AD) vector pAS2-1GW and the binding domain (BD) vector pACT2-GW via a Gateway LR recombination reaction (Invitrogen, Carlsbad, CA). BD vectors were introduced to the MATα *Saccharomyces cerevisiae* strain Y187 via the Lithium acetate transformation method (manufacturer's instructions MatchMaker; Clontech Palo Alto, CA), whereas AD constructs were transformed into the MATa strain AH109. The BD vector pACT2-GW confers the ability to grow on tryptophan free media and the AD vector pAS2-1-GW allows for selection on media deficient in leucine. Transformants were selected after 72 h of growth on SD media lacking

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the appropriate nutrient at 30 °C. BD constructs were assayed for autonomous activation in both Y187 and AH109 cells. Transformed Y187 and AH109 cells were mated at 20 °C for 48 h on YPD media prior to selection for diploids on SD media lacking both tryptophan and leucine. Interactions between BD and AD constructs were then assessed by selection upon media additionally lacking adenine and histidine. The addition of 40 μM x-α-gal (Glycosynth, Warrington, UK) to the media also allowed the activity of α-galactosidase to be assessed via production of a blue precipitate. Yeast selection was performed over a period of 72 h at 30 °C

2.3. Protein expression, antibody production

Coding sequences were amplified from cDNA by PCR using primers that also included GATEWAY (Invitrogen, Paisley, UK) sequences and then recombined into pDONR201 (Invitrogen, Paisley, UK) according to the manufacturer's guidelines, followed by recombination into pGAT4. pGAT4 is an ampicillin resistant pET based plasmid which adds a N-terminal 6xHIS to the recombinant protein. For expression in *Escherichia coli*, the strain BL21-DE3 (Stratagene, Amsterdam, The Netherlands) was used. Bacteria were cultured at 37 °C until an OD_{600} of 0.6, whereafter they were transferred to 15 °C and induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM overnight. HIS-tagged proteins were purified with the HisTrap purification system (Amersham Pharmacia, Uppsala, Sweden) according to the manufacturer's protocol. The purified AtAtg8a protein was dialysed into PBS, concentrated in a Vivaspin 5000 MWCO column (Vivascience, Lincoln, UK) and mice were immunised with 100 μg protein in PBS, with an equal volume of Freund's Incomplete Adjuvant (Sigma, Poole, UK). Mice were immunised 3× with two weeks intervals. Ten days after the last injection, the blood of the mice was collected, left at room temperature for 3 h and centrifuged in a table top centrifuge for 10 min ($16\,000 \times g$). The supernatant was tested by Western blotting on both *Arabidopsis* protein extract and bacterially expressed protein, and the optimal dilution was determined to be 1:100.

2.4. Co-sedimentation assay

PC-tubulin and purified AtAtg8 were centrifuged at $80\,000 \times g$ at 4 °C to remove aggregates. Reactions were set up in buffer (50 mM PIPES, 2 mM EGTA, 2 mM $MgSO_4$, 50 mM NaCl, 30% glycerol, and 0.1 mM GTP) and were allowed to polymerise at 30 °C for 30 min. After polymerisation, the reaction mixtures were centrifuged at $50\,000 \times g$ at 30 °C for 30 min. The supernatant was mixed with an equal volume of 2× SDS-sample buffer and boiled for 2 min. The pellet was washed with buffer and resuspended in an equal volume of 1× SDS-sample buffer as the final volume of the supernatant. Samples were separated by SDS–PAGE and stained with coomassie.

2.5. Co-localisation assay

PC-tubulin was polymerised with 10% glycerol and 1 mM GTP at 37 °C for 30 min. After polymerisation, bacterially expressed AtAtg8a was added, followed by a 30 min incubation at 37 °C. After incubation, glutaraldehyde was added to a final concentration of 0.5%, whereafter the reaction mixture was transferred to a coverslip, coated with 1 μg/ml poly-L-lysine and allowed to dry for 1 h. The dried coverslips were washed 2× 15 min in PBS and 15 min in PBS + 1% BSA (blocking buffer). The AtAtg8 antibody was applied 1:100 diluted in blocking buffer for 30 min at room temperature, followed by two washes of 15 min in blocking buffer. After washing, 1:300 diluted Goat-anti-mouse-IgG-Alexa 488 (Molecular Probes, Leiden, The Netherlands) in blocking was applied for 30 min, followed by two washes of 15 min in blocking buffer. Finally, microtubules were labelled by application of 1:100 diluted anti-β-tubulin clone tub 2.1, directly conjugated to Cy3 (Sigma) for 30 min. After washing twice in PBS, samples were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) and analysed in a Zeiss LSM510 META microscope.

3. Results

3.1. Identification of components of the autophagic pathway in Arabidopsis

We have searched the *Arabidopsis* genome for proteins that are homologous to proteins that are involved in post-translational modifications of the yeast Atg8 protein. In yeast Atg8 is processed by a series of post-translational modifications, mediated by a number of well characterised proteins. The first processing step of yeast Atg8 is the Atg4 mediated hydrolysis of the petide bond to the carboxy-terminal arginine, which leaves a conserved C-terminal glycine residue [10]. After this reaction, Atg8 is activated by the formation of a thioester link with a cysteine residue of the E1 analogue Atg7 (Apg7/Cvt2) [18], whereafter it is transferred to a cysteine residue of Atg3 (Apg3/Aut1), an E2 analogue. Atg3 changes Atg8 into a membrane-associated form by forming an amide bond with phosphatidylethanolamine [18]. In addition, Atg7 functions as an activator of Atg12 (Apg12), to allow its conjugation to Atg5 (Apg5). This complex is essential for membrane closure of autophagosomes. Fig. 1 gives a graphic overview of the

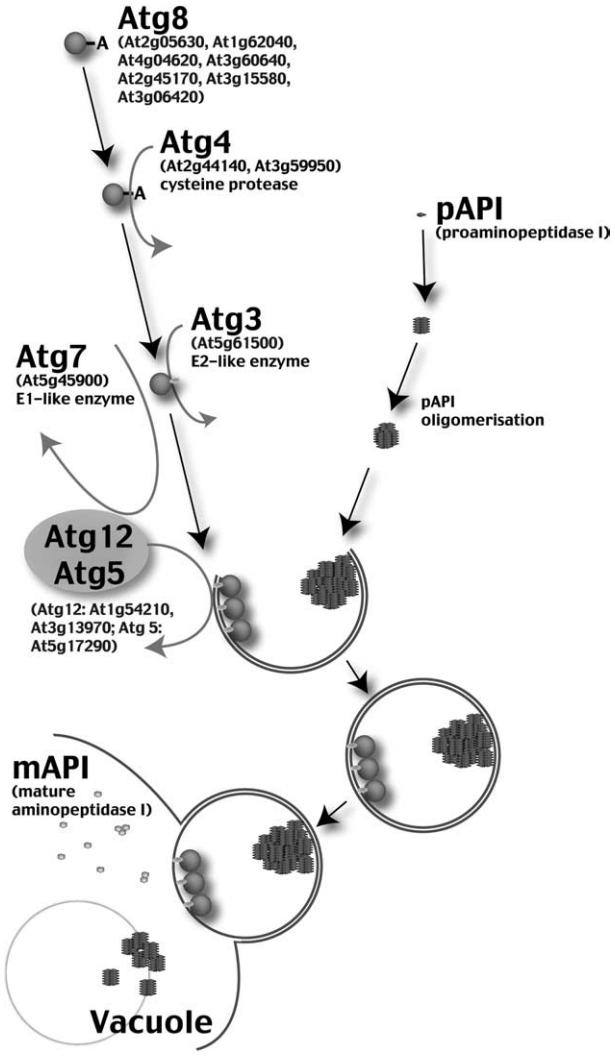

Fig. 1. Schematic overview of the post-translational modifications of Atg8 in yeast. Gene numbers of the *Arabidopsis* homologues to the proteins involved are given. Atg4 is a cysteine protease that cleaves off the C-terminal arginine residue of Atg8. Atg3 is an E2-like enzyme that is essential for conjugation of the C-terminal glycine of Atg8 to phosphatidylethanolamine. Atg7 is an E1-like ATP-dependent enzyme that activates Atg12 for conjugation to Atg5 and Atg8 to phosphatidylethanolamine. When the autophagic pathway is functional, mature aminopeptidase I appears in the vacuole.

pathway, including the gene symbols of *Arabidopsis* genes, homologous to the genes in yeast.

3.2. *The* Arabidopsis *Atg8 gene family*

In *Arabidopsis*, a family of nine Atg8 genes, termed AtAtg8a to 8i, has been identified [3]. The proteins encoded by this family show between 41% and 99% identity with each other and from 47% to 73% identity with the yeast protein. The AtAtg8 proteins also show considerable identity (between 33% and 41% identity) with light-chain 3 (LC3) from the mammalian MAP1A and 1B; the Golgi membrane transport modulator protein Gate 16 (between 48% and 60%) and to GABARAP (42–59%).

To test if the AtAtg8 proteins are functional homologues of yeast Atg8, we complemented an *atg8*Δ yeast strain with two different AtAtg8 genes. The cDNA coding sequences of AtAtg8a (At4g21980) and AtAtg8d (At2g05630) were cloned into pYES2-GW plasmids (see Section 2), which were transformed into an *atg8*Δ yeast strain and selected with the appropriate selection markers. Fig. 2A shows that the maturation of proaminopeptidase I in yeast, an Atg8 dependent process [19], is somewhat reduced, but that the *AtATG8a* and *AtATG8d* genes functionally complement the yeast *ATG8* gene.

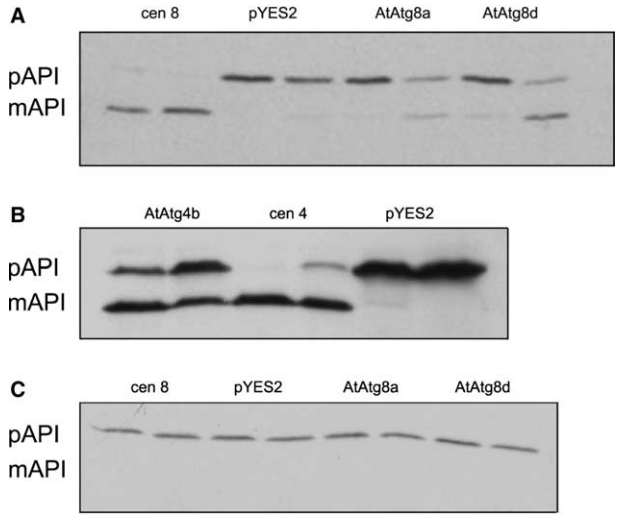

Fig. 2. Complementation of *atg8*Δ and *atg4*Δ yeast strains with *Arabidopsis* homologues. Transport of proaminopeptidase I (pAPI) to the vacuole, where it matures, only takes place when a functional autophagy pathway is present. The band with the higher molecular mass represents pAPI and the band with the lower molecular mass is matured aminopeptidase I (mAPI). In the first lane of every complementation, the maturation of aminopeptidase I in unstarved yeast is displayed and in the second lane the yeast has been starved for 4 h on 1% K-acetate. (A) Complementation of the yeast *atg8*Δ strain with yeast Atg8 (cen 8), the pYES2 plasmid only and AtAtg8a and 8d. Maturation of aminopeptidase I only takes place with the cen 8 construct, containing the yeast *ATG8* gene and to a lesser extent with the AtAtg8a and 8d constructs, indicating that these genes complement Atg8 partially. pAPI does not mature in the pYES2 only control, indicating that it does not complement *atg8*Δ. (B) Complementation of an *atg4*Δ yeast strain with AtAtg4b, Atg4 (cen 4) and empty pYES2. Maturation of aminopeptidase I only takes place with the cen 4 construct, containing the yeast Atg4 gene and the AtAtg4b construct, indicating that these genes complement the *atg4*Δ phenotype. pAPI does not mature in the pYES2 only control, indicating that Atg4 is essential for autophagy. (C) An *atg4*Δ *atg8*Δ yeast strain was not complemented with AtAtg8a or 8d. The failure of AtAtg8a and 8d alone to complement the double mutant indicates that Atg4 is essential for the function of AtAtg8a and 8d during yeast autophagy.

To further test if AtAtg8a and 8d are in the *Arabidopsis* autophagy pathway, we analysed Atg4, which is essential for maturation of Atg8 in yeast. Atg4 interacts with Atg8 in yeast; a direct interaction between their *Arabidopsis* homologues would strengthen the hypothesis that both *Arabidopsis* gene families are in the autophagy pathway. Two homologues of the yeast *ATG4* gene have been identified in *Arabidopsis* [3]. The protein products of these genes are 65% homologous to each other and 25% (AtAtg4a; At2g44140) and 27% (AtAtg4b; At3g59950) to yeast Atg4. The *ATG4* genes contain a cysteine peptidase domain consensus sequence. The AtAtg4 proteins are 32% and 28% homologous to this domain. To test for functional complementation of yeast Atg4, we complemented an *atg4*Δ yeast strain with one of the AtAtg4 genes (AtAtg4b) as described above (Fig. 2B) and found that Atg4 complemented the yeast deletion almost completely under the starvation conditions that induce autophagy.

To identify any interaction between AtAtg4b and the AtAtg8 proteins AtAtg8a and 8d, we performed a two-hybrid assay. Fig. 3 shows that the AtAtg4 and AtAtg8 proteins do interact. In order to assess whether this interaction is functionally important, we tested whether yeast *atg8*Δ *atg4*Δ double mutants could be complemented with AtAtg8 only. Fig. 2C shows that AtAtg8a and 8d did not complement the double mutant. From these data, we conclude that both AtAtg8a and 8d require the action of an Atg4 cysteine proteinase, similar to yeast Atg8.

The striking homology of Atg8 proteins to mammalian microtubule binding proteins, such as MAP1 LC3 and GABARAP, prompted us to express two HIS-tagged AtAtg8 proteins (AtAtg8a and 8d) in *E. coli* and to perform a co-sedimentation assay with these proteins and microtubules. Fig. 4 shows that both AtAtg8 proteins interact strongly with microtubules in

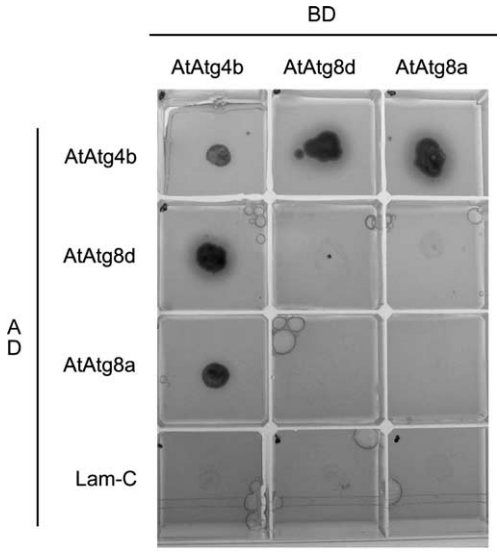

Fig. 3. AtAtg8a and 8d interact with AtAtg4b in a yeast two-hybrid screen. A yeast two-hybrid assay was used to test interaction between AtAtg8a and 8d and AtAtg4b, both in a BD and AD plasmid. All three proteins were tested against themselves and against Lamin C (Lam-C) as a negative control. Both AtAtg8 genes interact strongly with AtAtg4. AtAtg4 appears to weakly interact with itself.

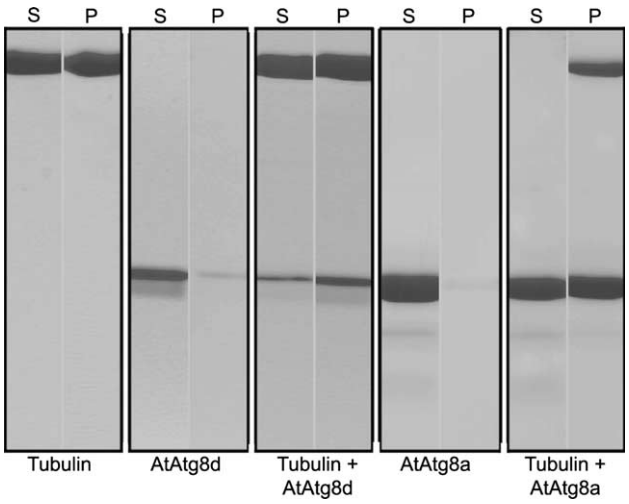

Fig. 4. Co-sedimentation assay of AtAtg8 proteins with microtubules. The supernatants (S) and pellets (P) of reactions are displayed. Controls with AtAtg8a and 8d proteins only and no microtubules do not sediment during centrifugation. In the microtubules only sample about half of the tubulin polymerises and sediments. When AtAtg8 and microtubules are mixed, a large amount of AtAtg8 co-sediments with the microtubules, indicating microtubule binding.

this assay. To confirm this result, we carried out an in vitro binding assay. Fig. 5 shows that microtubules are decorated by AtAtg8a. These data show that AtAtg8 proteins bind microtubules in vitro.

4. Discussion

Here we have identified two gene families in *Arabidopsis*, members of which are able to function in the yeast autophagic pathway. One of these families (AtAtg8) binds to microtubules in vitro. These data strongly suggest that a similar pathway occurs in plants and that AtAtg8 may involve microtubules in this pathway.

We have complemented yeast *atg8*Δ and *atg4*Δ mutants with similar *Arabidopsis* genes. Moreover, we have shown that AtAtg8a and 8d and AtAtg4b interact with each other directly in a yeast two-hybrid screen, as do their yeast orthologues [17]. In addition, the *atg8*Δ *atg4*Δ double mutant, which is defective in autophagy, similar to the single mutations, cannot be complemented with AtAtg8a or 8d alone, indicating that Atg4 activity is required for functional autophagy. Therefore, it is likely that the *Arabidopsis* AtAtg8 and AtAtg4 perform a

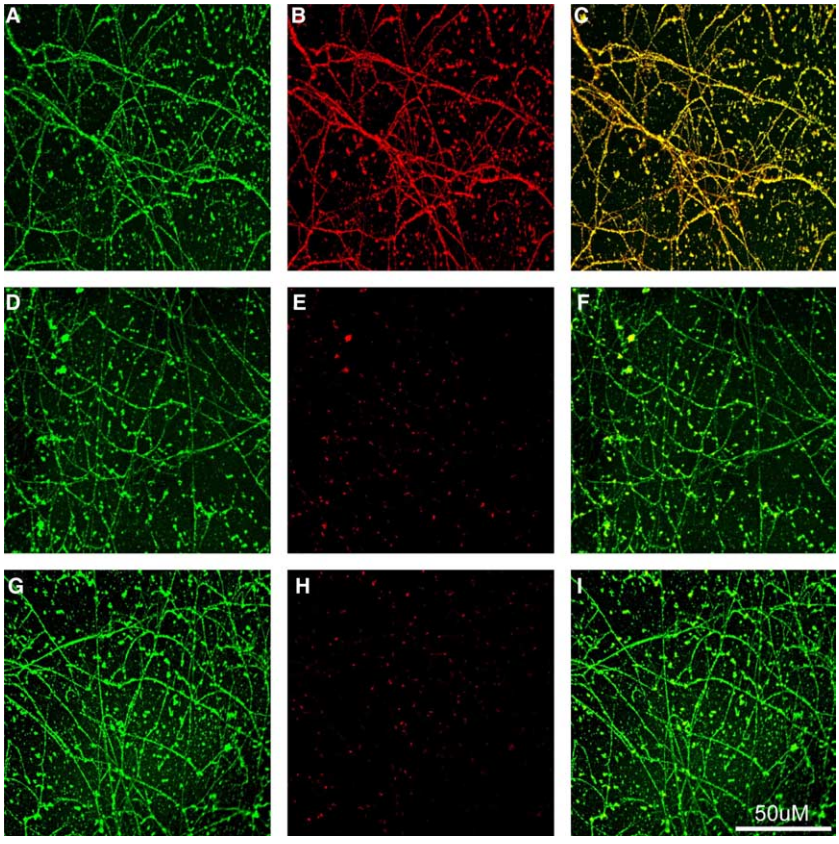

Fig. 5. Co-localisation assay of AtAtg8a protein with microtubules. The green images (A–D–G) are microtubules, stained with a CY3-conjugated anti-tubulin antibody, the red images (B–E–H) show the localisation of AtAtg8a and the last column of images (C–F–I) shows an overlay of the green (microtubules) and red (AtAtg8) images. The images in the first row (A–B–C) are images of microtubules with AtAtg8a. The images in the second row (D–E–F) represent microtubules without AtAtg8a protein, but with anti-AtAtg8 primary and secondary antibody. The images in the third line are images of microtubules with AtAtg8a, with secondary antibody only (without AtAtg8 antibody). In both negative controls, the AtAtg8 signal on microtubules is absent. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

similar function to that of their yeast orthologues in an autophagy pathway in plants.

Bacterially expressed AtAtg8a and 8d co-sedimented with microtubules in vitro and AtAtg8a localised to microtubules in an in vitro binding assay, indicating that this protein has the ability to bind microtubules. There is no evidence for microtubule binding of Atg8 in yeast, but both in vitro and in vivo interaction with microtubules of the mammalian GABARAB protein, which is homologous to Atg8, has been demonstrated [20]. In addition, it has been shown that MAP1A and 1B LC3 regulate the interaction of MAP1 to microtubules [12]. So AtAtg8 may be like other microtubule binding proteins that appear to have dual roles in the cell. In this case, as part of the autophagic pathway which may involve microtubules or as a microtubule interacting protein perhaps regulating the binding of, as yet unknown, MAPs to microtubules. For example, EF1α and eIF4F both have dual roles in cells: they are both involved in translation but are also both MAPs [21] and translation has been suggested to occur on a cytoskeleton scaffold [22] thereby linking both roles.

In conclusion, our data suggest that the autophagic pathway is functionally conserved in plants. As AtAtg8 binds microtubules and as Atg8 has been shown to localise to autophagic vesicle membranes [7,9,14], we suggest that AtAtg8 may be involved in linking the autophagy pathway to the microtubule network and that the microtubules are responsible for the relocation of autophagosomes to the vacuole.

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