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In silico modeling of the *Moniliophthora perniciosa* Atg8 protein

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ABSTRACT. Autophagy is defined as an intracellular system of lysosomal degradation in eukaryotic cells, and the genes involved in this process are conserved from yeast to humans. Among these genes, *ATG8* encodes a ubiquitin-like protein that is conjugated to a phosphatidylethanolamine (PE) membrane by the ubiquitination system. The Atg8p-PE complex is important in initiating the formation of the autophagosome and thus plays a critical role in autophagy. *In silico* modeling of Atg8p of *Moniliophthora perniciosa* revealed its three-dimensional structure and enabled comparison with its *Saccharomyces cerevisiae* homologue ScAtg8p. Some common and distinct features were observed between these two proteins, including the conservation of residues required to allow the interaction of α-helix1 with the ubiquitin core. However, the electrostatic potential surfaces of these helices differ, implying particular roles in selecting specific binding partners. The proposed structure was validated by the programs PROCHECK 3.4, ANOLEA, and QMEAN, which demonstrated 100% of amino acids located in favorable regions with low total energy. Our results showed that MpAtg8p contains the same functional domains

(3 α-helices and 4 β-sheets) and is similar in structure as the ScAtg8p yeast. Both proteins have many conserved sequences in common, and therefore, their proposed three-dimensional models show similar configuration.

Key words: Basidiomycetes; Autophagosome; Protein homology

INTRODUCTION

Eukaryotic cells employ autophagy (ATG, a general term for catabolic processes involving the lysosomal/vacuolar pathway) to degrade damaged or obsolete organelles and proteins. ATG is an evolutionarily conserved mechanism for degradation of biomolecules and is generally defined as a lysosome-dependent mechanism of intracellular degradation that is used for the turnover of cytoplasmic macromolecules in starving cells (Yorimitsu et al., 2007). Three forms of autophagic pathways have been described, chaperone-mediated autophagy (independent of vesicle formation), macro- and micro-autophagy (dependent on vesicles) in plants, mammals and fungi (Shpilka et al., 2011; Li and Vierstra, 2012). Both macro- and micro-autophagy involve the movement of membranes. Whereas in micro-ATG the transport of material destined for degradation occurs via invagination of the lysosomal membrane, macro-ATG involves the formation of double membranes of autophagic vacuoles, also known as autophagosomes, which transport cytoplasmic cargo to lysosomes for degradation (Shpilka et al., 2011; Li and Vierstra, 2012).

In filamentous fungi, ATG is involved in different cellular pathways such as cellular degradation, nutrient recycling, cellular differentiation, and cell death (Pinan-Lucarré et al., 2003; Pollack et al., 2009). Pinan-Lucarré et al. (2003) showed that ATG-mutants of *Podospora anserina* had compromised differentiation due to the absence of internal nutrient supply, lacking provision of new 'building blocks' for cellular differentiation while ATG-mutants of *Aspergillus nidulans* had abnormal development of conidiophores, even though forming aerial hyphae (Kikuma et al., 2006).

Molecular studies on ATG began more than a decade ago, starting primarily from genetic screenings in the budding yeast *Saccharomyces cerevisiae* and the methylotrophic yeasts *Pichia pastoris* and *Hansenula polymorpha* (Suzuki and Ohsumi, 2007; He and Klionsky, 2009). So far, 34 ATG genes have been identified in *S. cerevisiae*, including *ATG8*, a gene with a central role in the ATG pathway (Kirisako et al., 2000; Kikuma et al., 2006; Xie et al., 2008; Noda et al., 2010). Atg8 proteins constitute one of the 62 highly conserved eukaryote-specific protein families. While yeast and other fungal species have a single *ATG8* gene, multi-cellular animals, green plants and some protists have several (Shpilka et al., 2011).

In different organisms, Atg8p is a ubiquitin-like protein that undergoes cleavage of its C-terminal arginine residue by the cysteine protease Atg4p (Kirisako et al., 2000; Kabeya et al., 2004) exposing a C-terminal glycine residue. The C-terminally cleaved Atg8p is activated by Atg7p, an E1-like protein and is subsequently transferred to Atg3p, an E2-like enzyme, through a thioester bond. Later, Atg8p is conjugated to phosphatidylethanolamine (PE) by a covalent bond (Ichimura et al., 2000; Kirisako et al., 2000; Shpilka et al., 2011; Li and Vierstra, 2012), a reaction facilitated by the E3-like Atg12p-Atg5p complex; the conjugated Atg8p can also be de-conjugated by Atg4p (Kirisako et al., 2000). Ubiquitination by Atg8p is a key

step in the autophagic pathway, and yeast null-mutant *atg8* cannot conduct ATG (Zhang et al., 2007; Xie et al., 2008; Shpilka et al., 2011).

In the basidiomycete *M. perniciosa*, ATG may play a key role during its infection of *Theobroma cacao* since recycling of nutrients and differentiation are essential to successfully complete the fungal life cycle. This phytopathogenic fungus, the causal agent of witches' broom disease in *T. cacao*, has a hemibiotrophic life cycle, i.e., two distinct phases of growth that can also be observed *in vitro*: a biotrophic-like phase (monokaryotic cells) and a necrotrophic-like phase (dikaryotic cells) (Silva et al., 2002). To develop the spore-derived monokaryotic hypha, cells undergo dikaryotization, which allows further differentiation and production of basidiocarps that, by releasing basidiospores, complete the fungal life cycle (Silva et al., 2002; Scarpari et al., 2005).

Mycelia grown in different carbon sources (either glucose or glycerol) show different expression of Mp*ATG8*, and this alters the autophagy-dependent intracellular nutrient turnover. Glucose seems to trigger the expression of Mp*ATG8*, especially after mutagen-induced damage to cellular macromolecules. Also, Mp*ATG8* is continuously expressed during the different stages preceding basidiocarp formation and during later steps of fungal differentiation (Santos et al., 2008).

Since Mp*ATG8* gene expression has already been determined (Pungartnik et al., 2009), modeling of MpAtg8p is important to infer or verify the function of MpAtg8p, by comparison with the structures of known Atg8 proteins. When MpAtg8p is essential for ATG in *M. perniciosa*, the structure of the model could be useful in defining targets for new fungicidal molecules against witches' broom disease by *de novo* design methods (Andrade et al., 2009). The availability of genome sequences of the basidiomycete *M. perniciosa* and the ascomycete yeast *S. cerevisiae* allowed us to compare *ATG8*-encoded protein ScAtg8p from *S. cerevisiae* with its putative homologue MpAtg8 protein of *M. perniciosa*.

MATERIAL AND METHODS

Identity among basidiomycetes

The sequence of the Atg8p obtained from the database of *M. perniciosa* of NCBI (ACD93204.1) was submitted to the BLASTp algorithm (Altschul et al., 1997) tool database, using non-redundant sequential parameter of search. From the sequences obtained, we selected those belonging to the phylum Basidiomycetes (Table 1).

Alignment, template and construction of the three-dimensional (3-D) model

Initially, the MpAtg8p sequence was subjected to the BLASTp program, restricted to the Protein Data Bank (PDB) (http://www.pdb.org/). Conserved regions and motifs were found by aligning the found template by TCOFFEE with the Atg8 protein sequences (Notredame et al., 2000). 3-D models were constructed using the Swiss-PdbViewer 3.7 software (Guex and Peitsch, 1997; Guex et al., 1999; Mcguffin and Jones, 2003) following a standard protocol: a) load template pdb file; b) align primary target sequence with template; c) submit modeling request to Swiss Model Server. PyMol was used for presentation.

Validation

The structure derived from homology modeling was submitted to the validation process, using the programs PROCHECK 3.4 (Laskowski et al., 1993), ANOLEA (Atomic Non-Local Environment Assessment) (Melo et al., 1997; Melo and Feytmans, 1997), and QMEAN (Ramachandran et al., 1963) (http://swissmodel.expasy.org/).

RESULTS AND DISCUSSION

Identity of the MpAtg8p sequence with Atg8p of other basidiomycetes

With a sequence of 127 amino acids (aa), MpAtg8p has high similarity with Atg8 proteins of different species of basidiomycetes (Table 1), pointing to the highly conserved function of this protein. According to Nakatogawa et al. (2007), the aa alanine 76, jointly with seven other aa residues, Ile32, Lys48, Leu50, Arg65, Asp102, Phe104, and Tyr106, is highly conserved in Atg8p and, when modified, significantly reduces the efficacy of autophagy. The aa Tyr49, Leu50, Phe77, and Phe79 have been described as essential for the functionality of Atg8p since mutational changes at these positions affect the protein's activity. Tyr49 and Leu50 are positioned in a functional domain of Atg8p. After binding the lipid, Atg8p modifies its conformation and displays the Phe77 and Phe79 residues within a hydrophobic region, which allows interaction with Atg4p, a step necessary for removal of Atg8p from the membrane, delivering it to the cytoplasm. Residues Tyr49 and Leu50 are essential for the maintenance of the autophagic route, where they are involved in the lipidation process (Kirisako et al., 2000; Sugawara et al., 2004; Ichimura et al., 2004; Amar et al., 2006; Nakatogawa et al., 2007). In MpAtg8p, all these residues are conserved (Figures 1 and 2), and we may, therefore, deduce that the similarity found in aa sequence of MpAtg8p with that of ScAtg8p yeast and the similarity in their 3-D protein structure lead to likewise similarity in cellular function (Geng and Klionsky, 2008).

Table 1. Identity of the MpAtg8 amino acid sequence with Atg8p of other basidiomycetes.

Basidiomycetes	GenBank No.	Identity with Atg8p of *Moniliophthora perniciosa* (%)
M. perniciosa	ACD93204.1	100%
Coprinopsis cinerea	XP_001833603.1	98%
Laccaria bicolor	AAB53650.1	98%
Piriformospora indica	CCA70209.1	98%
Schizophyllum commune	XP_003035093.1	97%
Serpula lacrymans var. *lacrymans*	EGN96038.1	97%
Ustilago maydis	XP_761714.1	96%
Sporisorium reilianum	CBQ69980.1	96%
Melampsora larici-populina	EGG01516.1	94%
Mixia osmundae	GAA95663.1	92%

Preliminary results involving heterologous expression of a single copy of *MpATG8* in a sporulation-deficient diploid yeast *atg8/atg8* null mutant showed that sporulation could be restored, indicating heterologous expression of a fully functional protein (Pereira ACF, Pungartnik C, and Brendel M, unpublished results).

Figure 1. Multiple sequence alignment of Atg8p homologues. Amino acid sequences of Atg8p of yeast, *Moniliophthora perniciosa* and mammalian homologues are aligned using the Clustal_W program. Underlined residues represent conserved amino acids and asterisks mean identical amino acids in the five Atg8 proteins.

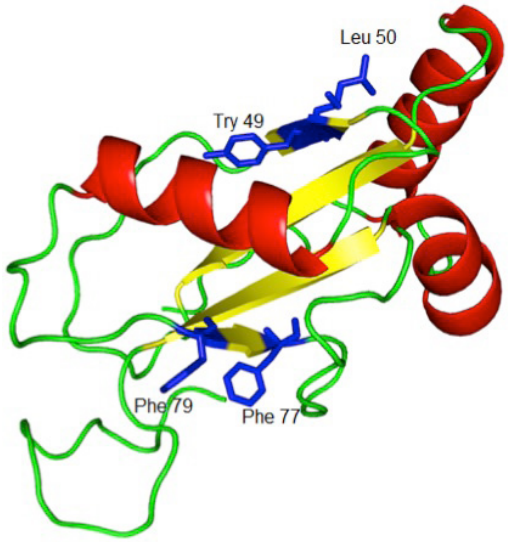

Figure 2. Three-dimensional model of MpAtg8p. Amino acids Tyr49, Leu50, Phe77, and Phe79 are marked in blue.

Alignment, template and construction of the 3-D model

The autophagic pathway of *M. perniciosa* is not very well described, except for some molecular data concerning protein expression (Santos et al., 2008; Pungartnik et al., 2009), in which the putative MpAtg8p is shown to be expressed during all phases of fungal growth with its induction related to oxidative stress. Therefore, the construction of a 3-D model of MpAtg8p permits the prediction of cellular function as well as provides a better understanding of domains present in the formed structure.

Sequence similarity of aa between ScAtg8p and MpAtg8p and prediction of secondary structure is shown in Figure 1, while the 3-D structure is shown in Figure 2. All proteins classified as Atg8p have one helicoid N-terminal domain [NHD, consisting of three α-helices, α1 to α3] and a second ubiquitin-like C-terminal domain [UDL, consisting of four β-sheets, β1 to β4] (Paz et al., 2000; Coyle et al., 2002; Sugawara et al., 2004; Nakatogawa et al., 2007). Similarity between the two proteins was 78% and MpAtg8p had regions characteristic of the ubiquitin family, which contain two domains consisting of three α-helices and four β-sheets, respectively (Figure 1). After analysis by SWISS MODEL (SwissPdb Viewer v.3.7) the 3-D arrangement of both domains could be identified (Figure 2). The model of MpAtg8p features a central structure formed by four β-sheets (β1 to β4), which is flanked by three α-helices (α1 to α3) (Figure 2). The first α-helix in the N-terminal region of ScAtg8p (which does not belong to the ubiquitin domain) is absent in MpAtg8p (Figure 2). The main part of the structure (aa 30 to 110) contains the four β-sheets (β1-β4) and two α-helices (α2 and α3) and resembles the characteristic ubiquitin structure owing to its significant aa sequence homology with the UBL protein family (Coyle et al., 2002). The β4 sheet of MpAtg8p C-terminal region is similar to those observed in mammalian homologues of the respective Atg8p microtubule-associated protein light chain 3 [LC3], Golgi-associated ATPase enhancer of 16 kDa [GATE-16] and receptor-associated protein [GABARAP] (Wang et al., 1999; Sugawara et al., 2004).

Alignment of the predicted MpAtg8p 3-D structure with its mammalian homologues GABARAP, LC3, and ScAtg8p yeast is shown in Figure 3. Their involvement in ubiquitination-like processes resembles that described for yeast and is similar to Atg4p, Atg3p and Atg7p (Tanida et al., 2003; Kabeya et al., 2004; Tanida et al., 2006). When comparing the 3-D structure of our model with that of known crystals of Atg8 [ScAtg8p, LC3 and GABARAP], a similarity can be inferred (Figure 3).

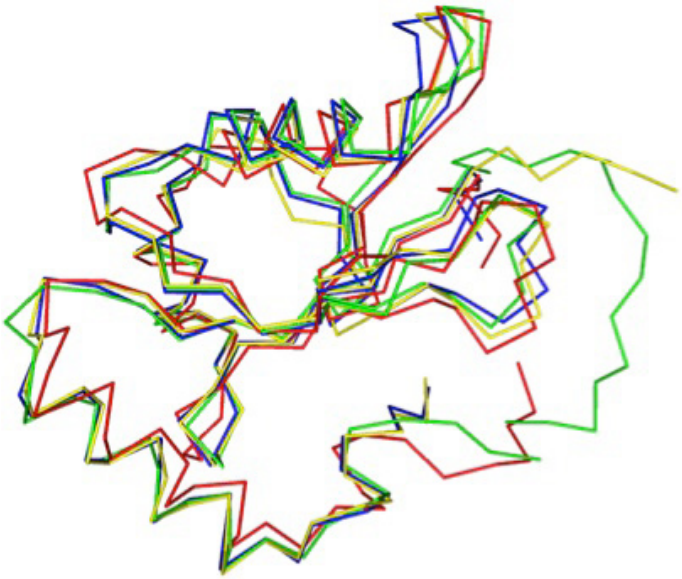

Figure 3. Alignment of the predicted MpAtg8p three-dimensional structure (red) with its mammalian homologues GABARAP (yellow), LC3 (green) and with yeast ScAtg8 (blue).

Modeling features for the yeast ScAtg8p as well as for the proteins LC3, GATE-16 and GABARAP include one α-helix in the first portion of the N-terminal region (Sugawara et al., 2004; Amar et al., 2006; Nakatogawa et al., 2007), whereas MpAtg8p lacks this structure (Figure 4). Compared with its homologue template (ScAtg8p), the N-terminal region of MpAtg8p contains Lys4, Asp7 and His9, differing from the ScAtg8p, which has Thr4, Ser7 and Tyr9 at the same positions. Since Nakatogawa et al. (2007) showed that deletion of the first N-terminal α-helix of ScAtg8p does not greatly impair the functionality of autophagy in this yeast, the observed aa variations in the N-terminus appear to be of minor influence.

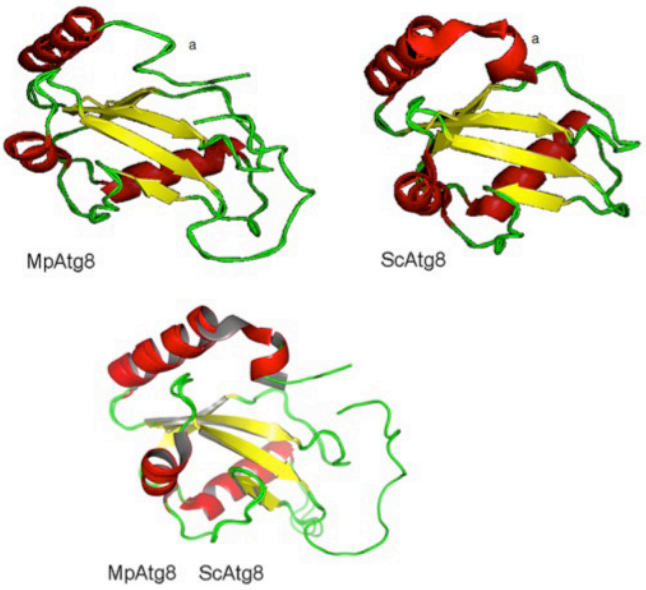

Figure 4. Structure of MpAtg8p, ScAtg8p and the three-dimensional structure of MpAtg8p/ScAtg8p alignment; "a" represents the initial N-terminal α-helix (aa 2-9).

Validation

While the comparison of sequences allowed prediction of the probable 3-D model of MpAtg8p, *in silico* validation of the proposed model is required. To validate the modeling for homology, it had to be ascertained whether the main chain and the aa that compose the lateral chains are in an energy favorable position and, therefore, in suitable structural conformation, when compared to already known 3-D structures. These validation processes were carried out using the softwares QMEAN, PROCHECK 3.4 and ANOLEA (Ramachandran et al., 1963; Melo et al., 1997; Melo and Feytmans, 1997; Benkert et al., 2011; Chen et al., 2011).

The assessment with QMEAN yields values between 0 and 1, and the closer the score is to 1 the higher the model's reliability. The validation of MpAtg8p using QMEAN showed a score of 0.75 (Benkert et al., 2011), supporting the model shown in Figure 2.

The Ramachandran plot as presented by PROCHECK represents the most favored regions for an aa position (Figure 5) and the ANOLEA plot presented by PROCHECK shows

energy distribution within MpAtg8p (Figure 6). Validation through the PROCHECK permitted the confirmation of the stereo-chemical quality of the model's structures, comparing them, aa for aa with other well-defined domain structures with a 2.0-Å resolution (Pereira ACF, Pungartnik C and Brendel M, unpublished results). The product of this analysis is shown in a Ramachandran plot (Ramachandran et al., 1963) and showed that 100% of aa in our MpAtg8p model were located in energy-favorable regions (Figure 5). The distribution of aa was as follows: 93.8% in the most favored regions, 5.4% in additionally allowed favored regions, and 0.9% in generally allowed regions without any aa located in disallowed regions.

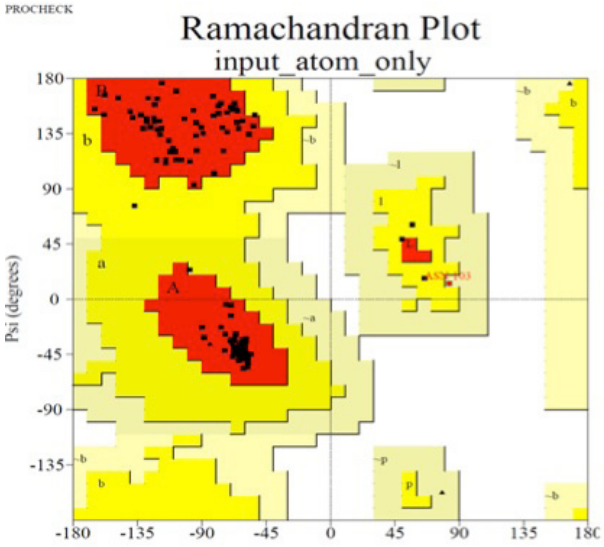

Figure 5. Ramachandran graphic as presented by PROCHECK. A, B, L (red regions) represent the most favored regions; a, b, l, and p (yellow region) represent additionally allowed regions; ~a, ~b, ~l, and ~p (light brown region) represent generally allowed regions.

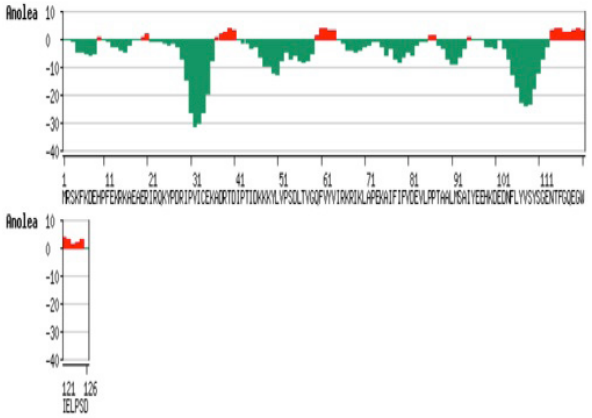

Figure 6. MpAtg8p energy. ANOLEA graphic presented by PROCHECK. The green bars represent regions of low energy.

Evaluation of the environment of atoms weighed by means of calculations of energy allows determination of possible regions of high energy within the protein, which are related to structural instability. Structural validation by the ANOLEA program showed 105 aa with low energy and 22 with high energy and total energy of -584 E/kT, pointing to a prevalence of regions with low energy in the MpAtg8p (Figure 6). As our proposed model had a value of 0.75, as most of the aa were located in favorable regions (Figure 5) and as our calculations did not predict high-energy but a predominance of low-energy regions (Figure 6), we are confident that it possesses structural reliability, since it was further validated by other analyses (Figures 2-4).

Prediction of the 3-D structure of MpAtg8 would provide valuable insight into the molecular basis of protein function. Here, we present the first 3-D model of the protein MpAtg8 that resembles the characteristic ubiquitin structure owing to its significant aa sequence homology with the UBL protein family. The aa sequence of MpAtg8p is highly identical with that of Atg8 proteins of other species of basidiomycetes, pointing to the conservation of this protein, and gives our model structural reliability. Since MpAtg8p may have the same function as its Atg8p homologs, this protein, essential for a functional autophagic pathway, deserves further study to perhaps serve as a new target to combat witches' broom disease.

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