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Molecular characterization and expression analysis of *Acmago* and *AcY14* in *Antrodia cinnamomea*

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ABSTRACT

Mago nashi (*Mago*) and *Y14* proteins, highly conserved among eukaryotes, participate in mRNA localization and splicing, and as such play important roles in oogenesis, embryogenesis and germ-line sex determination during animal development. Here we identified *mago* (*Acmago*) and *Y14* (*AcY14*) homologues derived from *Antrodia cinnamomea*. *Acmago* encodes 149 amino acids and *AcY14* encodes 168 amino acids. Multiple amino acid sequence alignment as well as secondary and tertiary structure prediction showed that *AcMago* and *AcY14* have similar protein structure to the reported crystal structures of other *Mago* and *Y14* proteins. During fungal development both *Acmago* and *AcY14* genes were abundantly expressed in natural basidiomes. This is the first report of the molecular characterization and expression analysis of the *mago* and *Y14* genes from fungi.

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Introduction

The *mago* (*Mago nashi*, grandchildless) gene, which is highly conserved among eukaryotes, was first identified as a strict maternal effect gene in *Drosophila* (*Drosophila melanogaster*; Boswell et al. 1991). It not only mediates germ plasm assembly in *Drosophila* but also regulates hermaphrodite germ-line sex determination in nematodes (*Caenorhabditis elegans*; Li et al. 2000; Newmark & Boswell 1994). Additionally, the *mago* gene is required for pollen tube growth in *Arabidopsis* (*Arabidopsis thaliana*) and root development in the conifer *Taiwania* (*Taiwania cryptomerioides* Hayata; Chen et al. 2007; Chen & Chu 2008; Johnson et al. 2004). In a yeast two-hybrid screen, the *Mago* protein interacted with the RNA binding protein *Y14* (also called *Tsunagi*, connection or link; Zhao et al. 2000).

Furthermore, crystal structure analysis demonstrated that they form a stable heterodimer strongly associating with spliced mRNA (Lau et al. 2003; Le Hir et al. 2001a; Shi & Xu 2003). The *Mago*-*Y14* heterodimer is required to localize *oskar* mRNA and to establish the polarization of the oocyte during *Drosophila* oogenesis (Hachet & Ephrussi 2001; Micklem et al. 1997; Mohr et al. 2001). Moreover, the *Mago*-*Y14* heterodimer is the core component of the exon junction complex (EJC), and associates with nonsense-mediated mRNA decay (NMD) and cytoplasmic mRNA localization (Bono et al. 2004; Fribourg et al. 2003; Kataoka et al. 2001; Kim et al. 2001; Le Hir et al. 2001b; Palacios et al. 2004; Park & Muench 2007).

Antrodia cinnamomea, a resupinate to effused-reflexed basidiomycete with porous hymenium (Chang & Chou 2004), is well-known by its Chinese name “Chang-chih” where it is

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used as a folk medicine in Taiwan. Recent reports have stated that it possesses a wide range of biological functions, including antioxidative, vasorelaxatory, anti-inflammatory, antitumor, hepatoprotective, and antiangiogenic activities (Chu et al. 2008). However, *A. cinnamomea* does not produce basidiomes in artificial culture and only grows on the inner surface of the heartwood cavity of the evergreen tree, *Cinnamomum kanehirai* Hayata, an endangered species endemic to Taiwan (Chang & Chou 2004). In order to further the development of this fungus as a source of potentially useful drugs while preserving the rare trees that it grows on in the wild, we are developing an improved artificial growth system for *A. cinnamomea*.

To better understand the growth and development of *A. cinnamomea*, we examined the function of two essential genes, *Acmago* and *AcY14*. Multiple amino acid sequence alignment and secondary and tertiary structure predictions all suggest that *AcMago* and *AcY14* have similar protein structure to the reported crystal structures of *Drosophila* and human *Mago* and *Y14* proteins. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) analyses confirmed that both *Acmago* and *AcY14* genes were abundantly expressed in natural basidiomes.

Materials and methods

Strain and growth conditions

A. cinnamomea strain TFRIB 479 was identified and provided by Dr. Tun-Tschu Chang (Taiwan Forestry Research Institute). The culture conditions for *A. cinnamomea* were identical to Chang and Wang's method (Chang & Wang, 2005). Natural basidiomes were obtained from the infested wood of *C. kanehirai*. Liquid-cultured mycelia, solid-cultured mycelia, solid-cultured basidiomes and natural basidiomes were frozen in liquid nitrogen and stored at -80°C until used.

RNA isolation and RT-PCR

The total RNA of each sample was isolated according to the manufacturer's recommendations (Hopegen; Chu & Chang, 2007). For reverse transcription, each 20 μl reaction containing 1 μg total RNA, 0.5 μg oligo(dT)_{12–18}, appropriate buffer, 0.5 mM dNTP mix, 10 mM DTT, and 200 units of Superscript II reverse transcriptase (Invitrogen) was incubated at 42°C for 50 min. Approximately 0.3 μg of the first-strand cDNA products were used for PCR amplification. The PCR reaction was carried out for 20 cycles, with each cycle consisting of denaturation at 94°C for 1 min, annealing at 58°C for 30 s, and extension at 72°C for 1 min. The *TM82* gene of *A. cinnamomea*, obtained from microarray data (Chu et al. 2008), served as an internal control to monitor reaction efficiency and to ensure that the initial amounts of cDNA were equal. The *TM82* was amplified with (5'-ACTGTGAAACTGCGAATGGCTC-3') and (5'-GACTTGCCCTCCAATTGTTCTC-3') primers. The *Acmago* and *AcY14* were amplified using the specific primers *Mago*RTF (5'-GTAAAGAGATGTGGGTTGGTCC-3'), *Mago*RTR (5'-TTCGGCAAAAAAGTGAATGC-3'), *Y14*RTF (5'-TGCAGGCGTCAGAAACACGAGC-3') and *Y14*RTR (5'-TCTCCCGCGACCTTTTATAGGC-3').

Cloning of *Acmago* and *AcY14*

The partial coding regions of *Acmago* and *AcY14* were amplified using the specific primers *Mago*-F (5'-ATGACCTCCTACGACAACGATC-3') and *Mago*-R (5'-CATATGGGCTTTATCTTG AAGTGC-3'), and the degenerate primers *Y14*-F (5'-CCGTGCGCTCYATNGARGGHTGGATC-3') and *Y14*-R (5'-GGYCGBACAAABGCAAAGTCNAC-3'). To obtain full-length *Acmago* and *AcY14*, 5'-RACE and 3'-RACE were performed using a SMART RACE cDNA Amplification Kit (BD Biosciences) with reverse primers *Mago*-5' (5'-CTCTTTCACAACCAGTGGACCAACCC A-3') and *Y14*-5' (5'-CAAACCTGTCCATCAGATCCTCCTCAGTC G-3'), and forward primers *Mago*-3' (5'-CTGGTGCAGATCCAAGACAGTGAAGAC-3') and *Y14*-3' (5'-GGATGGATCGTGTGGTCACTAACGTGC-3'). The PCR products were cloned using the pGEM-T Easy vector system (Promega) and sequenced on an ABI Prism 3700 DNA Analyzer (Perkin Elmer).

Homologues of *Acmago* and *AcY14* and protein structure prediction

The homologues of *Acmago* and *AcY14* were identified using BLAST algorithms at NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>). The gene sequences of *Acmago* and *AcY14* were submitted to GenBank (accession no. EF583522 for *Acmago* and EF583523 for *AcY14*). The homologues of *Acmago* and *AcY14* are listed in [supplementary Table S1](#), and multiple sequence alignment was carried out with the ClustalW program (<http://www.ebi.ac.uk/embl/index.html>).

The secondary and tertiary structures of *AcMago* and *AcY14* proteins were predicted by the SWISS-MODEL Protein Modeling Server (<http://swissmodel.expasy.org/>) and the illustrations of the tertiary structure and interaction prediction were viewed and edited by the DeepView - SwissPDB-Viewer program.

Phylogenetic analysis

The homologues of *AcMago* and *AcY14* used for phylogenetic analysis are listed in [supplementary Table S1](#). The analysis was performed using phyIip3.6 software. One hundred BS replicates were generated using SEQBOOT, distances were calculated using PROTDIST and trees inferred using NJ and BS values calculated using CONSENSE. The dendrogram was obtained using TREEVIEW.

Results and discussion

Molecular characterization of *Acmago* and *AcY14*

The partial sequences of *Acmago* and *AcY14* obtained by the degenerate primers were used for designing 5'-RACE and 3'-RACE primers. The sequence thus obtained for *Acmago* was 535 bp long, consisting of a 450 bp coding region encoding 149 amino acids, and a 85 bp 3'-UTR (GeneBank accession no. EF583522). *AcY14* was 898 bp long, comprising a 288 bp 5'-UTR, a 507 bp coding region encoding 168 amino acids, and a 103 bp 3'-UTR (GeneBank accession no. EF583523). In comparison with

Acma shared 71–93 % identity with other basidiomycetes and was also highly conserved among different organisms with 33–96 % identity. As shown by the alignment analysis, Mago proteins were highly conserved among eukaryotic

Fig 2 – Multiple amino acid sequence alignment and secondary structure prediction of AcY14. The positions of the RNP2 and RNP1 conserved motifs are indicated. Identical and similar residues are shaded in black and light grey respectively. The black arrows show the β -strands while the grey bars refer to α -helices. The abbreviations and the accession numbers are as given in Supplemental Table S1.

kingdoms. A conserved leucine zipper motif was identified in the C-terminal portion of the AcMago protein.

Y14 proteins, however, appeared to be more varied among different organisms, and AcY14 only shared 23–91 % identity between homologues. Y14 proteins had well-conserved RNA binding motif (RNP1 and RNP2) in the middle of the protein (Figs 1 and 2), and two arginine-serine dipeptide repeats that were characteristics of the SR splicing factors were found in the C-terminal portion of the AcY14 protein (Fu 1995).

Protein structures and interaction predictions between Acmago and AcY14

The predicted protein structure (Fig 3A) of AcMago shows six β -strands (β 1– β 6) and three α -helices (α 1– α 3), which is consistent with the reported crystal structures of the *Drosophila* (Shi & Xu 2003) and the human Mago proteins (Lau et al. 2003). The predicted secondary structure of AcY14 protein consisted of seven β -strands (β 1– β 7) and two α -helices

Fig 3 – Tertiary structure and interaction predictions of *AcMago* and *AcY14* proteins. (A, B) Front view of the conserved interaction surface of the predicted tertiary structures of *AcMago* and *AcY14*. (C) Interaction interface between *AcMago* and *AcY14* proteins. The identified residues represent the main residues of *AcMago* and *AcY14* protein involved in the predicted interaction.

($\alpha 1$ - $\alpha 2$), and the RNP2 and RNP1 sequence motifs located on $\beta 2$ and $\beta 5$ respectively (Fig 3B), again similar to the crystal structures of the *Drosophila* (Shi & Xu 2003) and the human Y14 proteins (Lau et al. 2003). Moreover, some residues in *AcY14*, such as Lys102 and Tyr104 located at $\beta 5$ (RNP1), were predicted to interact with Leu139 and Asp131 located at $\alpha 3$ of *AcMago* (Fig 3C; Fribourg et al. 2003).

Expression of the *AcMago* and *AcY14* during different stages

The variation of *AcMago* and *AcY14* expression in different *A. cinnamomea* samples, namely liquid-cultured mycelia, solid-cultured mycelia, solid-cultured basidiomes and natural basidiomes, was analyzed using RT-PCR with specific primers. A

389-bp DNA fragment, corresponding to the partial coding region and the 3'-noncoding region of the *AcMago* cDNA, and a 316-bp DNA fragment, corresponding to the partial coding region of the *AcY14* cDNA, were obtained. RT-PCR analysis indicated that the *AcMago* and *AcY14* genes were strongly expressed in natural basidiomes (Fig 4). There has been no previous report associating *mago* and Y14 genes with development in fungi. The particular roles of these genes and the associated protein interactions in fungal development merit further examination.

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Supplementary data

Supplementary data associated with this article can be found in the online version, at doi: [10.1016/j.mycres.2009.01.012](https://doi.org/10.1016/j.mycres.2009.01.012).

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Fig 4 – Expression patterns of *AcMago* and *AcY14* transcripts. Total RNAs were extracted from liquid-cultured mycelia (AL), solid-cultured mycelia (AM), solid-cultured basidiome (AF), and natural basidiome (AT) of *Antrodia cinnamomea*. The gene *TM82* of *A. cinnamomea* was used as an internal control.

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