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ORIGINAL PAPER

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# Cloning and sequencing of the *Aa-Pri1* gene specifically expressed during fruiting initiation in the edible mushroom *Agrocybe aegerita*, and analysis of the predicted amino-acid sequence

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**Abstract** A gene (*Aa-Pri1*) specifically expressed during fruiting initiation of the basidiomycete *Agrocybe aegerita* was cloned. The total length of the *Aa-Pri1* gene was 492 bp including a class-II intron of 54 bp size located at nt +125; the open reading frame encoded for a 145-aa protein of 16 093 Da. CCAAT (–156) and TATAAAT (– 83) boxes, and $T(A)_5T(A)_2$ (+593) and $T(A)_3T(A)_4T(A)_6T$ (+608) putative polyadenylation sequences were identified. The putative transcription start point was located at position 49. The Aa-Pri1 transcript was abundant only during fruiting initiation and was undetectable in the other stages of development. The Aa-Pri1 protein was hydrophilic, with a 20-aa hydrophobic motif in the NH2-terminal part, determining a putative $\alpha$-helix. Two putative glycosylation sites were identified. Aa-Pri1 protein activity may be controlled by the phosphorylation of several residues by different protein kinases.

**Key words** *Agrocybe aegerita* · Morphogenetic gene · Fruiting · Differentiation

## Introduction

The basidiocarps or fruit bodies of basidiomycetes are the most complex structures produced by fungi; knowledge of the genes triggering their fruiting initiation and maturation is of interest both for the elucidation of eukaryotic developmental control and for the industrial cultivation of mushrooms, since the yield of the crop depends on fruiting control. In mushrooms, most investigations have been based upon genetic analyses (for review see Esser et al. 1979) and upon the study of the influence of environmental factors on fruiting (Sietsma et al. 1977; Raudaskoski and Viitanen 1982). However, the regulation of the genes specifically involved in fruiting is still not well known.

In basidiomycetes, three genes have been characterized as specific for fruiting initiation: the *priA* (Kajiwara et al. 1992) and *priBc* (Endo et al. 1994) genes from *Lentinus edodes* and the *Sc1* gene from *Schizophyllum commune* (Dons et al. 1984). A different case is the *FRT1* gene expressed during vegetative growth and fruiting in *S. commune* since its ectopic genomic integration induces fruiting (Horton and Raper 1995). The *Sc7* (Shuren et al. 1993) and *mfbAc* genes (Kondoh et al. 1995) are over-expressed during the last stages of fruit body maturation in *S. commune* and *L. edodes*, respectively. The *hypA* (De Groot et al. 1996) and *ABH1* (Lugones et al. 1996) genes isolated from *Agaricus bisporus*, and the *Sc4* gene (Shuren and Wessels 1990) from *S. commune*, are expressed from initiation to full maturation, indicating their role in the expansion and/or maintenance of the fruit body.

The study of new morphogenetic genes specifically involved in primordia differentiation seems of particular interest for investigating fruiting regulation. In the edible mushroom *Agrocybe aegerita*, the genetic control of fruiting is well known (Esser and Meinhardt 1977; Meinhardt and Esser 1981). Moreover, this mushroom which is able to differentiate fruit bodies on synthetic medium, and whose homologous transformation has also been performed (Noël and Labarère 1994), provides a good tool to study the function and regulation of cloned morphogenetic genes.

Eight cDNA clones specific for primordial differentiation have previously been isolated in *A. aegerita* (Salvado and Labarère 1991). In this paper we report the cloning of a new gene (*Aa-Pri1*) specifically expressed during fruiting initiation. The nucleotide sequences of the cDNA and the cloned genomic DNA were compared in order to determine the possible presence of introns. The 5′ and 3′ flanking regions of the gene were analysed in the search

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for regulatory signals and for transcription start points. The specific expression of the *Aa-Pri1* gene was confirmed. The deduced amino-acid sequence was analysed and compared with the proteins in databases.

## Materials and methods

*Strains and culture conditions*. The *A. aegerita* dikaryotic strain used was from a subculture of a basidiocarp fragment of the wild-type strain SM51 (*A1B1/A2B2*). Fruit bodies at various stages of development were obtained as previously defined (Salvado and Labarère 1991). The vegetative mycelium was cultivated on liquid CYM medium (Raper et al. 1972) in Roux flasks, without shaking, at 25°C in the dark. The *Escherichia coli* strain JM83 was cultivated as described by Yanish-Perron et al. (1985).

*DNA isolation and analysis*. Total DNA was isolated according to Noël and Labarère (1989). Digested DNA was separated in a 0.8% agarose gel and transferred to a nylon membrane (Hybond-$N^+$, Amersham Corporation) according to the manufacturer's procedure. Hybridizations of digested DNA with the labelled probes were performed in standard conditions according to Maniatis (1982).

*RNA isolation and analysis*. Total RNAs were isolated using the hot-phenol procedure (De Vries et al. 1980). RNAs were fractionated in a 1.4% agarose-formaldehyde gel according to Maniatis et al. (1982) and blotted onto a nylon membrane by capillary transfer using 1.5 M NaCl/0.5 M NaOH as transfer buffer. Northern hybridizations were performed at 42°C in the standard hybridization buffer in the presence of 50% formamide. The nylon membranes were washed for 20 min with 1×SSC/0.1% SDS at 25°C and for 10 min with 0.2×SSC/0.1% SDS at 55°C. Poly(A) RNAs were prepared using the polyAtract mRNA system (Promega), according to the manufacturer's instructions.

*Preparation of radioactive probes*. EMA inserts released after *Bam*HI digestion of pEMA plasmids were purified from agarose-gel electrophoresis by the "Gene Clean" system (BIO 101). The *Pleurotus cornucopiae* 18*s* rDNA probe was obtained by *Sal*I-*Eco*RI digestion of the pPcR1 insert (Iraçabal and Labarère 1994). Probes were labelled by random priming with 25 μCi $\alpha$-$^{32}$P-dCTP (3000 Ci/mmol, Amersham Corporation) to a specific activitiy of $10^7$–$10^8$ cpm/μg, using the Primer-a-Gene Labelling System Kit (Promega).

*Genomic library construction and screening*. The genomic DNA isolated from the vegetative mycelium was digested with *Pst*I and separated on 0.8% agarose gels. The gel segment containing fragments from 2.9 to 3.3 kbp was cut out. The DNA was purified using the "Gene clean" system (Bio 101) and ligated to the *Pst*I-linearized pUC18 vector, which was de-phosphorylated by bacterial phosphatase alkaline treatment. The ligation mixture was used to transform *E. coli* JM83 cells, and transformant clones were screened by colony hybridization using the labelled EMAa-4 cDNA insert as probe.

*Sequencing and sequence analysis*. Nucleotide sequences were determined on both strands using overlapping deleted subclones generated by the method of Henikoff (1984) with the Erase-a-base kit (Promega). Gaps were eliminated using specific oligonucleotides as sequencing primers. Sequencing was performed as described by Sanger et al. (1977) using the Sequenase version 2.0 kit (United States Biochemical) . Amino-acid sequences were determined using the universal genetic code and compared with proteins from GenBank and EMBL databases using the Blast search algorithm (Altschul et al. 1990). The Isoelectric and Bestfit programs of the Genetics Computer Group package (GCG - Wisconsin package, v. 9.0) were used to determine the pI and to compare the amino-acid sequences, respectively. The hydrophobicity plot and molecular weight were both determined with the DNA Strider (1.2) program based on the Kyte-Doolittle algorithm. The GenBank accession number of the *Aa-pri1* nucleotide sequence is AF004297.

*Primer extension*. The transcription start points were determined by primer extension according to Kajiwara et al. (1992) with minor modifications: (1) the poly(A) RNAs (4.4 μg) were annealed for 5 min at 70°C with 2.2 μg of an 18-mer oligonucleotide (5′-GAG CAT AGG CCC TTT CAT) corresponding to the antisense strand from nt 17 to 34, (2) reverse transcriptase (67 units of AMV Reverse Transcriptase, Promega) and 25 μCi of $\alpha$-$^{32}$P-dCTP (3000 Ci/mmol, Amersham Corporation) were added and incubation was carried out at 42°C for 60 min. After phenol/chloroform extraction and precipitation, the extension products were air-dried, separated in a 7 M urea/6% polyacrylamide electrophoresis gel and visualized by autoradiography. The sizes of the extension products were determined by comparison with the sequencing ladders derived from the bacteriophage M13mp18.

## Results

### Cloning of an *A. aegerita* genomic DNA fragment hybridizing to the EMAa-4 cDNA insert

Hybridization of the digested *A. aegerita* total DNA with the pEMAa-4 probe corresponding to a mRNA specific for primordial differentiation revealed single hybridizing bands in all cases, with estimated sizes of 2.4, 2.7 and 3.1 kb for *Eco*RI, *Hin*dIII and *Pst*I digestions, respectively. To clone the genomic fragment carrying the gene corresponding to the EMAa-4 insert, the 2.4-kb *Eco*RI fragment was ligated to the *Eco*RI-linearized pUC18 vector. The recombinant plasmids were used to transform *E. coli* cells, and a recombinant clone was identified after colony hybridization with the labelled EMAa-4 insert as a probe. The 2.4-kb *Eco*RI fragment was then subcloned in both orientations, and recombinant plasmids were used as templates for DNA sequencing, after construction of overlapping deleted subclones by Exonuclease III treatment.

### Analysis of the nucleotide sequences

The complete nucleotide sequences of the 2.4-kb genomic *Eco*RI fragment and of the hybridizing cDNA were determined on both strands. Analysis of the genomic sequence revealed an open reading frame of 492 nt, homologous to the cDNA sequence.

Alignment of the genomic and corresponding cDNA sequences identified a class-II 54-bp intron located from nt + 125 to nt + 179. The 3′ intronic splice site (CAG) bears the typical consensus sequence for splicing (YAG; Y = C or T) (Gurr et al. 1987). However, at the 5′ boundary, the intron had the nucleotide pair GC instead of the classical GT. The putative internal sequence required for lariat formation (TACTTAC) was similar to the consensus sequence NNCTRAY (N = A, C, G or T; R = A or G; Y = C or T) reported for filamentous fungi (Unkles 1992), with the exception of the T at position 5.

The *Aa-Pri1* gene used C in 66% of codon endings. Likewise, when a purine is required in the wobble posi-

tion, 66% of the codons used G and its codon bias (49 codons used for translation) was less marked than in highly expressed fungal genes. On the seven nt of the ATG-surrounding sequence, four (nt-3 = A, nt-1 and -2 = C, nt+1 = G) matched the consensus sequence CC(A/G)-CC**ATG**GC of Kozak (1984).

In the 5′ flanking region of the genomic sequence, a TATA-box (TATAAAT) at nt –83, and a CAAT-box (CCAAT) at nt –156, were identified. In the 3′ flanking region, two sequences similar to the consensus polyadenylation sequence AATAAA were located at nt +593 [$T(A)_5T(A)_2$] and nt +608 [$T(A)_3T(A)_4T(A)_6T$]. Primer extension (Fig. 1) revealed three putative transcription start points (tsp) at nt –49, –85 and –91. The tsp at nt –49 was surrounded by a $TCA^{+1}$TTCC sequence matching the consensus initiator sequence $YYA^{+1}$NWYY (N = A, C, G or T; W = A or T; Y = C or T) (Javahery et al. 1984).

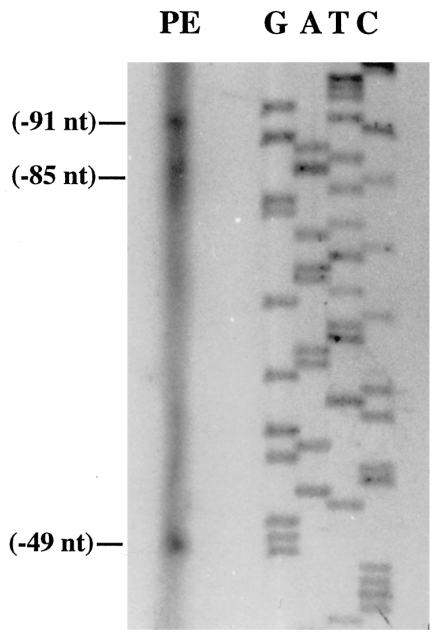


**Fig. 1** Transcription start points of the Aa-Pri1 transcript, determined by primer extension (PE) using an 18-mer primer corresponding to the antisense strand from nucleotide 17 to 34. Sizes of the extension products were determined by comparison with the sequencing ladders (GATC) derived from the bacteriophage M13mp18 DNA

## Deduced amino-acid sequence of the Aa-Pri protein

The *Aa-Pri1* gene encoded an acidic protein (pI = 5.7) of 145 amino acids with a calculated molecular weight of 16 093 Da. The deduced Aa-Pri1 protein was rich in glycine (11%), serine (9.6%), asparagine (8.9%), aspartic acid (7.5%) and lysine (7.5%). The protein was predominantly hydrophilic, although one hydrophobic region of 20 aa (from aa12 to aa32) determining a putative α-helix was predicted in the NH2-terminal sequence (TopPredII program). The Aa-Pri1 amino-acid sequence had 39.1% homology and 46.8% similarity with the Asp-hemolysin (Fig. 2) in *Aspergillus fumigatus* (Ebina et al. 1994).

Two putative N-glycosylation sites were located at positions 73 (NASS) and 91 (NKTI). Six putative phosphorylation sites were identified: (1) one for a cyclic AMP-dependent protein kinase (KRNT) starting at lysine[106], (2) two for a casein kinase II protein (SNKD) starting at serine[3] and serine[76] and (3) three for a protein kinase C starting at serine[3] (SNK), threonine[93] (TIR) and serine[105] (SKR).

## *Aa-Pri1* gene expression during the life cycle

To study the expression of the *Aa-Pri1* gene, total cellular RNAs were isolated from four stages of development: from vegetative mycelia to mature fruit bodies. For each stage, total RNAs were subjected to Northern-blot analysis using the labelled EMAa-4 cDNA insert as a probe (Fig. 3A). The same amount of total RNAs (30 μg) was loaded onto each lane, as verified by ethidium bromide staining of the gel before transferring. Northern analysis revealed a strong signal corresponding to a 600-b RNA band in the primordial extract (lane 3); vegetative mycelia (lane 1), aggregates (lane 2) and mature fruiting bodies (lane 4) did not contain detectable amounts of the *Aa-Pri1* transcript.

As a control, the stochiometric transfer of the four RNA preparations was examined by de-hybridization and reprobing the blot with the *P. cornucopiae* 18*s* rDNA (see

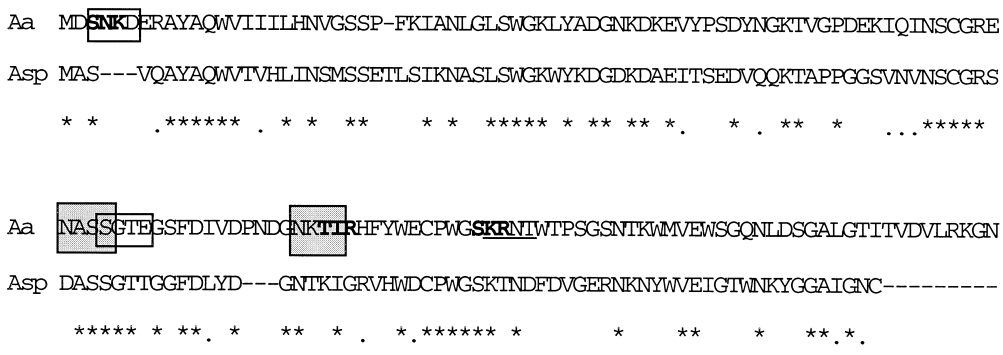


**Fig. 2** Comparison of the Aa-Pri1 protein with the Asp-hemolysin from *A. fumigatus* (GeneBank accession number: D16501). Identical and similar amino acids are indicated by (*) and (.) respectively; putative glycosylation motifs are indicated by *large shaded boxes*; the sites phosphorylated by the cAMP-dependant protein kinase are *underlined*, by casein kinase II are *boxed* and by protein kinase 5 are in *bold letters*

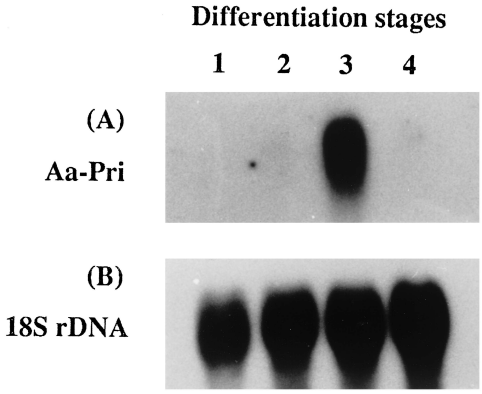


**Fig. 3A,B** Expression of the Aa-Pri1 transcript during fruiting. Total cellular RNAs were isolated from the vegetative mycelia (*lane 1*), aggregates (*lane 2*), primordia/immature fruiting bodies (*lane 3*), and mature fruit bodies (*lane 4*). The Northern hybridization experiment (**A**) was performed using the EMAa-4 cDNA insert as probe. **B** Northern obtained after re-probing the blot with a *P. cornucopiae* rDNA probe

Materials and methods) which did not exhibit differential expression during the life cycle; 18*s* rDNA signals were detected in all RNA blots with similar intensities (Fig. 3B). Since the RNA concentrations in each sample were similar, it was obvious that the *Aa-Pri1* gene was actively transcribed only at the stage of primordia/immature fruit bodies.

## Discussion

The *Aa-Pri1* gene (492 bp), specifically transcribed at the stage of primordia/immature fruit bodies, possessed a short class-II intron 54-bp long. Short introns of 59 and 52 bp, similarly located in the 5′ part, were described in the *Aspergillus nidulans pyrG* and *Fusarium solani cutA* genes, respectively (Gurr et al. 1987). At the 5′ boundary, instead of the classical GT, the *Aa-Pri1* intron had the nucleotide pair GC like other fungal introns present in the *des-1* and *qa-IS* genes from *Neurospora crassa* (Gurr et al. 1987), the *pgaI* gene from *Aspergillus niger* and the *ras* gene from *L. edodes* (Unkles 1992).

TATA and CAAT boxes were located at nt –83 and nt –156. Similar sequences have been identified in many fungal genes; for example, at nt –96 and –188 in the *cbh2 Trichoderma reesei* promoter (Unkles 1992), and at nt –111 and –167 in the *priA L. edodes* promoter (Kajiwara et al. 1992). The transcription start point located at nt –49 downstream from the putative TATA box appears to be the more probable; moreover; this tsp begins with the most common dinucleotide pair (CA) found in eukaryotic tsp (Breathnach and Chambon 1981) and is surrounded by a strong initiator sequence. At the 3′ end of the gene, the poly(A) tail starts 145 nt dowstream from the predicted translation stop codon, and two polyadenylation sequences were found upstream of the polyadenylation site.

The Aa-Pri1 protein has 39.1% homology with the Asphemolysin of *Aspergillus fumigatus* (Ebina et al. 1994). Fungal hemolysins have been reported in *Gyromitra esculenta* (Gray 1973), *Tricholoma populinum* (Lindequist et al. 1989) and *Laetiporus sulfureus* (Konska et al. 1994); moreover, a cytolytic protein has been purified from *Pleurotus ostreatus* (Bernheimer and Avigad 1979), and cytolytic activities have been reported for *Amanita rubescens* and *Amanita phalloides* (Seeger and Wachter 1981). However, the functions of these molecules in fungal cells are not clear. Nevertheless, based on the ability of hemolysins to interact with membrane receptors (Thelestam and Möllby 1979) and on the presence of a hydrophobic region in the NH2-terminal sequence of the Aa-Pri1 protein, it can be hypothesized that the binding of the Aa-Pri1 protein with specific membrane receptors in the hyphae allows for their aggregation and compaction to form the primordia. Moreover, the property of binding to the cellular and/or the nuclear membrane has also been reported for the priA protein from *L. edodes*, which plays a role during fruiting initiation (Kajiwara et al. 1992). However, the mechanism of the putative binding seems to be different for the Aa-Pri1 protein, since no C-terminal Cys-Aaa-Aaa-Xaa box was found.

It is of particular interest that the Aa-Pri1 protein contains several residues that could act as substrates for phosphorylation by protein kinases and several putative phosphorylation sites for cAMP-dependant protein kinase, protein kinase C, and casein kinase II. A possible role of cyclic AMP through cAMP-dependent protein kinases has been proposed in the fruiting of *Coprinus macrorhizus* (Ishikawa and Uno 1977). Moreover, it is known that (1) high levels of cAMP are closely related to fruiting initiation in *L. edodes* (Takagi et al. 1988), (2) a putative cAMP-dependent protein kinase phosphorylation site was found in the *priBc* protein preferentially synthesized during the primordial stage (Endo et al. 1994), and (3) protein kinases seem to be involved in cellular growth (Hanks et al. 1988).

Northern hybridizations with total RNAs extracted from four stages of development clearly showed that the *Aa-pri1* gene is specifically expressed during the primordia/immature fruit body stage, and that it is not expressed (or else not at a detectable level) during the vegetative growth or the late stages of fruit body maturation. In Basidiomycetes, three other genes have been shown to be specifically expressed during the same stage: the *priA* and *priBc* genes from the mushroom *L. edodes* (Kajiwara et al. 1992; Endo et al. 1994) and the *Sc1* gene from the fungus *S. commune* (Dons et al. 1984; Wessels et al. 1991). It is of interest to note that, like the pri Bc protein of *L. edodes*, the Aa-Pri1 protein possessed a cAMP-dependent phosphorylation site, suggesting that the corresponding structural gene could be regulated by the amount of cAMP in the cells. In this sense, future studies involving gene-disruption experiments and the expression of the Aa-Pri protein should be conducted to study the in vivo function of the protein.

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