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The gene coding for the DOPA dioxygenase involved in betalain biosynthesis in *Amanita muscaria* and its regulation

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Abstract Genomic and cDNA clones derived from the gene (*dodA*) coding for DOPA dioxygenase, a key enzyme in the betalain pathway, were obtained from the basidiomycete *Amanita muscaria*. A cDNA library was established in the phage λZapII and *dodA* clones were isolated using polyclonal antibodies raised against the purified enzyme. Their identity was confirmed by comparison of the deduced amino acid sequence with the sequence of several tryptic peptide fragments of DOPA dioxygenase. The gene coded for a 228-amino acid protein that showed no homology to published sequences. The coding region was interrupted by five short introns. Regulation was shown to occur at the transcriptional level; the mRNA accumulated to high levels only in the coloured cap tissue. *dodA* was found to be a single-copy gene in *A. muscaria*. To our knowledge, this is the first gene from the betalain pathway to be cloned. It encodes a type of aromatic ring-cleaving dioxygenase that has not been previously described.

Key words *Amanita muscaria* · Betalain · Extradiol ring-cleaving dioxygenase · Basidiomycete · Molecular cloning

Introduction

Betalains are a class of yellow or violet vacuolar pigments characteristic of plants of the order Caryophyllales and also of a few fungal species belonging to the genus *Amanita*, e.g. *A. muscaria*, and *Hygrocybe* (Besl et al. 1975; Gill and Steglich 1987; Clement et al. 1994). Originally referred to as "nitrogen-containing anthocyanins" their structure has been elucidated and the biosynthetic pathway is now well characterised as to the chemical intermediates involved (for reviews see Mabry 1980; Piattelli 1981; Steglich and Strack 1990). However, very few of the enzymes involved in betalain synthesis have been purified and characterized so far, in spite of the importance of betalains as natural food colourants. The only enzyme activities that have been described from higher plants are enzyme preparations that catalyse the glucosylation of betanidin (Heuer et al. 1996) and enzymes involved in betalain degradation (Kumon et al. 1990), but none of the genes have been cloned.

In plants, betalain biosynthesis is subject to complex regulation and the pigments accumulate only in certain tissues and at specific stages of development. Their synthesis has been shown to be regulated by light and cytokinins (Biddington and Thomas 1973; Kochhar et al. 1981; Kishima et al. 1995). Beet cell lines expressing different colour phenotypes have been established and the factors affecting the type and quantity of betalains produced have been analysed (Girod and Zryd 1987; Girod and Zryd 1991b; Leathers et al. 1992). Genetic analysis of betalain biosynthesis in the ornamental plant *Portulaca grandiflora* indicated that three loci were sufficient (Trezzini and Zryd 1990). All this makes betalains an interesting model for the study of secondary metabolism.

Recent work on the enzymology of betalain biosynthesis has focused on the mushroom *Amanita muscaria*. In this basidiomycete the accumulation of betalain and muscaflavin pigments is restricted to the cuticle of the cap and is subject to developmental regulation. There is a rapid increase in pigment levels during the process of development from very young mushrooms, which are still entirely covered by the universal veil, to mature specimens. The fast growth and the rapid accumulation of pigments make *A. muscaria* an interesting organism for the isolation of the enzymes and genes involved in betalain synthesis. The enzymes that catalyse the first two steps in betalain biosynthesis, a tyrosinase and a

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DOPA dioxygenase, have been purified from this fungus and characterized (Girod and Zryd 1991a; Müller et al. 1996). *A. muscaria* accumulates not only betalains, but also muscaflavin in the coloured tissues of its cap (Von Ardenne et al. 1974; Musso 1979). Betalamic acid, the chromophore of all betalain pigments, and muscaflavin are both derived from the precursor DOPA by extradiol ring cleavage, followed by intramolecular recyclization. Cleavage between carbons 4 and 5 gives rise to an unstable seco-DOPA and then to betalamic acid, while cleavage between carbons 2 and 3 gives rise to muscaflavin (Terradas and Wyler 1991). Such extradiol ring-cleaving dioxygenases are well known from bacteria that degrade aromatic compounds, but not from eukaryotes (Hayaishi et al. 1975; Harayama and Kok 1992).

In this work we describe the cloning and the regulation of the gene *dodA* coding for the DOPA dioxygenase from the basidiomycete *A. muscaria*. To the best of our knowledge, this is the first gene from the pathway to be cloned. Moreover, it encodes a type of ring-cleaving dioxygenase not described previously.

Materials and methods

Biological material, bacterial strains, DNA techniques and chemicals

A. muscaria carpophores were collected under fir trees in the Jorat forest near Lausanne (Switzerland). The fungi were divided into three classes: young (still entirely covered by the universal veil), medium (expanding, domed cap) and mature (convex cap). The stipe and the betalain-containing cap tissue were separated, frozen in liquid N_2 and stored at −80° C.

Routine molecular biological techniques were performed as described in Sambrook et al. (1989).

Phage λZapII and its host *E. coli* XL1-Blue (Stratagene) were used for cloning procedures. A cDNA library was established using a kit from Pharmacia Biotech. Restriction enzymes were purchased from New England Biolabs and used according to the instructions of the suppliers. DNA sequences were determined according to the procedure of Sanger et al. (1977), using Sequenase Version 2.0 (United States Biochemicals-Amersham). *E. coli* tRNA and oligo (dT) cellulose were from Boehringer Mannheim.

Purification of DOPA dioxygenase and antibody production

DOPA dioxygenase was purified essentially as described by Girod and Zryd (1991a), except that affinity chromatography was replaced by hydrophobic interaction on C8-Sepharose (Pharmacia) and preparative SDS-PAGE was used as the last purification step. Antibodies were raised in white New Zealand rabbits. Crushed gel slices containing about 100 μg of DOPA dioxygenase were injected subcutaneously as described (Harlow and Lane 1988). The injection was repeated after 10 and 40 days, and serum was collected after 45 days.

Isolation of tryptic fragments and sequence analysis

After preparative SDS-PAGE DOPA dioxygenase was transferred to a PVDF membrane as described by Harlow and Lane (1988) and digested with 1% trypsin (wt/vol) for 24 h at 37° C. The fragments were eluted using 80% formic acid and dried under a stream of N_2. The peptides were resuspended in a small volume of 0.1% trifluoroacetic acid and separated by reversed-phase HPLC on a Vydac C18 column. Individual peaks were collected and analysed on an Applied Biosystems Model 470A gas-phase sequenator.

RNA extraction and construction of a cDNA library

The faintly yellow cap tissue from young *A. muscaria* specimens was ground to a fine powder in liquid N_2 and total RNA was extracted using a buffer containing guanidium thiocyanate (Wadsworth et al. 1988). Polyadenylated RNA was purified on oligo (dT) cellulose and 5 μg were used to make a cDNA library. Part of the cDNA (0.1 μg) was ligated with 1 μg of de-phosphorylated λZapII arms and packaged using a Gigapack Gold II kit (Stratagene) according to the instructions of the supplier. The library (10^6 plaque forming units) was plated on *E. coli* XL1-Blue and 10^5 clones were screened using anti-DOPA dioxygenase serum diluted 1/1000 and alkaline phosphatase-labelled secondary antibodies (Sigma). Positive clones were purified and subjected to in vivo excision to obtain the plasmid pBluescript-DodA.

DOPA dioxygenase activity of the fusion protein was measured in permeabilized bacteria in 10 mM DOPA, 5 mM DTT at pH 8.6 using 100 mM EDTA as buffer, and the formation of yellow products was monitored at 424 nm.

Isolation of *dodA* genomic clones and Southern blot analysis

A. muscaria genomic DNA was extracted using cetyltrimethyl ammonium bromide as described by Rogers and Bendich (1988) and digested with various restriction enzymes. The fragments were separated by electrophoresis through an agarose gel and transferred to a nylon membrane (Zeta-probe, Biorad). The filters were processed according to the instructions of the suppliers and hybridised overnight with a radiolabelled probe made from the coding part of the cDNA using a random hexamer primer kit from Pharmacia. Hybridisation was in 3 × SSPE, 0.5% SDS, 5 × Denhardt's, 0.1 mg/ml tRNA at 55° C.

For construction of the genomic library the DNA was digested with *Eco*RI. The approximately 5-kb fragments corresponding to the size class containing the DOPA dioxygenase gene were isolated by preparative electrophoresis through an agarose gel, and 300 ng of the fraction was ligated with 1 μg dephosphorylated λZapII arms. The DNA was packaged and the phage were plated as described above. Replicas were made on Zeta-probe membranes and the library (5×10^5 pfu) was screened using a radiolabelled probe as described for Southern blot analysis.

Northern blot analysis

Total RNA (2 μg each) was extracted from the stipe and from the yellow cap tissue of young specimens as described above. The RNA was subjected to electrophoresis through a formaldehyde-containing agarose gel (Sambrook et al. 1989) and was then transferred to a nylon membrane. Hybridisation conditions were the same as described for the Southern blots.

Results

Isolation of cDNA clones for DOPA-4,5-dioxygenase from *A. muscaria*

DOPA dioxygenase was purified from the coloured tissue of expanding, domed caps of *A. muscaria*. We determined the amino acid sequences of five tryptic peptides, corresponding to about one-quarter of the

polypeptide. Comparison with the sequences published in the Swissprot and EMBL databanks indicated that there was no significant similarity with known sequences.

A cDNA library was established in an expression vector (phage λZapII) using poly(A)+ RNA from the cap tissue of young specimens that had not yet begun to synthesize betalains. Part of the library (10^5 clones) was screened with anti-DOPA dioxygenase antibodies that had been raised in rabbits, and 20 positive clones were obtained, indicating that the DOPA dioxygenase mRNA was abundant and that the *dodA* gene was highly expressed. These clones all had inserts of approximately 750 bp, and expressed active DOPA dioxygenase; the lysed bacteria converted DOPA to bright yellow products that were identified as betalamic acid and muscaflavin by HPLC (Müller et al. 1997a). DNA sequence analysis of several clones showed that they were identical, and the deduced amino acid sequence exactly matched the sequence of the tryptic fragments. Apparently a single DOPA dioxygenase gene is expressed exclusively, or at least predominantly. The *dodA* cDNA clones coded for a 612-bp open reading frame (ORF) followed by a 62-bp untranslated 3′ region (Fig. 1). The first ATG in the sequence was not in a favourable context for optimal protein synthesis (Kozak 1991) and would have given rise to a 185-amino acid protein. The apparent molecular mass of DOPA dioxygenase estimated by SDS-PAGE was 24.7 kDa, suggesting that the cDNA clones lacked about 100 bp of the coding region at the 5′ end. Repeated screening of the library yielded more clones, but none was complete.

Fig. 1 Nucleotide sequence and deduced amino acid sequence of the *dodA* gene from *A. muscaria* and its product. Only the sense strand is shown in the 5′ to 3′ orientation, numbered from the first nucleotide of the presumed initiation codon. Intervening sequences are in *lower case*. The sequences of the peptide fragments that were determined by Edman sequencing are *underlined*. The 3′ sequence after the stop codon corresponds to that of the cDNA and the corresponding fragment was not sequenced in the genomic clone. The nucleotide sequence has been deposited in the EMBL databases under accession number Y12886

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                                acatgcgcttcgtcactcaaatatatccattggtttcgcgacacgctggttccgaacccagagagtctcgc  -501
gactctcttcagaaattctcccaacttgcgttccttcaacacccctttggtgcccgcggcagaggactgcgacagttagccatgacggattctttcattc  -401
ctctctgacgactatccatgcagaggccaggattctggactatgctagtcctatcccacctcatctcatgaaccttccttctctacgccagatgtccttc  -301
gttgttcctctttcgccgggcagacgcacgttggcgcagtttcctcgagtggcatggttcacgtttacagcatatccaagctttgatttaccttgcaggg  -201
aaccacttccagtccgacttggcctacattgcatcatcatgccgcaaccttcttagactcgatcttgctttctcccattggacggcatatgccgtttcct  -101
gttagcctcccgcctacagtggaacatctgggcatcttttgtactcagggtcagatcgccaactaccagacgttcttctctcgtttagataggatcgagt  -1

ATGGTGCCAAGCTTCGTTGTATACAGTTCTTGGGTAAACGGACGTCAGAGATATATTCGACAAGCATTTGCATCAATTCTGgtctggcacgaggcgacta  100
M  V  P  S  F  V  V  Y  S  S  W  V  N  G  R  Q  R  Y  I  R  Q  A  F  A  S  I  L

agaggtagtggggttagattcatgaatcatagaggggcatgtatagcgtagctaaggatagcactggcagttccacagTTCTATATAATCCGTGATACGA  200
                                                                               F  Y  I  I  R  D  T  T

CTCTCTCGTTCCCCTCACATACTACCATGTCCACCAAGCCAGAGACTGACCTTCAAACTGTCCTCGACAGCGAAATCAAgtttgtctagtttgtctccact 300
  L  S  F  P  S  H  T  T' M  S  T  K  P  E  T  D  L  Q  T  V  L  D  S  E  I  K

agcaaaaactctgctgacttgtgtacagGGAATGGCACTTTCgtcagtcgaccaagcaacgactaacttatgtagctcaaattgaccctgcaactaatag  400
                             E  W  H  F  H

ACATCTACTTTCATCAGAACAACGCCGCAGAGCATCAAGCTGCGCTTGAGCTTCGTGACGCGGTTCTGAGGCTCAGACAAGACGGCGCATTCGTCGCCGT  500
  I  Y  F  H  Q  N  N  A  A  E  H  Q  A  A  L  E  L  R  D  A  V  L  R  L  R  Q  D  G  A  F  V  A  V

TCCCTTGTTCCGCGTTAACATGGACCCCATGGGTCCTCATCCTGTCGgtcagtagtcatcgcaatcacaccaccagtccttgaacaagtcccttgactct  600
 P  L  F  R  V  N  M  D  P  M  G  P  H  P  V  G

caagGTTCTTATGAGATCTGGGTTCCGTCTGAAACGTTCGCTTCCGTGTTCTCCTACTTGTGCATGAACAGAGGGAGATTAAGCATCCTTGTGCATCCTT  700
    S  Y  E  I  W  V  P  S  E  T  F  A  S  V  F  S  Y  L  C  M  N  R  G  R  L  S  I  L  V  H  P  L

TGACACGCGAAGAACTCAGAGACCATGAAATTCGTAATGCCTGGATAGGACCCTCTTTCCCACTCAATCTCGCCAACCTACCGATCAAGAGTGATGAGAT  800
  T  R  E  E  L  R  D  H  E  I  R  N  A  W  I  G  P  S  F  P  L  N  L  A  N  L  P  I  K  S  D  E  I

CCCTTTGCAATATCCAAGCCTCAgtatgtcatctcaccactctctggcggccctcagtgacaaattgttgcagAGCTTGGGTACTCATCGACAGCGCATA  900
 P  L  Q  Y  P  S  L  K                                                    L  G  Y  S  S  T  A  H  K

AGATGTCATTGGAAGAAAGGCGGAAATTAGGCGACGATATAGAAGCAGTGCTTAGGGGAGAGAAAGAGGCGGCCAGAGCGCCCCATCGAGATGCAtagag  1000
  M  S  L  E  E  R  R  K  L  G  D  D  I  E  A  V  L  R  G  E  K  E  A  A  R  A  P  H  R  D  A  *

ctacattcgattgtctatattgctactgacatagggtaatgttggaattttctgccc 1057
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Isolation of genomic clones and structure of the *dodA* gene

A partial, size-selected, genomic library was constructed in order to characterise the 5′ end of the coding sequence and at the same time learn about the gene structure. Digestion of the genomic DNA with the enzyme *Eco*RI gave a single 5-kb fragment on Southern blots. *Eco*RI fragments of this size were isolated by preparative agarose gel electrophoresis and cloned into λZapII. Screening of the library with a radiolabelled probe derived from the coding region of the *dodA* cDNA yielded twelve positive clones, two of which were subjected to sequence analysis. The positions of the introns and the transcription initiation site were determined by comparison with the *dodA* cDNA sequence (Fig 1). The splice sites were in agreement with the fungal consensus sequences (Unkles 1992). The putative transcription initiation site in the *dodA* gene was in a favorable context for a highly expressed gene (Kozak 1991, 1992). The gene itself is 1003 bp long and codes for a 228-amino acid polypeptide. The analysis of the deduced amino acid sequence indicated that DOPA dioxygenase is a typical soluble protein, exhibiting only one region of clearly hydrophobic character (Kyte and Doolittle 1982).

The *dodA* gene contains six short exons varying in length between 14 and 219 bp. The five introns are short; their lengths varying between 49 and 99 bp. The sequence of the exons matches that of the cDNA clone exactly, except for the part coding for the first 27 amino acids that was not represented in the cDNA. Upstream of the *dodA* gene was a 570 bp region containing several short ORFs, but these are not in a favourable context for expression and are probably not transcribed.

Southern blot analysis: *dodA* is a single-copy gene

Genomic DNA from *A. muscaria* was digested with restriction enzymes and subjected to Southern blot analysis (Fig 2). Enzymes that did not cleave within the gene gave a single hybridising band, indicating that DOPA dioxygenase is encoded by a single-copy gene in *A. muscaria*. The enzyme *Nco*I cleaves once within the cDNA sequence and gave two bands. Hybridisations at lower stringency failed to reveal any bands suggestive of the existence of isogenes.

Northern blot analysis: regulation of DOPA dioxygenase synthesis occurs at the RNA level

Total RNA from the stipe and the coloured layer of the cap of young and medium *A. muscaria* specimens was analysed by Northern blot hybridisation (Fig 3). *dodA* transcripts were found to be 850 bases long and accumulated only in betalain-producing tissues; transcript was detectable in the stipe but at levels at least 10 times lower than those in the coloured layer.

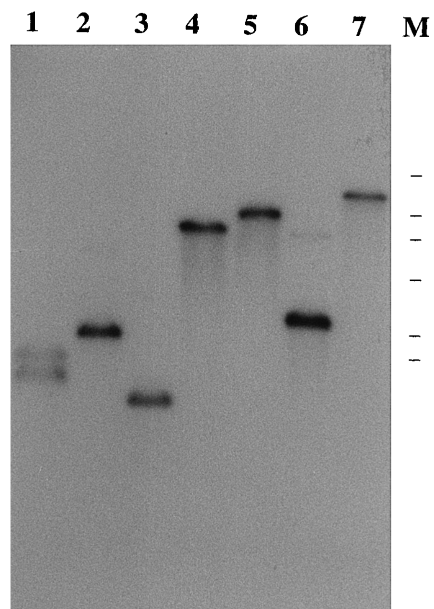

Fig. 2 Southern blot analysis of genomic DNA from *A. muscaria* using a probe derived from the coding part of the cDNA. The DNA was digested with the following restriction enzymes: lane 1, *Nco*I; lane 2, *Bgl*II; lane 3, *Hin*dIII; lane 4, *Pst*I; lane 5, *Bam*HI; lane 6, *Eco*RV; lane 7, *Xho*I; M, size markers (23 130, 9416, 6557, 4361, 2322, 2027 bp). Hybridization was at 65° C in 3 × SSPE

Discussion

The biosynthetic pathway leading to betalain pigments was elucidated by radiotracer experiments (Impellizzeri and Piattelli 1972) and the factors regulating betalain accumulation in plants have been studied intensively (Biddington and Thomas 1973; Girod 1991b; Leathers et al. 1992). Nevertheless, the plant enzymes involved in the pathway remain mostly unknown, and the tyrosinase and DOPA dioxygenase that catalyse the first two steps in the pathway have been isolated from the basidiomycete *A. muscaria* (Girod and Zryd 1991a; Müller et al. 1996).

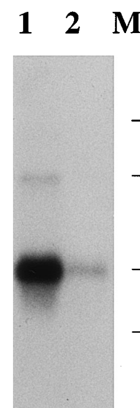

Fig. 3 Northern blot analysis of total RNA from stipe and cap. Each lane contained 2 μg of total RNA. Lane 1, cap; lane 2, stipe; M, RNA size markers (2800, 1898, 872, 562 bases). Hybridization was at 65° C in 3 × SSPE; the probe was derived from the coding part of the cDNA

An *A. muscaria* cDNA library made from young, barely coloured cap tissue yielded 20 *dodA* clones of similar length, containing a 612-bp ORF, but they all seemed to be truncated at the 5′ end. The construction of a size-selected, partial library of genomic DNA identified the true 5′ end and yielded additional information regarding the upstream sequences and the exon-intron structure of the gene. The *dodA* gene is 1003 bp long and codes for a 228-amino acid polypeptide, the first 27 of which were not encoded by the cDNA clones. Nevertheless the fusion protein that was produced in *E. coli* showed the same activity and specificity as did native DOPA dioxygenase (Müller 1997a), and the cDNA clone was stably expressed in tobacco and restored betalain synthesis in white *P. grandiflora* petals (Müller 1997b), suggesting that DOPA dioxygenase can be used for the modification of flower colour or as marker enzyme. Clearly, the first 30 amino acids are not important for the folding and stability of the polypeptide and are not necessary for enzymatic activity.

The putative start codon of the *dodA* gene lies in a favourable context for high-level expression (Kozak 1991, 1992). The structure of the gene is typical for fungal genes (Unkles 1992); its six short exons are interrupted by five short introns. In addition, the clones contained a 570-bp upstream region, but it was not possible to identify familiar promoter elements. Fungal genes often do not exhibit typical TATA boxes or CAAT boxes, suggesting that there must be other, more subtle signals (Gurr et al. 1987).

Southern blot analysis indicated that DOPA dioxygenase is encoded by a single-copy gene. The situation in higher plants is less clear; genetic analysis of *P. grandiflora* has indicated that a single locus codes for DOPA dioxygenase (Trezzini and Zryd 1990), while several alleles have been reported from red beet (Eagen and Goldman 1996).

Northern blot analysis showed that regulation of betalain synthesis occurs at the RNA level and that DOPA dioxygenase transcripts accumulate to high levels only in betalain-producing tissues. The fact that DOPA dioxygenase activity, antigen and mRNA accumulate only in betalain-producing tissues indicates that DOPA dioxygenase is specifically involved in betalain biosynthesis, although it is unclear how this benefits the fungus.

A search in the EMBL or Swissprot databanks for protein or DNA sequences similar to those derived from the *dodA* clones gave negative results, and a direct comparison of the deduced amino acid sequence with those of extra-diol ring-cleaving dioxygenases from bacteria indicated low overall sequence identity. These enzymes have a broad substrate specificity and share a number of potential substrates with the *A. muscaria* DOPA dioxygenase, and their size and subunit structure is similar to that of the fungal enzyme (Harayama and Kok 1992). The sequences of many bacterial extradiol dioxygenases are known, and amino acid residues important for ligand binding and catalytic activity have been identified (Harayama and Kok 1992; Han et al. 1995), but it was not possible to match the conserved residues from the bacterial enzymes with the fungal sequence. Thus, DOPA dioxygenase represents a new type of dioxygenase. To our knowledge this is the first gene from the betalain pathway to be cloned. It will be interesting to compare its sequence with those of its plant homologues.

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