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Independent duplications of α-amylase in different strains of *Aspergillus oryzae*

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ABSTRACT

Aspergillus oryzae is a filamentous fungus that has arisen through the ancient domestication of *Aspergillus flavus* for making traditional oriental foods and beverages. In the many centuries *A. oryzae* has been used for fermenting the starch in rice to simple sugars, it has undergone selection for increased secretion of starch-degrading enzymes. In particular, all *A. oryzae* strains investigated thus far have two or more copies of a gene encoding α-amylase, whereas *A. flavus* has only one. Here we investigate the duplications leading to these copies in three *A. oryzae* strains. We find evidence of at least three separate duplications of α-amylase, an example of parallel evolution in a micro-organism under artificial selection. At least two of these duplications appear to be associated with activity of transposable elements of the *Tc1/mariner* class. Both involve a 9.1 kb element that terminates in inverted repeats, encodes a putative transposase and another putative protein of unknown function, and contains an unusual arrangement of four short internal imperfect repeats. Although "unusual *Mariners*" of this size have previously been identified in *A. oryzae*, *Aspergillus fumigatus* and *Aspergillus nidulans*, this is the first evidence we know of that at least some of them are active in modern times and that their activity can contribute to beneficial genetic changes.

1. Introduction

Aspergillus oryzae is a filamentous fungus that has been used for centuries in the production of traditional oriental foods and beverages. In particular, it is used in the fermentation of rice for *sake* production, during which it secretes large amounts of amylases for the saccharification of starch into glucose for subsequent fermentation by yeasts. Great quantities of *A. oryzae* amylases are also prepared and used in food production industries (Berka et al., 1992).

Alpha-amylase (EC 3.2.1.1) catalyses the hydrolysis of internal α-1,4-glycosidic bonds in starch and related molecules and is the major secreted amylase in *A. oryzae* solid-state culture (Oda et al., 2006). The *A. oryzae* α-amylase is known as Taka-amylase and has been studied extensively at the levels of transcription initiation (Tanaka et al., 2000; Tani et al., 2000), protein folding (Kawata et al., 1998), crystal structure (Swift et al., 1991), glycosylation (Eriksen et al., 1998), secretion kinetics (Santerre Henriksen et al., 1999), and reaction kinetics (Batlle et al., 2000); there have also been many investigations of the optimal conditions for α-amylase production and activity.

Given the importance of α-amylase in rice fermentation and the length of time *A. oryzae* has been used to ferment rice, it is unsurprising that *A. oryzae* has experienced considerable selection for increased α-amylase secretion (Hara et al., 1992). In 1989 four groups independently reported that *A. oryzae* has multiple copies of α-amylase. Using Southern blotting, two copies were detected in *A. oryzae* NBRC 30105 (Tsukagoshi et al., 1989) and NRC401013 (Gines et al., 1989), and three copies in *A. oryzae* RIB40 (Tada et al., 1989) and DSM63303 (Wirsel et al., 1989). To our knowledge, no-one has reported an *A. oryzae* strain with fewer than two α-amylase copies. The genome of *A. oryzae* RIB40 was subsequently sequenced, confirming it had exactly three copies of α-amylase on different chromosomes (Machida et al., 2005). These copies have almost identical nucleotide sequences, differing at only three sites across a region spanning 3.2 kb. *A. oryzae* has arisen through the ancient domestication of *Aspergillus flavus* (Geiser et al., 1998), which has only one α-amylase gene (Fakhoury and Woloshuk, 1999). Thus it is likely that the additional copies of α-amylase have arisen through gene duplication in *A. oryzae* during its domestication.

Gene duplication can result from a variety of genetic changes associated with transposable elements (reviewed in Gray, 2000). Although transposable elements have long been considered genetic parasites, the last two decades have seen the description of many examples of beneficial genetic changes mediated by transposable elements (reviewed in Sinzelle et al., 2009). Transposable elements may be especially beneficial as a source of genetic variation in filamentous fungi such as *A. oryzae* that lack a known sexual cycle; indeed, transposition of transposable elements of the *Tc1/mariner*

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superfamily has been shown to be enhanced by stress conditions in *A. oryzae* (Ogasawara et al., 2009). Members of the *Tc1*/*mariner* superfamily are the predominant transposable elements in *A. oryzae*, comprising approximately half of all transposable elements and repetitive sequences (Galagan et al., 2005 Supplementary data §5). They characteristically integrate at TA dinucleotide sites, and their excision leaves duplicates of the TA integration site separated by two or three base pairs (reviewed in Plasterk et al., 1999).

Here we argue for the involvement of *Tc1*/*mariner*-type transposable elements in the duplications of α-amylase in *A. oryzae* strains NBRC 30105 and RIB40. In addition, we show that *A. oryzae* DAR3699, a strain used in soy fermentation (FRR Culture Collection catalogue, http://www.foodscience.afisc.csiro.au, accessed 2/16/2008) and shown to be an effective degrader of starch (Jin et al., 1999), also has two copies of α-amylase but that its second copy has arisen independently of that of the other strains previously characterised. Thus in an example of parallel evolution, duplication of an α-amylase gene has occurred at least three times in different *A. oryzae* strains.

2. Materials and methods

2.1. Strains

A. oryzae strains used were DAR3699 from the FRR culture collection (http://www.foodscience.afisc.csiro.au), NBRC 30105 (also called JCM02239) from the NITE Biological Resource Center (http://www.nbrc.nite.go.jp/NBRC2/NBRCDispSearchServlet), and RIB40 (also called ATCC42149 or NBRC 100959), also from the NITE Biological Resource Center.

2.2. Southern hybridization

Southern hybridization was performed using the DIG High Prime DNA Labelling and Detection Kit (Roche) according to the manufacturer's instructions. The probe to detect α-amylase bound a region of the α-amylase coding sequence and was constructed using the primers Ao_amy_F (AGGGAATGGGCTTCACAG) and Ao_amy_R (GGCGTTGAGGAGTGGATAG) and Taq polymerase (NEB). The probe to detect the 9.1 kb element bound a region indicated in Fig. 1 and was constructed using the primers 9kb_detect_F (ATGCCTCCACCCTCAACG) and 9kb_detect_R (CCCTCACGGACACTCTTGCT) and Taq polymerase (Roche).

2.3. PCR

Primers used for showing *A. oryzae* DAR3699 lacks a copy of α-amylase on chromosome 5 in the location where one is present in *A. oryzae* RIB40 were Ao_amy_C5_F (CTCATGGGCAGGAAACTTGG) and Ao_amy_C5_R (AGACCGAAGGACTTGAAACACC). This was done using Taq polymerase (NEB) according to the manufacturer's instructions. All other PCR was performed using Taq polymerase (Roche) according to the manufacturer's instructions. Primers used in generating Fig. 3A were Ao_amy_copy1_fwd (CCAGGCTCGCACTATGTATG), Ao_amy_copy2_fwd (GGACGGGATTGGATGAGG), Ao_amy_copy3_fwd (CAATAGTCATCTAACGCCTCG), and Ao_amy_copyX_rev (CGGCTGCTCGGTCTACTAC) with an annealing temperature of 55 °C, an extension time of 45 s, and 30 cycles. Primers used to investigate chromosome 3 of *A. oryzae* NBRC 30105 were Ao_amy_copy2_fwd given above and Ao_amy_C3_R (CGATAATACCACTCCCCAAAGC), used with annealing temperatures from 50 to 70 °C, an extension time of 200 s, and 40 cycles.

2.4. Sequence alignment

Fig. 2 was created by aligning 300 nt of each sequence using MUSCLE alignment with a terminal gap open score of zero, but otherwise using the default parameters of Geneious (Drummond et al., 2010). Other alignments used in creating Figs. 2 and 5 but not shown were generated using CLUSTAL.

2.5. Amylase assays

Fresh spores were point-inoculated onto 1% Aspergillus nitrogen-free media (Cove, 1966) containing 10 mM urea and 1% soluble starch (BDH). After two days' growth at 30 °C, the plates were

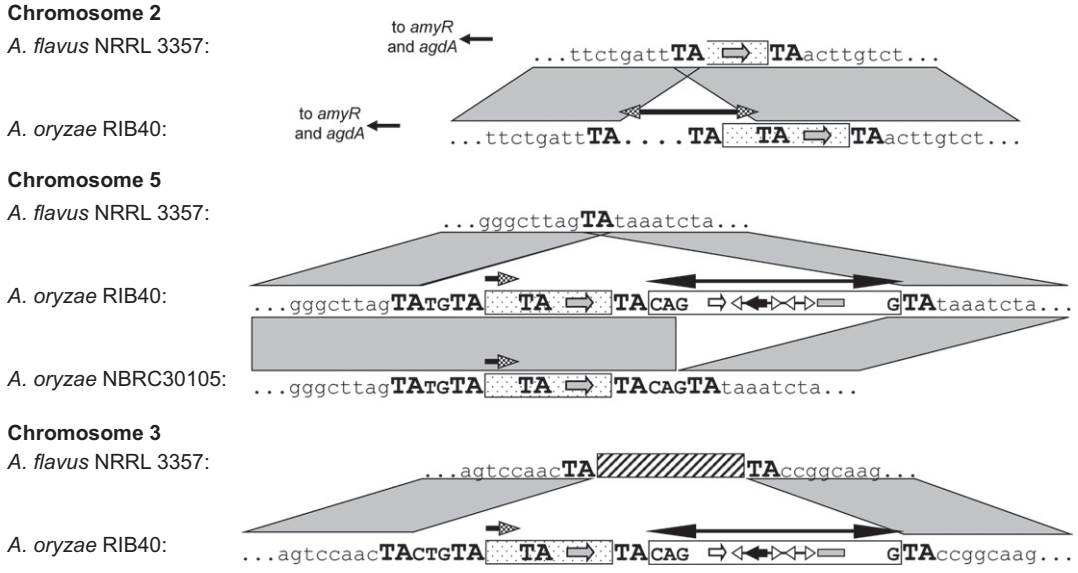

Fig. 1. Schematic of chromosomal regions about α-amylase genes in *A. oryzae* RIB40 and corresponding regions of other strains. Not to scale. Grey shaded areas indicate regions of homology on the corresponding chromosome between strains. TA dinucleotides are indicated in large capital letters. Other nucleotides mentioned in the text are indicated in smaller capital letters. Nucleotides not mentioned in the text are written in lower case. Corresponding pairs of inverted repeats are indicated by chequered, black, or white triangles. The repeats in the Aot1 complex indicated by white triangles all terminate in TA dinucleotides, which are not depicted due to space constraints. Gray arrow, α-amylase gene; white arrow, gene for a putative transposase *tpnA*; black arrow, putative gene of unknown function; grey box, region to which probe for detecting 9.1 kb element binds; speckled box, 3.2 kb conserved region in *A. oryzae* or part thereof in *A. flavus*; white box, 9.1 kb conserved *Mariner*-like element; striped box, 2.8 kb region unique to *A. flavus*.