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AT-rich sequences from the arbuscular mycorrhizal fungus *Gigaspora rosea* exhibit ARS function in the yeast *Saccharomyces cerevisiae*

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Abstract

Autonomous replicating sequences are DNA elements that trigger DNA replication and are widely used in the development of episomal transformation vectors for fungi. In this paper, a genomic library from the mycorrhizal fungus *Gigaspora rosea* was constructed in the integrative plasmid YIp5 and screened in the budding yeast *Saccharomyces cerevisiae* for sequences that act as ARS and trigger plasmid replication. Two genetic elements (*GrARS2*, *GrARS6*) promoted high-rates of yeast transformation. Sequence analysis of these elements shows them to be AT-rich (72–80%) and to contain multiple near-matches to the yeast autonomous consensus sequences ACS and EACS. *GrARS2* contained a putative miniature inverted-repeat transposable element (MITE) delimited by 28-bp terminal inverted repeats (TIRs). Disruption of this element and removal of one TIR increased plasmid stability several fold. The potential for palindromes to affect DNA replication is discussed.
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Keywords: Glomales; Repetitive sequences; Replicative plasmid; Origin of replication; Terminal inverted repeats; MITEs

1. Introduction

Autonomous replicating sequences (ARS) are DNA elements essential for chromosomal and plasmid replication. ARS elements of *Saccharomyces cerevisiae* are the best understood origins of DNA replication. In this organism, ARS activity depends on short (150–200 bp) elements, containing a degenerate ARS consensus sequence (ACS), 5′-(T/A)TTTA(T/C)(A/G)TTT(T/A)-3′ which is the binding site for the initiator origin recognition complex (ORC). The sequence requirement of this element is strict and point mutations in this sequence eliminate ARS function (Van Houten and Newlon, 1990). Recently, an exceptional ARS from *S. cerevisiae* has been characterized which depends upon a non-perfect match to the canonical 11-bp ACS (Theis and Newlon, 1997). The authors compared this essential ACS with those of other ARS elements and found an expanded 17-bp consensus sequence (EACS) which better identifies the essential sequence for ARS function than the conventional ACS.

In addition to the ACS, a variable number of 10–15 bp B elements, located at the 3′ terminus of the T-rich strand of the ACS and with no extended sequence conservation, have been recognized (Huang and Kowalski, 1996; Marahrens and Stillmann, 1992; Rao et al., 1994). B elements contribute to the binding sites for ORC and for the replication and transcription factor ABF1 (Diffley and Stillman, 1988; Rao and Stilman, 1995). Disabling of B elements by linker-substitution mutagenesis has been shown to reduce plasmid stability and yeast growth (Marahrens and Stillmann, 1992).

Arbuscular mycorrhizal (AM) fungi are obligate biotrophs that colonize the roots of most terrestrial plant species to form the most widespread symbiosis in nature. These fungi are believed to be ancient asexual organisms whose genomes evolve mainly clonally. The organization of their genomes is peculiar in that thousands of nuclei coexist in coenocytic mycelia and display unusually high-genetic polymorphism. Whether the genetic variation is distributed

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among polyploid-homokaryotic nuclei or heterokaryotic nuclei has been the subject of recent controversy (Hijri and Sanders, 2005; Kuhn et al., 2001; Pawlowska and Taylor, 2004).

With the exception of *Glomus intraradices* (Hijri and Sanders, 2004), AM fungal genomes are large and contain highly repetitive DNA sequences (Hosny et al., 1998; Zézé et al., 1994, 1996). A dispersed repetitive AT-rich DNA element (MYCDIRE) found in genomes of Glomales contains a match (10/11 bp) to the ACS of *S. cerevisiae* (Zézé et al., 1999). An ARS function for this element was proposed by the authors, but no experimental work was done to confirm this, mainly because of the difficulty of using AM fungi for genetic studies. Here, the unicellular fungus *S. cerevisiae* was used in conjunction with the non-replicating plasmid YIp5 to isolate ARS from the AM fungus *Gigaspora rosea*. Integration of plasmid DNA into yeast chromosomes is an inefficient process and transformation rates obtained with integrative vectors are notoriously low (Fincham, 1989). However, any genetic element that triggers the replication of the integrative vector and its consequent conversion to an episomal vector will transform yeast cells at a frequency about 1000-fold higher than integrative plasmids (Chan and Tye, 1980). Two *G. rosea* genetic elements with ARS function in *S. cerevisiae* were identified and characterized by sequence analysis and deletion studies. The heterologous characterization of these elements would represent the first step in studies focused on DNA replication in AM fungi. If such sequences maintain ARS function in the native species, they could be useful in the development of a transformation protocol for AM fungi.

2. Materials and methods

2.1. Strains

Spores of the arbuscular mycorrhizal fungus *G. rosea* BEG 9 produced in axenic conditions (Bécard and Fortin, 1988) were used for isolation of ARS elements. Unlike other AM fungi, this species has been reported to be refractory to endobacteria infection (Bianciotto et al., 2000) and so can be used to generate genomic libraries devoid of bacterial DNA. The *S. cerevisiae* strain BMA64n (*MAT*α, *his3-11*, *15*, *leu2-3*, *112*, *ura3-1*, *trp1*Δ*1*, *ade2-1*) was the recipient for testing ARS activity and mitotic stability. *Escherichia coli* DH5α (Invitrogen) was used for propagation and further manipulation of plasmids.

2.2. Isolation of ARS

A genomic library of the fungus *G. rosea* was constructed in the integrative shuttle vector YIp5 (Botstein et al., 1979). YIp5 is a pBR322 derivative containing the yeast selectable marker gene *URA3*. Genomic DNA from *G. rosea* spores was extracted according to Zézé et al. (1994). Ten micrograms of genomic DNA were partially digested with 10 U *Sau*3AI and dephosphorylated with a shrimp alkaline phosphatase (Roche) to avoid formation of chimeric fragments during the ligation process. The 5′ dephosphorylated *Sau*3AI fragments were ligated to *Bam*HI-linearized YIp5 and the resulting library used for yeast transformation as described by Schiestl and Gietz (1989).

From each *S. cerevisiae* transformant, replicating plasmids were rescued into *E. coli* following the methods of Robzyk and Kassir (1992). 10–100 ng of each plasmid were used to retransform yeast cells and transformation rates evaluated. Putative ARS elements were sequenced on both strands. One of them (*GrARS2*) was further manipulated by exploiting the internal *Sna*BI site, and two deletion derivatives, a 792-bp *Eco*RI–*Sna*BI fragment (Δ*GrARS2EB*) and a 644-bp *Sna*BI–*Sal*I fragment (Δ*GrARS2BS*), created by digesting pGrARS2 with *Eco*RI–*Sna*BI and *Sna*BI–*Sal*I, respectively, were subcloned back into YIp5.

2.3. Mitotic stability and plasmid retention

The mitotic stability of *GrARS*-based plasmid was determined from the fraction of plasmid-bearing cells in cultures grown under selection (Palzkill and Newlon, 1988). Culture suspensions were initially plated on non-selective (YPDA) and selective (uracyl-free medium) plates and the percentage of plasmid-containing cells determined. Plasmid retention was estimated by growing yeast transformants in the absence of selection over a 16-h period, corresponding to the completion of about 8 generations.

Sequence data from this article have been deposited in the GenBank/EMBL databases under Accession Nos. DQ056285 and DQ114976. ARS consensus sequences were detected with a Perl script written in Perl v.5.8.1 and run on a Unix machine.

3. Results

Transformation of the yeast strain BMA64n with the YIp5-based library of *G. rosea Sau*3AI genomic fragments produced eight yeast transformants. Replicating plasmids from two of them (pGrARS2 and pGrARS6) were successfully rescued in *E. coli* and characterized. pGrARS2 contained a 2200 bp insert, further subcloned as a *Eco*RI–*Sal*I 1413 bp fragment without loss of ARS function. pGrARS6 contained a 1664-bp insert.

Sequencing of *GrARS2* and *GrARS6* revealed that both elements were AT-rich, 72 and 80%, respectively. A perfect match to the *S. cerevisiae* ACS was found only in *GrARS6* (GrARS6ACS17, see Table 1); however, both *GrARS2* and *GrARS6* contained close (15/17 bp) matches (GrARS2ACS6, GrARS6ACS14, and GrARS6ACS17) to the 17-bp extended ARS consensus sequence (EACS), which is regarded to better fulfill the sequence requirement for replication function in yeast (Theis and Newlon, 1997). Furthermore, several 10/11 bp matches to the ACS were recognized in both *GrARS2* and *GrARS6* elements (Table 1).

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