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Hairpin dsRNA does not trigger RNA interference in *Candida albicans* cells

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Abstract

RNA interference/silencing mechanisms triggered by double-stranded RNA (dsRNA) have been described in many eukaryotes, including fungi. These mechanisms have in common small RNA molecules (siRNAs or microRNAs) originating from dsRNAs that, together with the effector protein Argonaute, mediate silencing. The genome of the fungal pathogen *Candida albicans* harbours a well-conserved Argonaute and a non-canonical Dicer, essential members of silencing pathways. Prototypical siRNAs are detected as members of the *C. albicans* transcriptome, which is potential evidence of RNA interference/silencing pathways in this organism. Surprisingly, expression of a dsRNA a hairpin *ADE2* dsRNA molecule to interfere with the endogenous *ADE2* mRNA did not result in down-regulation of the message or produce adenine auxotrophic strains. Cell free assays showed that the hairpin dsRNA was a substrate for the putative *C. albicans* Dicer, discounting the possibility that the nature of the dsRNA trigger affects silencing functionality. Our results suggested that unknown cellular events govern the functionality of siRNAs originating from transgenes in RNA interference/silencing pathways in *C. albicans*.

Keywords

Candida albicans; dsRNA; RNAi; RNase assay; Argonaute; Dicer

Introduction

RNA silencing pathways are conserved across many species. Two essential components of these pathways, Argonaute and Dicer, participate in cellular processes such as transposon silencing, viral defence, DNA elimination, heterochromatin formation, protection of the genome from repetitive elements and posttranscriptional gene silencing and regulation (Cerutti and Casas-Mollano, 2006; Drinnenberg *et al*., 2009; Malone and Hannon, 2009). The prototypical RNA silencing pathway, RNA interference (RNAi), is triggered by double-stranded RNA (dsRNA) that can arise endogenously by transcription of repetitive DNA, for example, or be experimentally introduced into the cell. The trigger dsRNA is cleaved by Dicer, a specialized endonuclease, into small interfering RNAs (siRNAs), which are

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subsequently loaded onto Argonaute and act as guides for silencing cognate mRNA molecules. Another silencing pathway commonly found in metazoans utilizes small non-coding microRNAs that are made as short stem-loop dsRNA precursors, preprocessed by a nuclear RNase III complex (Drosha-DGCR8), before final processing into microRNAs in the cytoplasm by Dicer (Rana, 2007).

RNA silencing pathways have been described in all four eukaryotic kingdoms, and many reports support an ancestral role of RNA silencing pathways in protection of the genome (Almeida and Allshire, 2005). In fungi, the RNA silencing machinery has undergone diversification and, in some cases loss, during evolution (Nakayashiki *et al*., 2006). RNA silencing pathways have been well described in the model fungi *Neurospora crassa* (Catalanotto *et al*., 2004; Cecere and Cogoni, 2009; Lee *et al*., 2009; Pickford *et al*., 2002) and *Schizosaccharomyces pombe* (Ekwall, 2004; Martienssen *et al*., 2005; Raponi and Arndt, 2003; Volpe *et al*., 2002, 2003; White and Allshire, 2008) but are notably absent in *Saccharomyces cerevisiae* (Drinnenberg *et al*., 2009). The genome of the fungal pathogen, *Candida albicans*, harbours a typical Argonaute homologue (orf19.2903; www.candidagenome.org), and a non-canonical Dicer (orf19.3796; Drinnenberg *et al*., 2009) lacking both a helicase and a PAZ domain. Recently, siRNAs were found as part of the transcriptome of *C. albicans* suggesting that RNAi pathways may exist in this organism. The authors also demonstrated Dicer activity in *C. albicans* extracts, but RNAi activity within cells was not addressed (Drinnenberg *et al*., 2009). RNAi in *C. albicans* cells has not been reported to date. Because important cellular processes are carried out by RNA silencing pathways and RNAi has been used as a tool to downregulate gene expression, we investigated the possibility of RNAi within *C. albicans* cells.

Materials and methods

Strains and growth conditions

The wild-type strain, SC5314 (Gillum *et al*., 1984) and the Ura-derivative, CAI4 (*hisG::ura3::hisG*/*hisG::ura3::hisG*; Fonzi and Irwin, 1993) were routinely maintained on yeast peptone dextrose medium (1% yeast extract, 2% peptone, 2% glucose; YPD). When necessary, uridine was added to a final concentration of 80 μg/ml to support the growth of CAI4.

Construction of the dsRNA *ADE2-yEGFP3-ADE2* expressing plasmid

Generation of pEAGA was performed in several steps essentially as outlined in Liu *et al*. (2002). The oligonucleotides used in amplifications are listed in Table 1. Nucleotides +1 to +414 of the *ADE2* gene were amplified by PCR utilizing an in-house recombinant *Taq* polymerase expressed in *E. coli* (Rustad *et al*., 2002) with pCRW3 (Srikantha *et al*., 1996) as source of *ADE2* sequences, and primers ADEFP and ADERG to add a *Pst*I restriction site at the 5′ end and a portion of *yEGFP3* to the 3′ end of the amplicon (fragment A). Fragment A was used as template with primers ADEFH and ADERVH in a PCR to incorporate *Hind*III sites at each end and an *Eco*RV site at the 3′ end (fragment B). One hundred base pairs (+1 to +100) of *yEGFP3* were amplified using primers ADEaG and GFPRH with pENO1GFP3 (Staab *et al*., 2003) as source of *yEGFP3* sequences to add a HindIII

restriction site at the 3′ of the amplicon (fragment C). Fragments A and C were used in a patch-PCR with primers ADEFP and GFPRH to generate a 530 bp amplicon (fragment D). Fragment D was digested with *Pst*I and *Hind*III and cloned into pENO1GFP3 after removing the *yEGFP3* open reading frame following digestion with *Pst*I and *Hind*III. Fragment B digested with *Hind*III was subsequently cloned at the *Hind*III site of the fragment D-containing plasmid, pENOGA. The relative orientation of the *ADE2* sequences in pENOGA clones was assessed by digesting with *Eco*RV and *Pst*I. Those recombinant plasmids (pEAGA) in which the *ADE2* sequences were in opposite orientation were kept for subsequent studies. The final *ADE2-yEGFP3-ADE2* construct had the constitutive enolase promoter driving expression of a hairpin dsRNA molecule and an *URA3* gene for positive selection in the Ura$^-$ strain CAI4 (Fonzi and Irwin, 1993).

C. albicans transformation

Linearized pEAGA was used to transform CAI4 spheroplasts as previously (Staab *et al*., 2003). Ura$^+$ transformants were selected on yeast nitrogen base [0.17% yeast nitrogen base without amino acids (Sigma), 0.5% ammonium sulfate, 50 mM glucose; YNB] agar plates without uridine. Stable transformants were verified for integration of the pEAGA at the *ENO1* locus by Southern blotting (Staab *et al*., 2003).

Northern blot analysis

ADE2 message levels in EAGA3 (pEAGA CAI4 transformant) were analysed by northern blotting. Total RNA (RNeasy RNA isolation Kit, Qiagen) was isolated from cultures grown in liquid YNB to mid-log phase at 30 °C. Ten micrograms of RNA from wild-type (SC5314), parent (CAI4) and EAGA3 were separated in standard formaldehyde gels and transferred to nitrocellulose (Brown *et al*., 2004). The immobilized RNAs were probed with a ^{32}P-labelled (Promega, Prime-a-Gene kit) PCR amplicon comprising bp +1 to +414 of *ADE2* (fragment B, see above). The hybridized membrane was washed three times in 0.2× SSC, 0.1% SDS at room temperature, and exposed to X-ray film at −80 °C for 4 days.

For detection of *CaAGO* expression, 4 μg of RNA from SC5314 grown in YNB was separated in formaldehyde gels and immobilized onto nitrocellulose as described above. The membrane was first probed with a ^{32}P-labelled (Promega, Prime-a-Gene kit) PCR (Platinum *Taq*, Invitrogen) product (bp +1 to +1427 relative to the ATG of orf19.2903) generated with oligonucleotides SI 5′-ATGAGTGATTTGGTTAAATTTTCAACACCAACC-3′ and B7, 5′-GGTTTCCCCATTGGTTTAGTGAG-3′ bp with SC5314 genomic DNA as template. The hybridized membrane was washed and exposed to X-ray film as above for 4 days at −80 °C. The membrane was stripped of the *CaAGO* probe (Sambrook *et al*., 1989), and hybridized with a PCR product generated from the *ACT1* locus (GenBank AACQ01000009, region 219902..220984) using the same oligonucleotides as for RT-PCR of the actin message (see below; for ACT237 and ACT994, see Table 1). The membrane was washed, and exposed to X-ray film at −80 °C for 2 days.

Detection of *ADE2* hairpin and *CaAGO* expression by reverse transcriptase-PCR (RT-PCR)

Expression of the *ADE2* dsRNA hairpin molecule in strain EAGA3 was detected by a reverse transcriptase reaction followed by PCR (RT-PCR) using a single oligonucleotide

predicted to anneal twice within the construct, and generate an amplicon. Total RNA was prepared (RNeasy RNA isolation kit, Qiagen) from CAI4 (parental strain) and EAGA3 (pEAGA CAI4 transformant) grown in YPD at 30 °C to mid-log phase. Approximately 600 ng of DNAse I (Promega)-treated RNA was used in RT reactions with 500 ng oligo $(dT)_{12-18}$, 10 nmol each dNTP, 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, and 200 U of Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen). PCR utilizing a recombinant *Taq* polymerase (Rustad *et al*., 2002) was performed with one tenth of the RT reaction and the single oligonucleotide ADE2-35 (Table 1), a sense primer of *ADE2* nt +35 to +58. Control PCRs were performed with oligonucleotides to actin, ACT237 and ACT994 (Table 1).

CaAGO expression was detected in RNA isolated from SC5314 grown in YNB to mid-log phase by first performing an RT reaction with 500 ng of DNase I-treated total RNA, followed by PCR (Platinum *Taq* polymerase, Invitrogen) with one-tenth of the RT reaction and oligonucleotides UF1, 5′-CCCTACCACGTCTCTGGC-3′ and B8, 5′-CGCTGGGGTTGGATTCAAATTG-3′ comprising bp −37 to +741 relative to the putative ATG in orf19.2903. Positive control reactions amplified a region of the actin message with oligonucleotides ACT237 and ACT994. The products of the RT-PCRs were subsequently separated in agarose gels and stained with ethidium bromide as per standard methods (Sambrook *et al*., 1989).

Cell-free RNase assays

The generation of siRNAs from radiolabelled dsRNA was performed essentially as described previously (Drinnenberg *et al*., 2009) except for the use of both a hairpin and a blunt-ended dsRNA substrates. SC5315 extracts from cells grown in YPD to OD_{600} 1.4 at 30 °C served as a source of Dicer activity. The hairpin substrate was produced by T7 transcription of the internal XbaI fragment of the *ADE2-yEGFP3-ADE2* construct (Fig. 1A) cloned at the *Xba*I site of pBluescript SK- (Stratagene/Agilent Technologies) and digested with *Not*I. *Not*I digests the plasmid once immediately downstream of the insert. The blunt-ended substrate (same *ADE2* sequences found from the *Xba*I site to nt 414) was the result of transcription from two amplicons bearing T7 promoter sequences at either end (Lanar and Kain, 1994). Plasmid pENOGA (see above) was used as template for two separate PCRs (High Fidelity Platinum *Taq*, Invitrogen) utilizing oligonucleotides T7-131 and A414, and T7-414 and A131 (Table 1). Radiolabelled transcripts were generated from the linearized plasmid and the T7 promoter-*ADE2* amplicons with use of a MAXIscript Kit (Ambion/Applied Biosystems) and a molar ratio of UTP to (α^{32}P)UTP of 32 : 1. The labelled substrates were gel-purified from a 6% acrylamide, 7 M urea gel (Invitrogen). The 20 µl reactions [5 µg cell extract, 40K cpm dsRNA substrate in reaction buffer (Drinnenberg *et al*., 2009) with 80U of Protector RNase inhibitor (Roche Applied Science)] were incubated at 30 °C for 2 h, followed by quenching with 50 mM sodium acetate, 10 mM EDTA, pH 5.5 and extraction with equal volume of acidic phenol–chloroform/isoamyl alcohol (25 : 24:1). One set of reactions was incubated at 4 °C to determine the requirement for ATP hydrolysis (Ye and Liu, 2008). The products of the RNase reactions were resolved by electrophoresis in 15% acrylamide, 7 M urea gels (Invitrogen). The size of the products was determined by

comparing their migration to RNA standards (Decade Markers, Ambion/Applied Biosystems).

Results and discussion

To test the possibility of RNAi in *C. albicans*, a plasmid was generated that expresses a dsRNA hairpin molecule that interferes with the *ADE2* message as previously described (Liu *et al*., 2002; Table 1). ADE2 was chosen as a target because disruption of this gene is non-lethal, and mutant colonies are readily distinguished on agar plates by their red colour (Poulter and Rikkerink, 1983). The plasmid bearing the ADE2 hairpin construct (pEAGA; Fig. 1A) was integrated at the *ENO1* locus in CAI4 (*ura3* Δ::*λimm434/ura3*Δ::*λmm434*; Fonzi and Irwin, 1993; Staab *et al*., 2003) by electroporation (Reuss *et al*., 2004). Verified transformants [via Southern blot analysis (Sambrook *et al*., 1989), data not shown] were analysed for adenine auxotrophy. We tested multiple transformants for growth in the absence of adenine on YNB minimal medium and determined that the colonies grew normally and did not exhibit the typical reddish colour of adenine auxotrophs (data not shown). Furthermore, the growth rate of these transformants on media lacking adenine did not differ from the parental strain or the Ura^+ wild-type strain, SC5314 (data not shown). Adenine auxotrophy is not a phenotype of *ADE2*/*ade2* strains but slowed growth has been reported for single *ADE2* deletions propagated in the absence of added adenine (Donovan *et al*., 2001), suggesting that *ADE2* message abundance must fall at or below the levels conferred by a heterozygous null genotype to observe any phenotype. Northern blot analysis showed that *ADE2* message levels were not affected by expression of the *ADE2* hairpin construct (Fig. 1B), consistent with the lack of an *ADE2*-related phenotype. We were confident that the *ADE2* probe detected the native message because we were unable to detect the *ADE2-yEGFP3-ADE2* hairpin (approximately 1.0 kb in length) using conventional formaldehyde gels and a probe consisting of *yEGFP3* sequences that separate the inverted *ADE2* repeats (data not shown), probably due to the extensive secondary structure of the predicted transcript. In addition, the migration of the single hybridized band at approximately 2.2 kb correlated well with the calculated size of the *ADE2* open reading frame (1707 bp; www.candidagenome.org, orf19.5906). Taken together, the results were consistent with the lack of RNAi in the cells.

Other laboratories have attempted RNAi in *C. albicans* by expressing 1.0 kb of inverted repeats of *TUP1* or *EFG1* driven by the *MAL2* promoter (unpublished results; personal communication, Richard Bennett, Brown University). Bidirectional transcription of the same *TUP1* and *EFG1* 1.0 kb fragments from opposing *MAL2* promoters also failed to produce transformants with defects associated with decreased *TUP1* or *EFG1* message. In addition, expressing a short transcript consisting of inverted repeats of 33 bp of *TUP1* separated by a short sequence of thymidines did not yield transformants having altered filamentation patterns that would suggest a decrease in message abundance. Bennett's negative results are congruent with ours, and suggest a general inability to induce RNAi by cellular expression of dsRNA in *C. albicans*. Others have achieved RNAi of the *ADE2* gene in *Cryptococcus neoformans* (Liu *et al*., 2002) and in *Histoplasma capsulatum* (Rappleye *et al*., 2004) via the expression of long hairpin dsRNAs. Thus, in other fungi, the highly conserved *ADE2* is amenable to depletion through an RNAi mechanism, implying that the *ADE2* target gene is

unlikely to be the culprit for RNAi failure. In summary, both published and published results support our findings of a lack of RNAi functionality in *C. albicans* triggered by exogenously introduced dsRNA.

Failure of dsRNA expression in the cells may explain the negative RNAi results. To investigate this possibility, we performed reverse transcription of total RNA prepared from EAGE3 (CAI4 transformed with pEAGA) followed by PCR (RT-PCR) utilizing a single oligonucleotide hybridizing within the *ADE2* gene fragments. A single oligonucleotide (ADE2-35, Table 1) was expected to hybridize twice, once within each inverted *ADE2* repeat, and generate an amplicon if the hairpin construct was transcribed. The RT-PCR confirmed hairpin expression (Fig. 2), as an amplicon of the predicted size together with a faster migrating DNA molecule (presumably a hairpin of one strand of DNA, the result of the secondary structure of the inverted repeats) was detected only in the EAGA RNA sample (Fig. 2, lane 2). Thus the lack of RNAi did not correlate with the failure to express the hairpin dsRNA. Based on our findings, we concluded that the absence of RNAi was probably related to the inability of the hairpin dsRNA to induce silencing of the cognate *ADE2* RNA in *C. albicans*.

Our results were puzzling in light of the recent report of evidence of RNAi products (siRNA) in *C. albicans* and other yeasts (Drinnenberg *et al*., 2009). Reconstitution of transgene RNAi in *Saccharomyces cerevisiae* is possible by introducing *Saccharomyces castellii AGO1* (Argonaute) and *DCR1* (Dicer) into the model yeast's genome (Drinnenberg *et al*., 2009). Analysis of the small-RNA libraries from *S. castellii* and *C. albicans* reveal that 68% of the sequence reads from *C. albicans* map to rRNA loci and ~6% of siRNAs map to retroelements, while in *S. castellii*, 41% map to retrotransposons (Ty elements) and repetitive elements, consistent with the predicted function of transposon control in the latter yeast (Drinnenberg *et al*., 2009). Although the abundance of rRNA siRNAs in *C. albicans* is not understood in terms of function, it may suggest that the RNA silencing pathway in *C. albicans* is not orthologous to that in *S. castellii*. Perhaps the nature of the dsRNA impacts subsequent RNAi functionality in *C. albicans*; the substrate used for RNase assays was a blunt-end dsRNA and not a hairpin molecule. To determine if *C. albicans* Dicer utilizes hairpin dsRNA as a substrate, we performed dsRNase assays with wild-type SC5314 (Drinnenberg *et al*., 2009). Uniformly radiolabelled hairpin and blunt-ended dsRNAs were tested as substrates for the generation of siRNAs (Fig. 3). SC5314 extracts (source of Dicer activity) accumulated siRNAs of approximately 23 nt in length when presented with both hairpin and blunt dsRNAs (Fig. 3), and this reaction was ATP-dependent (Fig. 3), as has been shown for other Dicers (Colmenares *et al*., 2007; Ye and Liu, 2008). The processing of the substrate dsRNAs into approximately 23 nt length fragments was specific to Dicer activity as the assays were performed in the presence of an RNase inhibitor to prevent RNase A, RNase B and RNase T2 activities (Roche Applied Science). These results showed that *C. albicans* is capable of utilizing hairpin dsRNA as substrate to generate putative siRNAs, and recapitulated Drinnenberg and colleagues' results utilizing a blunt-ended dsRNA substrate (Drinnenberg *et al*., 2009). Together, the data suggest that the presence of Argonaute and Dicer in *C. albicans* is insufficient to perform RNAi trigged by exogenously introduced dsRNA. Although we can conclude from our data that RNAi activity is lacking in

C. albicans, the possibility exists that a form of RNAi is viable in this organism based on the isolation of small non-coding siRNAs typical of Argonaute-associated guide RNAs (Drinnenberg *et al*., 2009). Perhaps *C. albicans* RNAi pathways are specific to the rDNA locus and transgene dsRNAs cannot serve as substrates. Further studies are needed to elucidate the significance of predominantly rDNA siRNAs in *C. albicans*.

Although RNase assays demonstrated the production of siRNAs from hairpin dsRNA, other factors must influence the functionality of these molecules to induce RNAi within the cells. The *C. albicans* Argonaute (*CaAGO*) is unlikely to be a pseudogene because transcription was detected by northern blotting and RT-PCR (Fig. 4). Because the biological functions of Argonaute and Dicer are unknown in *C. albicans*, the lack of endogenous RNAi triggered by the exogenous introduction and expression of dsRNA remains unclear. The genome of *C. albicans* is missing an RNA-dependent RNA polymerase (RdRP) homologue (Nakayashiki *et al*., 2006), an important component of silencing pathways in nematodes, plants and fungi. An identifiable homologue of an RdRP is also missing from the *S. castellii* genome and this organism is still capable of performing RNAi of transgenes, thus it appears that Argonaute and Dicer are sufficient for RNAi activity in *S. castellii* but not for *C. albicans*. This together with our negative data may imply that RNA silencing in *C. albicans* has devolved to an atypical, species-specific pathway that is no longer triggered by exogenously introduced dsRNA.

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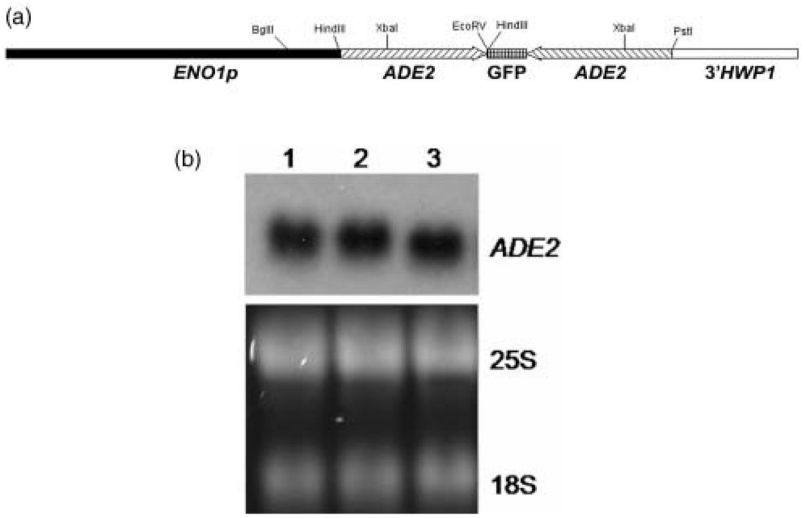

Figure 1.

Plasmid construct for expression of hairpin *ADE2* dsRNA within cells to interfere with the *ADE2* message. (a) Schematic of the *ADE2* hairpin construct. *ADE2* sequences from nt +1 to +414 were cloned in opposite orientation flanking 100 bp of yEGFP3, downstream of the enolase promoter (*ENO1p*) and with the 3′ untranslated region of *HWP1* (3′ *HWP1*) in pENO1GFP3. Arrows indicate 5′ to 3′ of *ADE2*. Relevant restriction endonuclease sites are shown above the construct. (b) Endogenous *ADE2* mRNA levels are not affected in a strain bearing the dsRNA plasmid construct. Top panel: northern blot analysis of the wild-type strain SC5314, lane 1; parental strain (CAI4), lane 2; and EAGA (CAI4 transformed with pEAGA), lane 3; probed for *ADE2* sequences with a ^{32}P-labelled PCR amplicon. Bottom panel: equal loading of total RNA was assessed by ethidium bromide staining of rRNA bands separated in standard formaldehyde gels used in northern blot analysis

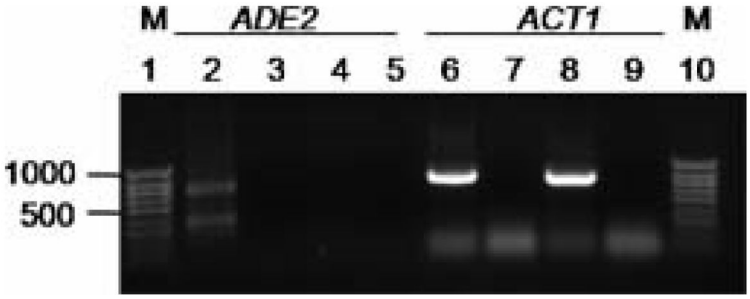

Figure 2.

Detection of *ADE2* dsRNA by RT-PCR. Total RNA from EAGA (lanes 2, 3, 6 and 7) and the parental strain, CAI4 (lanes 4, 5, 8 and 9), was reverse-transcribed and used in PCRs with a single oligonucleotide of *ADE2* sequences (ADE2-35; lanes 2, 3, 4, and 5). A PCR amplicon was detected only in reactions with RT templates from EAGA in addition to a faster migrating band probably due to secondary structure (hairpin) of the inverted repeat DNA product (lane 2). Controls: no RT reactions to exclude DNA contamination (lanes 3, 5, 7 and 9), and the amplification of an actin amplicon (lanes 6, 7, 8 and 9) that served as positive control for the RT-PCR. M, DNA size markers (HyperLadder IV, Bioline). The migration of the 1000 and 500 bp bands are indicated on the left

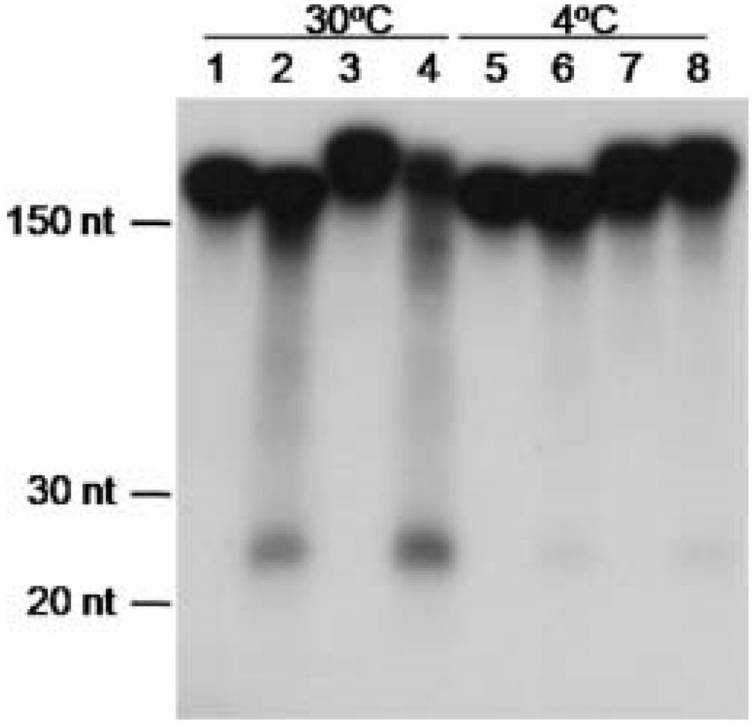

Figure 3.

Cell extract processing of blunt and hairpin dsRNA templates. A hairpin (*ADE2*-GFP-*ADE2*) or blunt (*ADE2* sequences only) radiolabelled substrate was incubated with SC5314 cell extracts, and the product resolved in a 15% acrylamide, 7 M urea gel. Lanes 1, 2, 5 and 6 were incubated with the blunt dsRNA; lanes 3, 4, 7 and 8 were incubated with the hairpin dsRNA. Odd-numbered lanes lacked cell extract. The RNase reaction is dependent upon ATP hydrolysis (4 °C reactions). The migration of RNA size markers is indicated on the left (Decade Markers, Ambion/Applied Biosystems)

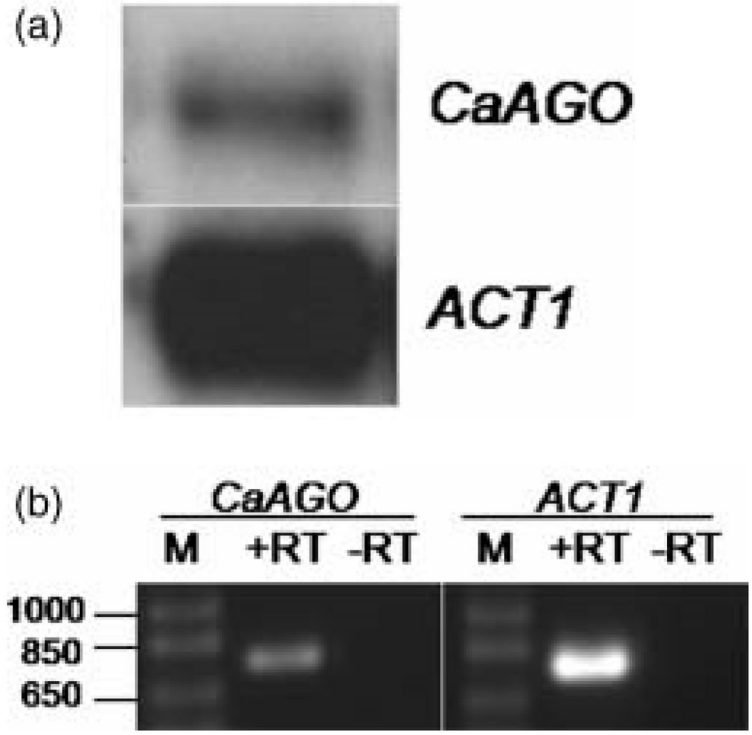

Figure 4.

Expression analysis of *C. albicans* Argonaute (*CaAGO*, orf19.2903). (a) Total RNA from SC5314 was probed with radiolabelled *CaAGO* and *ACT1* sequences by northern blotting. (b) Detection of *CaAGO* mRNA by RT-PCR. Amplification of a region of *ACT1* served as positive control for the RT reactions. Reactions lacking RT (–RT) controlled for DNA contamination of the samples. M, DNA size markers (1 kb Plus DNA Ladder, Invitrogen). The identity of the size markers in base pairs are indicated on the left

Table 1

Oligonucleotides used in this study

Primer	Sequence	Description
ADEFP	5′-AACTGCAGATGGATAGCAAAACTGTTGG-3′	dsRNA construct. *ADE2* nt 1–20; PstI site (underlined)
ADERG	5′-GAATAATTCTTCACCTTTAGACATCCCCAATGTGTAACAAGTCATCG-3′	dsRNA construct. *yEGFP3* nt 24–1 antisense (underlined); *ADE2* nt 414–392 antisense
ADEFH	5′-CCCAAGCTTATGGATAGCAAAACTGTTGG-3′	dsRNA construct. *ADE2* nt 1–20; *Hin*dIII site (underlined)
ADERVH	5′-CCCAAGCTTGATATCCCCAATGTGTAACAAGTCATCG-3′	dsRNA construct. *ADE2* nt 414–392 antisense; HindIII site (underlined) and *Eco*RV site (double underlined)
ADEaG	5′-**CGATGACTTGTTACACATTGGGG**ATGTCTAAAGGTGAAGAATTATTC-3′	dsRNA construct. *ADE2* nt 392–414 sense (bold), *yEGFP3* nt 1–20
GFPRH	5′-CCCAAGCTTCACCTTCACCGGAGACAGAAAATTTG-3′	dsRNA construct. *yEGFP3* nt 100–73 antisense; *Hin*dIII site (underlined)
ADE2-35	5′-GGAGGTGGCCAATTAGGTCGTATG-3′	RT-PCR. *ADE2* nt 35–58 sense
ACT237	5′-GAGAGTTGCTCCAGAAGAACATCC-3′	RT-PCR. *ACT1* nt 943–966 sense
ACT994	5′-CCAAGATAGAACCACCAATCCAGAC-3′	RT-PCR. *ACT1* nt 1700–1676 antisense
T7-A131	5′-CCAAGCTTC**TAATACGACTCACTATAGGG**TCTAGACGATCACGTTGATGGATCG-3′	*ADE2* sense oligo nt 131–155 with T7 promoter (bold) and *Hin*dIII site (underlined)
A414	5′-CCCCAATGTGTAACAAGTCATCGAC-3′	*ADE2* antisense oligo nt 414–389
A131	5″-TCTAGACGATCACGTTGATGGATCG-3′	*ADE2* sense oligo nt 131–155
T7-141	5′-CCAAGCTTC**TAATACGACTCACTATAGGG**CCCCAATGTGTAACAAGTCATCGAC-3′	*ADE2* antisense oligo nt 414–389 with T7 promoter (bold) and *Hin*dIII site (underlined)