Endogenous short RNAs generated by Dicer 2 and RNA-dependent RNA polymerase 1 regulate mRNAs in the basal fungus *Mucor circinelloides*

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DISCLAIMER:

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Running title: A new class of short RNAs regulate mRNAs in fungi

ABSTRACT

Endogenous short RNAs (esRNAs) play diverse roles in eukaryotes and usually are produced from double stranded RNA (dsRNA) by Dicer. esRNAs are grouped into different classes based on biogenesis and function but not all classes are present in all three eukaryotic kingdoms. The esRNA register of fungi is poorly described compared to other eukaryotes and it is not clear what esRNA classes are present in fungi and whether they regulate the expression of protein coding genes. However, evidence that some *dicer* mutant fungi display altered phenotypes suggests that esRNAs play an important role in this kingdom. Here we show that the basal fungus *Mucor circinelloides* produces new classes of esRNAs that map to exons and regulate the expression of many protein coding genes. The largest class of these exonic-siRNAs (ex-siRNAs) are generated by RNA-dependent RNA Polymerase 1 (RdRP1) and Dicer-like 2 (DCL2), although there is some redundancy between *dcl1/dcl2*. Ex-siRNAs act in *cis* and target the mRNAs of protein coding genes from which they were produced. Our results expand the range of esRNAs in eukaryotes and reveal a new role for esRNAs in fungi.

Keywords: fungus, short RNA, dicer, RdRP, siRNA

INTRODUCTION

The gene silencing pathways using endogenous short RNAs (esRNAs) 20-24 nucleotide (nt) in length are very diverse in eukaryotes (1). Most classes of esRNAs are produced from double stranded RNA usually by a member of the Dicer family and are incorporated into an effector complex containing a member of the Argonaute family (1). esRNAs that are produced from and target transposons seem to be the most ancient because these were found in fungi (2), plants (3) and animals (4 and 5). These esRNAs act in *cis* and may lead to DNA methylation and/or histone modifications (2 and 3). Another class of esRNAs, microRNAs (miRNAs), are produced from hairpin structure non-coding transcripts and target mRNAs in *trans* (6). miRNAs have been found in plants and animals and play a role in diverse processes including development and adaptation to environmental changes (6). Although miRNAs have not been found in fungi, *dicer* mutants of several fungi have been reported to be affected in vegetative and developmental processes (7, 8), which may suggest the existence of esRNAs with regulatory functions in this group of organisms. However, information on esRNAs in fungi is very scarce.

Mucor circinelloides, a basal fungus of the clade zygomycete, has been revealed as a model organism in the fungal kingdom for the study of RNA silencing and other processes due to the availability of molecular tools and its evolutionary distance from other fungal model organisms. The existence of a transgene-induced RNA silencing mechanism in *M. circinelloides* has been previously demonstrated (9) and the siRNA pathway has been dissected by identifying genes and proteins involved in the silencing mechanism. As a result, two *dicer* genes, *dcl1* and *dcl2*, have been identified (8, 10). The DCL2 protein plays

a major role in gene silencing induced by sense or hairpin transgenes, being responsible for the production of two different classes of antisense siRNAs, 21 nt and 25 nt long. Null *dcl2* mutants show a significant reduction in the production of asexual spores, which suggests a role for *dcl2* in the control of vegetative development (8). Similarly, mutants affected in the *dcl1* gene show defects in vegetative growth and hyphal morphology, even though this gene is not required for efficient gene silencing triggered by transgenes (10). Also two genes coding for RNA dependent RNA polymerases (RdRP) have been involved in the silencing mechanism operating in *M. circinelloides*. Gene *rdrp1* is required for sense transgene-induced silencing to generate dsRNA molecules while *rdrp2* participates in the amplification process (S.C., S.T-M and R.M.R-V, unpublished results).

Relatively little is known about the physiological roles of the fungal RNA-silencing pathways (7). Availability of *M. circinelloides* mutants affected in the silencing mechanism and the recent completion of its genome sequencing has provided the genetic and molecular tools required to investigate how esRNAs regulate gene expression in this basal fungus. We analyse here the esRNA content of *M. circinelloides* by deep sequencing of short RNAs (18-25nt) in the wild type and several mutant strains and identify a new class of esRNAs that target mRNAs of protein coding genes from which they were produced

MATERIALS AND METHODS

Strains and growth conditions

The leucine auxotroph R7B, derived from *M. circinelloides* f. sp. *lusitanicus* CBS 277.49 (syn. *Mucor racemosus* ATCC 1216b), was used as the wild-type strain. Strain MU406 is a *dcl1* null mutant derived from MU402, a uracil auxotroph derivative of R7B (8). Strain

MU410 is a *dcl2* null mutant derived from MU402 (10). Strain MU411 is a double *dcl1*/*dcl2* null mutant derived from MU410 (10). Strains MU419 and MU420 correspond to *rdrp1* and *rdrp2* null mutants derived from MU402, respectively (S.C., S.T-M and R.M.R-V, unpublished results). Cultures were grown at 26°C in complete YPG medium as described previously (10). The pH was adjusted to 4.5 for mycelial growth.

RNA analysis

Short RNA samples were extracted from mycelia grown for 48 h in YPG plates using the miRVana kit (Ambion), following the instruction of the supplier. cDNA libraries of short RNAs were generated and sequenced as described previously (11). Low and high molecular weight RNA was extracted from frozen mycelia and analysed by Northern blot as described previously (9).

Sequence analysis

Raw sRNA reads were processed by removing adaptor sequences with exact matches to the first eight bases of the 3' adaptor. Annotated exon regions were extracted from the *M. circinelloides* genome (v 1.0) along with annotated transposons and repeat regions. All remaining segments of the *M. circinelloides* genome were used as the intergenic set for this analysis.

sRNAs were mapped to exons, transposons/repeats and intergenic regions using PatMaN (12) and distinct sRNA producing loci were predicted in intergenic regions using a previously published method (13). miRNA prediction was carried out both on intergenic regions and the full genome using a modified version of the miRCat pipeline described

previously (14). In order to correct for variation in sRNA sample sizes and allow us to perform cross-sample expression analysis, sRNA counts for each of the intergenic, exonic and transposon loci were converted into reads per million genome matching reads. sRNA loci were said to be down-regulated in a given sample if the normalised locus abundance showed at least a fourfold decrease in comparison to the wild type sample. To increase the stringency of the analysis and avoid lowly expressed regions, any loci with a normalised abundance count of less than 50 in the wild type were excluded from the analysis.

RESULTS AND DISCUSSION

Short RNAs are primarily generated by DCL2 in *Mucor*

Two *dicer-like* genes (*dcl1* and *dcl2*) have been identified in *Mucor* and a possible role in the control of endogenous functions has been hypothesized for *dcl2* gene (8). In order to identify *bona fide* esRNAs we generated cDNA libraries of short RNAs from wild type (*dcl1*/*dcl2*), single mutant (*dcl1*$^{-}$/*dcl2* or *dcl1*/*dcl2*$^{-}$) and double mutant (*dcl1*$^{-}$/*dcl2*$^{-}$) strains (8 and 10) and sequenced them on the Solexa platform. Table 1 shows the normalized read counts for the different strains analyzed (GEO accession number is GSE18958). Non-redundant reads were mapped to the genome, whose sequence was available from the JGI (http://genome.jgi-psf.org/Mucci1/Mucci1.home.html). In fact, this is the first global analysis reported from the *Mucor* sequencing project. esRNAs that mapped to the genome within less than 200 bp from each other and had a minimum abundance of five (Simon to check) were grouped together and the regions covered by these related esRNAs were designated as loci (as previously described in 13). A total of 24111 loci were identified all

over the *Mucor* genome by this criterium. All esRNA loci were analyzed by comparing accumulation of esRNAs in the wild type and *dcl* mutants. Many loci produced esRNAs only in sense orientation at a similar rate in wild type and all mutant strains and accumulation of esRNAs from some of these loci was analysed by Northern blot. These esRNAs were either not detectable or the probes detected a smear between 15-2000 nt but not bands with distinct sizes between 20-25 nt (data not shown). Therefore we concluded that reads from these loci are most likely to be degradation products of ribosomal, transfer and messenger RNAs.

To identify DCL-dependent esRNAs loci we selected those that showed at least a fourfold decrease in the normalized esRNAs abundance in one of the *dcl* mutants in comparison with the wild type strain. Nine hundred sixty seven (please Simon check total number of loci) differentially expressed loci were identified, which were grouped based on the annotation of the locus: intergenic, transposon or exon. (Table 2 Simon please check these numbers!! at the end of this file). Twelve loci showed a decrease in *dcl1*$^{-}$ and 833 were down-regulated in *dcl2*$^{-}$, five of which were suppressed also in *dcl1*$^{-}$. The majority (97%) of the 840 loci producing fewer esRNAs in either *dcl1*$^{-}$ or *dcl2*$^{-}$ or in both also produced fewer esRNAs in the double mutant. In fact, the read numbers were usually lower in the double mutant than in any of the single mutants demonstrating some redundancy for the two *dcl* genes (Supplementary Figure 1). In addition, a further 127 (please Simon, check; must include exons and intergenic regions) loci showed differential expression only in the double mutant. Despite the redundancy between *dcl1* and *dcl2*, the sequencing data suggested that DCL2 is the primary protein that generates the majority of esRNAs in *M. circinelloides* although from a very small number of loci mainly DCL1 produces esRNAs.

The DCL proteins have similar hierarchy in transgene-induced silencing, where DCL2 also plays a more prominent role in the generation of siRNAs than DCL1 (8).

Short RNAs are produced from transposons but there is no evidence for miRNA genes

In order to investigate the distribution of DCL-dependent esRNAs across the different loci categories, we calculated the normalized abundance of these esRNAs per 1 kb of exonic, intergenic and transposon regions in the wild type and *dcl* mutant strains. Supplementary Figure 2 shows the results obtained in the four samples analysed. Results clearly show that esRNAs are not formed at random across the genome but they are enriched in exonic regions compared with intergenic regions and transposons. In fact, only 207 out of 967 esRNA producing loci, down-regulated in at least one of the *dcl*$^{-}$ strains, were annotated transposons or repeats (Table 2). This is in contrast with other organisms, such as *Schizosaccharomyces pombe* (15), *Saccharomyces castellii* and *Candida albicans* (16), where most esRNAs are produced from repeats and transposons.

Intergenic and intronic loci are the prime candidates for producing miRNAs. Therefore, we asked whether any of these loci can be folded into a stem-loop structure characteristic of miRNA loci. None of the 441 *dicer* dependent intergenic and intronic loci fulfilled the criteria of miRNA loci, although several esRNAs from intergenic regions were confirmed by Northern blot (Figure 1A). We also tested all 24111 esRNA producing loci identified in the initial analysis (including the ones that are not down-regulated in *dcl* mutants) but none of them had the features of miRNA genes. It is likely that miRNAs are

not present in *Mucor* although we cannot exclude the possibility that they are expressed in different growth condition or developmental stages.

The apparent lack of miRNAs in fungi and the presence of transposon derived esRNAs in basal fungi, plants and animals suggest that life evolved esRNAs to silence transposons and than each branch of life has utilised this machinery to silence other targets.

Short RNAs are generated from exons and regulate mRNA accumulation

A main class of esRNAs mapped to exons (Table 2 and Supplementary Figure 2), and we call them exonic-siRNAs (ex-siRNAs) based on their unusual location. In total, 324 exonic loci were identified, which correspond to 276 genes, since exons of the same gene separated by more than 200 bp were considered different loci (see loci definition above). To validate the sequencing data, accumulation of selected ex-siRNAs was analysed by Northern blot (Figure 1B), and in all cases the presence of distinct bands around 20-25 nt was confirmed. Some loci produced almost exclusively antisense ex-siRNAs, whereas others produced ex-siRNAs in both orientations. Results also confirmed the reduced expression of ex-siRNAs in mutant strains compared to wild type, as well as that DCL2 is the primary *dicer* in *Mucor*. The Northern blots corroborated the redundancy between the two *dcl* genes because the ex-siRNA signals were often weaker in the double mutant than in the single mutants. Sometimes different sized ex-siRNAs were detected from the same locus, which seems to be a characteristic of the *M. circinelloides* DCL enzymes. In fact, transgene-induced gene silencing is also associated with two different size classes of siRNAs, 21 nt and 25 nt long, both in the wild type and the *dcl1*$^{-}$ mutant (9 and 10).

Accumulation of ex-siRNAs does not necessarily mean that they are functional as for example some non-conserved plant miRNAs do not have target mRNAs (17). To test whether the exon mapping *Mucor* ex-siRNAs are functional and affect gene expression, accumulation of the mRNAs was analysed by Northern blot analysis. All the four tested mRNAs that showed reduced ex-siRNA expression in *dcl2*$^{-}$ accumulated at an increased level in the *dcl* mutant strains compared to the wild type (Figure 2, loci 4-9) confirming that the *Mucor* ex-siRNAs are functional. The effect of ex-siRNAs on mRNA accumulation is most likely post-transcriptional, since no significant methylation is associated with gene silencing in *Mucor*, and siRNAs generated in transgene-induced silencing have been demonstrated to act post-transcriptionally (9). In addition to the four mRNAs we also tested the accumulation of a potential RNA transcript corresponding to an intergenic region, which produced less esRNA in *dcl*$^{-}$ strains than in wild type. A transcript was detected from this intergenic locus and it also showed a higher accumulation in the *dcl2*$^{-}$ single and *dcl1*$^{-}$*/dcl2*$^{-}$ double mutants relative to the wild type (Figure 2, locus 3), supporting that the different esRNAs of *Mucor* are functional.

Biogenesis of exonic endogenous siRNAs

Since most exons that generated less ex-siRNAs in *dcl2*$^{-}$ than in wild type produced antisense ex-siRNAs (some almost exclusively), we hypothesised that an RNA dependent RNA polymerase (RdRP) must be involved in ex-siRNAs biogenesis. Two different RdRP proteins have been implicated in *M. circinelloides* transgene-induced gene silencing (S.C., S.T-M and R.M.R-V, unpublished results), and we tested the involvement of these proteins,

RdRP1 and RdRP2, in the biogenesis of ex-siRNAs by deep sequencing of esRNAs from *rdrp1*$^{-}$ and *rdrp2*$^{-}$ strains. The normalised reads of ex-siRNAs from the 324 exons that accumulated less ex-siRNA in, at least, one *dcl* mutant compared to the wild type strain are shown in Supplementary Table 2, which also shows the strand bias (sense/antisense) of the ex-siRNAs produced by the wild type and all mutant strains. The accumulation of ex-siRNAs from each exon in the different mutant strains was compared to the wild type and the fold difference is shown in Supplementary Table 3, in which exons are ordered according to the fold change of ex-siRNAs in the *dcl2*$^{-}$ strain. Four different patterns in the changes of ex-siRNAs accumulation in the different mutants can be easily identified and we call these almost perfectly separated groups class 1-4. Table 3 shows a summary of the properties of each of the different ex-siRNA classes.

Classes 1 and 2 include all ex-siRNAs that are *dcl2*-dependent. The first nucleotide of the 5' end of these ex-siRNAs exhibits a strong preference for uracil (92%) (Table 3). This preference has been also shown for Argonaute-bound guide RNAs of animal, plants and other fungi (16). The majority of the *dcl2*-dependent ex-siRNAs also showed reduced levels in the *rdrp1* mutant but not in the *rdrp2*$^{-}$ strain and define class 2. This is the largest group of ex-siRNAs, with 222 exons that represent 68.5% of the 324 ex-siRNA producing exons. Most likely, these ex-siRNAs act *in cis*, since mRNAs transcribed from these exons are up-regulated in the absence of the ex-siRNAs (Figure 2). There is not a strong strand bias among these ex-siRNAs, most exons producing a mixed sense and antisense ex-siRNAs, which is expected considering that a large number of ex-siRNAs are produced from each exon (Supplementary Figure 1). The requirement for RdRP1 and DCL2 for the biogenesis of this ex-siRNA class suggests that mRNAs from those loci are turned into

dsRNA by RdRP1 and then processed by DCL2. The involvement of these two proteins in the biogenesis of the majority of ex-siRNAs can be extended to esRNAs derived from transposons and intergenic regions, as also in these loci categories most of the *dcl2*-dependent esRNAs require *rdrp1* (Figure 3).

A very small group of the *dcl2*-dependent ex-siRNAs, made up by ex-siRNAs from only 9 exons belonging to five genes, does not require RdRP1 but most of them depend on RdRP2. These ex-siRNAs, which identify class 1, are the most down-regulated in the *dcl2*$^{-}$ strain (Supplementary Table 3). Accumulation of these ex-siRNAs is strongly up-regulated in the *rdrp1*$^{-}$ mutant, which may suggests that both RdRPs can compete for binding the mRNA templates but only RdRP2 can turn them into dsRNA. Consequently, lack of RdRP1 may allow RdRP2 to make more dsRNA that, in turn, would result in a higher ex-siRNA level.

We have investigated the biological functions of the genes that produce less ex-siRNAs in the *dcl2* mutant (class 1 and 2), trying to link those functions with the phenotype of the *dcl2* mutant strain (8). However, the high number of genes affected in the *dcl2* mutant makes difficult to summarize their roles and to reveal their biological significance. Nonetheless, it can be emphasized that many of those genes are annotated as encoding for proteins involved in signal transduction and information storage and processing (Supplementary Table 3), which may indicate the involvement of these ex-siRNAs in the regulation of different cellular processes.

A significant group of ex-siRNAs producing exons (88 out of 324) are classified in class 3 and correspond to ex-siRNAs that are down-regulated only in the double *dicer*

mutant but not in *dcl1*$^{-}$ or *dcl2*$^{-}$ (Supplementary Table 3). This suggests that dsRNA produced from these exons can be processed by either DCL1 or DCL2. Also, both RdRP enzymes are involved in the biogenesis of these ex-siRNAs, because they are down-regulated in the absence of either of the two *rdrp* genes. However, the most peculiar feature of the class 3 ex-siRNAs is that they show a very strong strand bias, being almost all of them exclusively sense to the mRNAs. Besides that, the class 3 ex-siRNA molecules show a random spread of size distribution that is not observed in the *dcl2*-dependent classes 1 and 2, which produce predominantly 23-24 nt sRNAs (Supplementary Figure 3). This suggests that class 3 ex-siRNAs are not produced by a canonical RNA silencing mechanism. Instead, we can speculate that mRNA molecules with discrete dsRNA regions produced by sequential or combined activity of RdRP1 and RdRP2 must be processed by either DCL1 or DCL2 and after the initial cleavage, these mRNAs are degraded by non-specific RNases, probably because they lose their cap and/or polyA tail. The ability of human Dicer to cleavage ssRNA in a partial dsRNA molecule has been described recently (18). Most of the genes corresponding to the class 3 are highly expressed, as denoted by the sequencing of high numbers of ESTs. Further experiments are needed to identify what other features, besides high expression, are required for the mRNA to enter this pathway.

Finally, class 4 is a tiny group of ex-siRNAs that derive from only five exons. These are the only ex-siRNAs that are down-regulated in *dcl1*$^{-}$ but not in *dcl2*$^{-}$. Only one of these exons shows a reduced accumulation in the double *dcl* mutant, although two others are just below the fourfold threshold in the *dcl1*$^{-}$*/dcl2*$^{-}$ strain (Supplementary Table 3). We cannot rule out the possibility that some of the ex-siRNAs of this class is only an artefact of the sequencing, since it is difficult to explain why the double *dcl* mutant is different from *dcl1*$^{-}$.

However, we can point out that one of the exons included in this class (ID: 27711) encodes a conserved protein that in other fungi, such as yeast, co-localise with other proteins in sites of polarized growth (tip of the hypha). This, together with the fact that other exons code for proteins involved in mitochondria metabolism and ribosome function may help to understand the phenotype of the *dcl1* mutant, that is, abnormal hyphal morphology and a decrease in the growth rate.

The biogenesis and function of the class 2 ex-siRNAs, the largest group of ex-siRNAs we have identified in *M. circinelloides*, somewhat resemble that of endogenous siRNAs in animals and plants. However, there are clear differences between these ex-siRNAs and previously described esRNAs. The most similar group is the endogenous siRNAs in *C. elegans*, which regulate the expression of protein coding genes. However, these are mainly generated directly by the RdRP activity without the participation of a Dicer enzyme (19, 20, 21). The 24mer heterochromatin siRNAs in plants are produced by Dicer from an RdRP generated dsRNA, but these siRNAs predominantly target transposons and repeat elements (22). They are therefore similar to the *Mucor* esRNAs mapping to transposons. Endo-siRNAs found in *Drosophila* and mouse oocytes are similar to class 2 ex-siRNAs because some of these target protein coding genes, but this class of esRNAs is produced from complementary transcripts without RdRP activity (4). The class 2 ex-siRNA pathway involves elements from all these different pathways. Thus, dsRNA is generated by *Mucor* RdRP, similarly to plants (heterochromatin-siRNA), the dsRNA is cleavage by Dicer, as occurs in plants (heterochromatin-siRNAs) and higher animals (endo-siRNA) and the generated ex-siRNAs target protein coding genes *in cis*, which is similar to animals

(endo-siRNA and secondary siRNAs). Therefore, the ex-siRNA pathway in *Mucor* uses all these known elements but in a new and unique combination.

A new class of esRNA, qiRNAs, was recently reported to be involved in DNA-damage response in another filamentous fungus, *Neurospora crassa* (23). Here we identified another class of esRNAs that potentially regulate up to 276 mRNAs, suggesting that this layer of regulation is extensive in *Mucor* and potentially in other fungi. Indeed, the *dcl* mutants are affected in hyphal morphology and colony growth (*dcl1*$^{-}$) (10) and in the production of asexual spores (*dcl2*$^{-}$) (8). We identified ex-siRNAs in fungi grown in optimal condition but qiRNAs are induced by DNA damage (23) and it is also well documented that plant short RNAs are involved in stress responses (24). Therefore it is possible that expression of other genes is also regulated by ex-siRNAs when the fungus grows in sub-optimal conditions or responds to different signals to execute complex developmental processes, such as asexual sporulation or sexual interaction. Finally, although we cannot rule out that miRNAs will be identified in other classes of fungi, the apparent lack of miRNAs in fungi raises the question whether miRNAs were present in a common eukaryotic ancestor and lost in fungi or appeared independently in plants and animals. The first option is supported by the presence of miRNAs in green alga (13) and the latter is supported by the differences in miRNA biogenesis, degree of complementarity to the target mRNAs and lack of sequence homology between animal and plant miRNAs.

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Table 1 Sequencing of short RNAs in different *Mucor* strains

	WT	*dcl1*$^-$	*dcl2*$^-$	*dcl1*$^-$/*2*$^-$	*rdrp*$^-$	*rdrp*$^-$
Total Reads	6421725	5326957	6155439	10899332	11088786	10846130
After adaptor removal (total)	5701786	4604676	5049070	9303440	10178115	9877133
After adaptor removal (unique)	1362728	1357556	1354177	3027587	1929224	2654222
Mapping to genome (total)	4317874	3619529	3912401	7094985	8347666	7880896
Mapping to genome (unique)	777409	808650	890709	2121808	1272679	1512498

The table shows the read numbers of different categories for each strain we have analysed.

Table 2 Number of loci down-regulated at sRNA level in *dcl1*$^{-}$ and *dcl2*$^{-}$ mutants.

	Down-regulated in		
Type of loci	*dcl1*$^{-}$	*dcl2*$^{-}$	*dcl1*$^{-}$/*dcl2*$^{-}$
transposons	0	207	205
intergenic regions	7	401	441
exons	5	225	319

Number of loci is shown for each category that displayed a fourfold or higher reduction in the number of esRNAs in the different mutant strains compared to the wild type. Loci showing reduction in esRNAs in more than one mutant strains are counted for each mutant. Please note that the two transposon loci that are not down-regulated in *dcl1*$^{-}$/*dcl2*$^{-}$ but reduced in *dcl2*$^{-}$, are just below the fourfold threshold in the double mutant.

Table 3. Summary of the specific features of different classes of ex-siRNAs

		Average Log2 fold change from WT					
ex-siRNA class	Strand bias	*dcl1*$^{-}$	*dcl2*$^{-}$	*dcl1*$^{-}$*/2*$^{-}$	*rdrp1*$^{-}$	*rdrp2*$^{-}$	% 5' U
Class 1	-0,78	0,34	**-12,84**	**-8,86**	3,74	-1,33	92,18
Class 2	-0,34	0,55	**-3,79**	**-8,72**	**-5,02**	1,36	92,12
Class 3	0,90	-0,47	0,12	**-3,21**	**-4,52**	**-3,50**	8,39
Class 4	0,83	**-2,45**	-0,26	-1,02	**-2,61**	**-2,42**	28,28

Strand bias indicates orientation to exons, where 1 corresponds to all sRNAs in the same orientation as the exon, 0 to equal mixture of sRNAs on both strands and -1 to all sRNAs antisense to exons. Numbers in bold indicate significant down-regulation in the corresponding mutants relative to wild type. The percentage of uracil (U) in the 5’ most base in the sRNAs for each class is also indicated.

Figure legends

Figure 1: Accumulation of esRNAs in wild type and *dcl* mutant *Mucor* strains.

Northern blots of esRNAs from intergenic regions (A) and exons of protein coding genes (B). Low-molecular weight RNA was extracted from wild type, *dcl1*$^-$, *dcl2*$^-$ and *dcl1*$^-$/*dcl2*$^-$ double mutant strains, separated on 15% denaturing polyacrylamide gels, transferred to membranes and probed with riboprobes or end labelled DNA oligonucleotides specific to each locus. For exact probe sequences see Supplementary Table 1. Ethidium bromide stained images of gels below the radiograms show equal loading of lanes. Panel B shows the accumulation of antisense and sense esRNAs separately. The exon loci correspond to the following proteins: locus 4: ID 80452, serine/threonine kinase; locus 5: ID 82197, no domains found; locus 6: ID 77050, no domains found; locus 7: ID 78553, low similarity to transposase 21 protein; locus 8: ID 85423, Zn-finger CCHC containing protein; locus 9: ID 95230, no domains found. Ten picomoles per lane of 23-mer to 27-mer DNA oligonucleotides in antisense and sense orientation were used as size markers and to control the hybridization specificity. In all cases, the RNA probes hybridised to these controls.

Figure 2. Accumulation of mRNAs in wild type and *dcl* mutant *Mucor* strains.

Northern blots of high molecular weight RNAs corresponding to an intergenic region (locus 3) or protein coding exons (loci 4, 5, 8 and 9). Total RNA (50 µg) extracted from wild type and mutant strains were separated in 1.2% denaturing agarose gel, transferred to

membranes and hybridised with gene specific or rRNA probes (Supplementary Table 1). The locus numbers correspond to the loci on Figure 1.

Figure 3. *rdrp1* and *rdrp2* dependence of DCL2 generated esRNAs.

The pie charts show the percentage of DCL2 dependent loci that also show reduced level of esRNAs in *rdrp1*$^{-}$ and *rdrp2*$^{-}$ strains.

Supplementary Figure 1. Genome browser shots of selected loci.

The nine loci analysed on Figure 1 were selected to show esRNA accumulation in wt, *dcl1*$^{-}$, *dcl2*$^{-}$, *dcl1*$^{-}$/*dcl2*$^{-}$, *rdrp1*$^{-}$ and *rdrp2*$^{-}$ strains. Arrows represent esRNA sequence reads. Thickness of the arrows indicates the abundance of read on a log scale. Color of arrows refers to the length of the sequence (pink <19 nt, red: 20-21 nt, green: 22-23 nt, blue: 24-25 nt and grey> 25nt). Orange arrows represent the position and orientation of probes used for esRNA detection by Northern blot (for exact probe sequences see Supplementary Table 1).

Supplementary Figure 2. Normalized abundance per 1 kb of regions down-regulated in the *dcl* mutants.

(Please, add the legend)

Supplementary Figure 3. Size distribution per classes of ex-siRNAs in the wild type strain.

The percentage of raw reads corresponding to different length of cloned and sequenced esRNA in the wild type strain was calculated for classes 1 to 3 of ex-siRNAs. (Please, add whatever it is necessary)