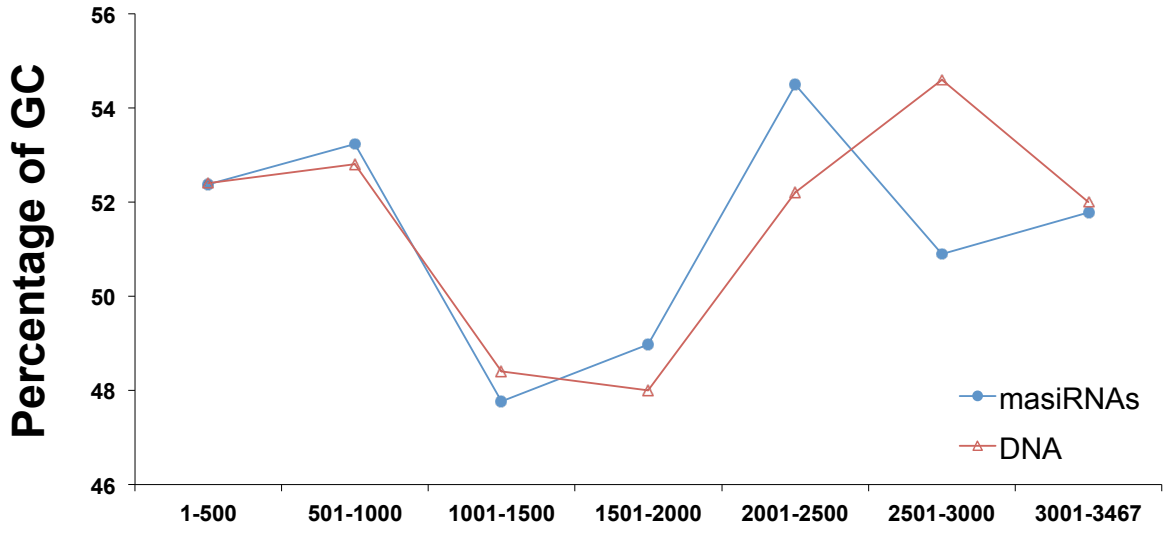

Figure S1 The GC content of masiRNAs vs. DNA. Unlike certain plants, small RNAs generated from *Neurospora* (during meiotic silencing) do not have a significantly higher GC content than that of the genomic region. Intervals of 500 nt from the *r*-target (unpaired) region (9281458–9284924; 3467 bp in total) are shown. The weighted mean is used to calculate the GC content of masiRNAs.

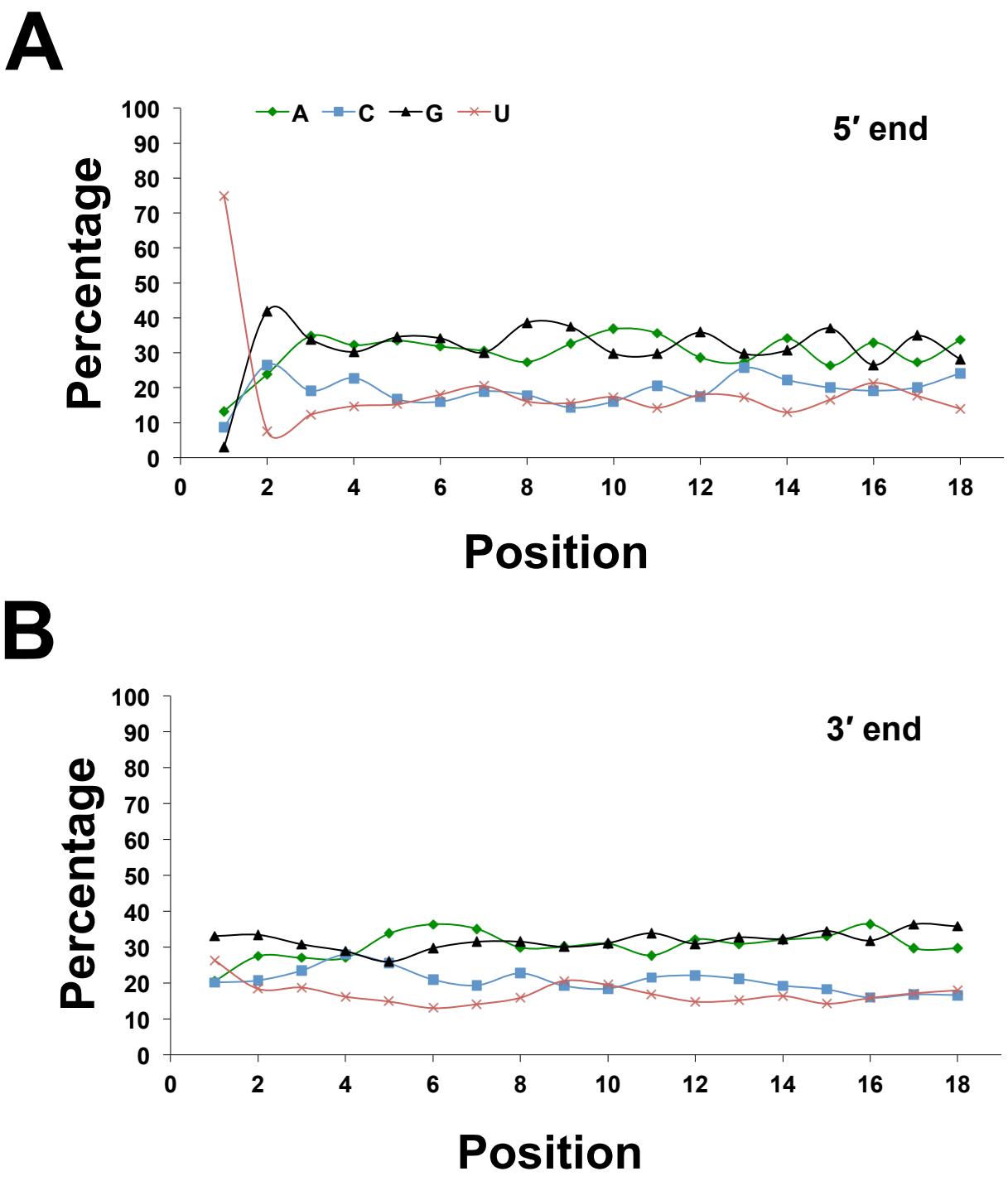

Figure S2 The nucleotide frequency of the (A) 5' and (B) 3' end of masiRNAs. The collected *r*-specific masiRNAs exhibit a preference for 5' U (74.9%).

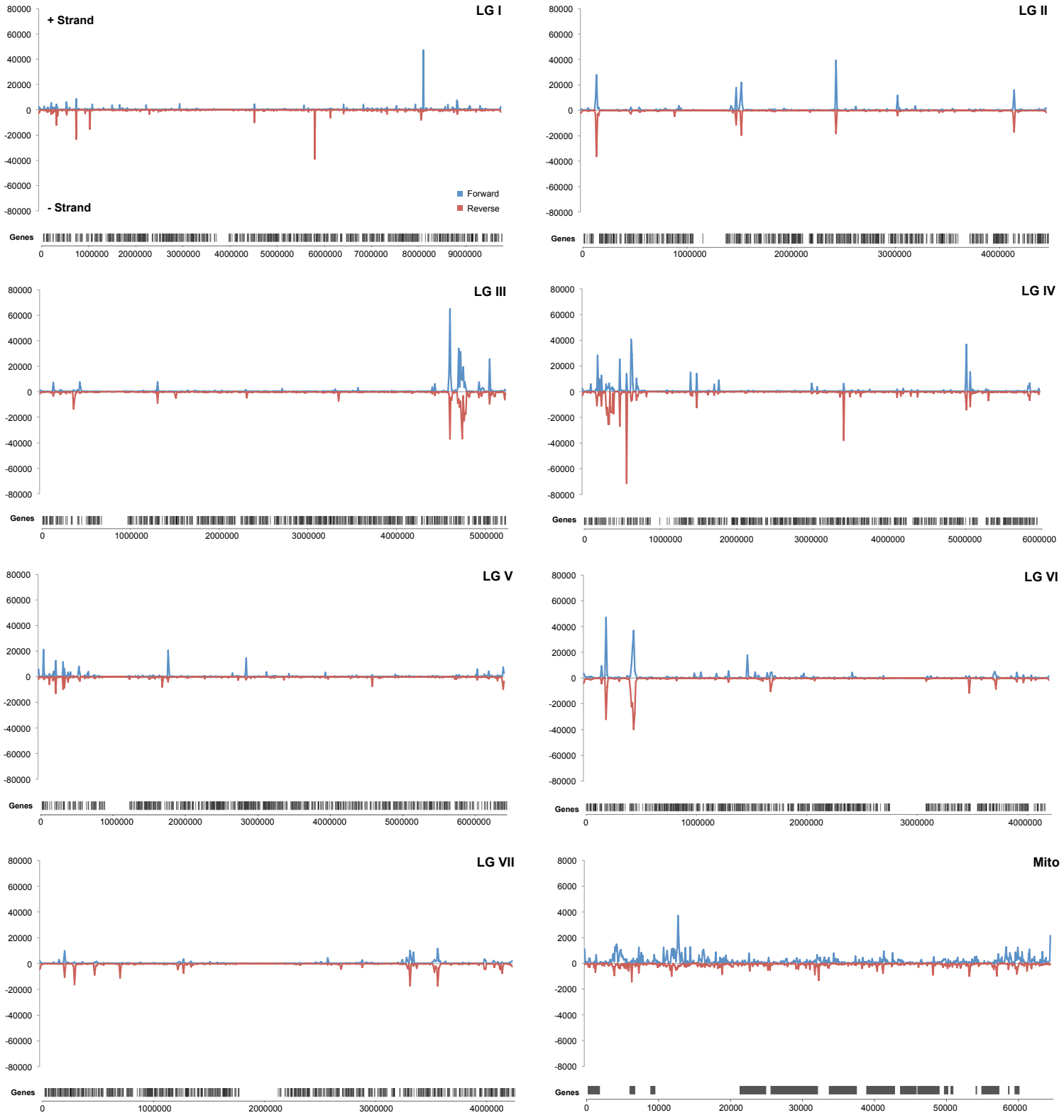

Figure S3 The distribution of endogenous perithecial small RNAs (epsRNAs) across the seven linkage groups (LG; in 10-Kb windows) and the mitochondrial genome (Mito; in 100-bp windows) in the paired (control) cross. Y-axes, number of small RNAs.

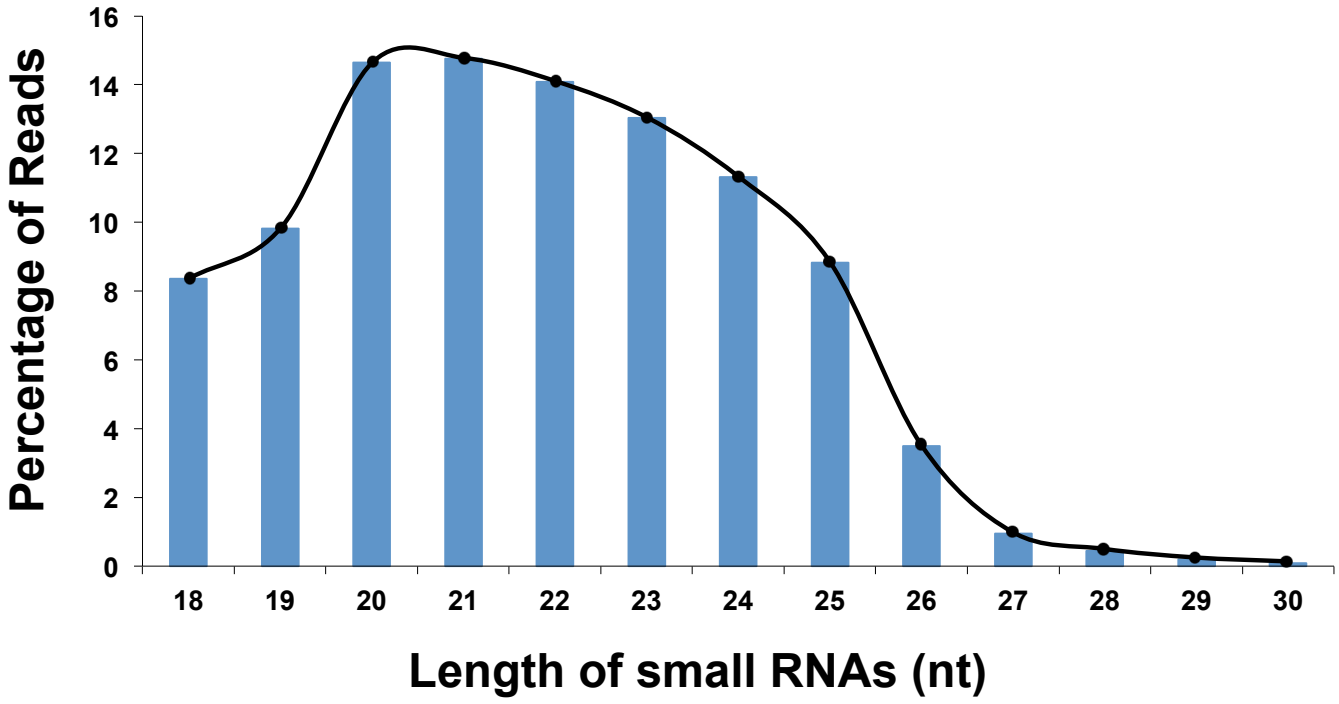

Figure S4 The length distribution of epsRNAs detected from two control cross sequencings (pooled). The control cross contains no artificially unpaired regions. The abundance of these endogenous RNAs peaks at 20–21 nt and drops off gradually thereafter.

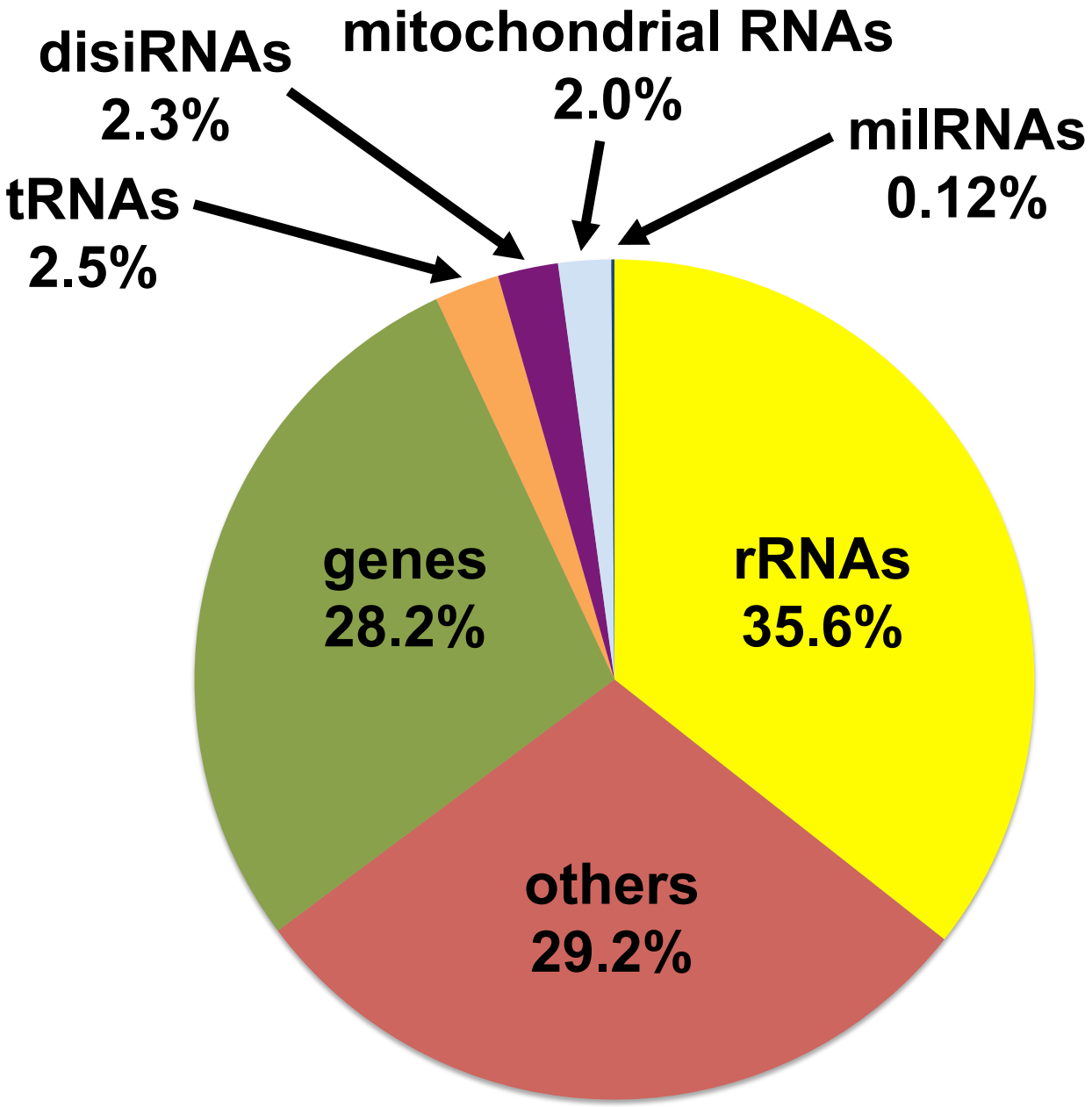

Figure S5 The composition of epsRNAs. disiRNAs, dicer-independent small interfering RNAs; milRNAs, microRNA-like RNAs; rRNAs, RNAs produced from rDNA repeats and their intergenic regions.

File S1

Materials and Methods

Manipulation of Neurospora strains

Strains used in this study include P9-42 (Oak Ridge wild type *a*), P12-02 (r^{Δ}::*hph a*), and F2-01 (*fl A*). Mutant alleles used, including r^{Δ} (which contains an *hph*-mediated gene replacement; Colot *et al*. 2006), were obtained from the Fungal Genetics Stock Center (McCluskey 2003) and are described in the *Neurospora* Compendium (Perkins *et al*. 2001) and e-Compendium (http://www.bioinformatics.leeds.ac.uk/~gen6ar/newgenelist/genes/gene_list.htm). Culturing and crossing media were prepared as previously described (Vogel 1964; Westergaard and Mitchell 1947). Standard procedures for growth, crosses, and other *Neurospora* manipulations were used during the course of this study (Davis and de Serres 1970).

Small RNA library preparation

Half a gram of four-and-a-half-day-old perithecia (fruiting bodies) were harvested as previously described (Shiu and Glass 1999). Small RNAs were extracted on a glass fiber filter using the *mir*Vana miRNA Isolation Kit (Ambion, Austin, TX). cDNA libraries were constructed with the Small RNA Sample Prep Kit v.1.5 (Illumina, San Diego, CA). Each purified library was evaluated using the Experion automated electrophoresis system (with the DNA 1K chip; Bio-Rad, Hercules, CA), quantified with the Qubit fluorometer using the Quant-iT dsDNA HS Kit (Invitrogen, Carlsbad, CA), and diluted according to Illumina's standard protocol for sequencing on the Genome Analyzer (GA) II. One sample was used per sequencing lane.

Illumina sequencing

cDNA molecules were ligated to adapters at both ends and subsequently attached to the solid surface of a reaction chamber (flow cell), which had been coated with oligonucleotides complementary to adapter sequences. Each DNA fragment bent over and the unattached adapter annealed with an oligonucleotide primer, forming a "bridge". After repeated cycles of polymerase-based denaturation and extension (bridge amplification), a "cluster" of homogeneous molecules (~1000 copies) was produced for each DNA fragment. A single flow cell contains 8 lanes (channels), and each can accommodate several millions of clusters. The molecules in each cluster were sequenced using a four-color DNA sequencing-by-synthesis approach that utilizes reversible terminators with removable fluorescent dyes. A high-resolution image of the flow cell was captured after each sequencing cycle, allowing the reading of nucleotides added to each individual cluster.

Computational analyses

Flow cell image files from the Illumina Genome Analyzer (GA) II were processed using the GA Pipeline v.1.4 software, yielding 10.2 ± 1.2 million reads per sequencing run (lane). These data are available through the NCBI Sequence Read Archive (SRA) database (SRX247455–247456). The 3'-adapters of the Illumina sequences were trimmed and all sites of alignment of the remaining sequence to the reference genome were identified using the program SOAP (Li *et al*. 2008), allowing for no mismatches or base call ambiguity in either adapter or insert. The reference genome (*N. crassa* OR74A, NC10) was obtained from the Broad Institute website (http://www.broadinstitute.org/annotation/genome/neurospora/MultiDownloads.html). Computation was performed using the High Performance Computing resources at the University of Missouri Bioinformatics Consortium. Downstream processing of the results of the analysis by SOAP was performed using custom Linux shell scripts. DNAPlotter and Tablet were used for whole genome sequence and alignment visualization, respectively (Carver *et al*. 2009; Milne *et al*. 2010).

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Table S1 Distribution of small RNAs along the *r* gene

Cross	Region	No. of small RNAs	Length (bp)	No. of sequencings	No. of small RNA/bp per sequencing
r-unpaired (experimental)	Upstream	106	400	3	0.0883
	Target	4848	3467	3	0.4661
	Downstream	50	288	3	0.0579
r-paired (control)	Upstream	29	400	2	0.0363
	Target	313	3467	2	0.0451
	Downstream	18	288	2	0.0313

Table S2 masiRNA-generating hot spots within the *r* gene

Position	Orientation	Run 1	Run 2	Run 3	Total	Surrounding bases (5' to 3')
9281704	Forward	9	9	14	32	UCAGACGCGCUGCGAAGCAUA
9281741	Forward	22	24	21	67	UCUUAUCGGGUAUCACGGAAA
9281743	Forward	18	17	25	60	UUAUCGGGUAUCACGGAAAGU
9281821	Forward	16	10	14	40	GUUGUACCCUUGGUGGAAGUA
9281881	Forward	16	20	22	58	CUCCCUCCUUUGCGUGGGAGA
9282118	Forward	51	52	81	184	GUAUUGGUGGUCAGAAGGGAC
9282627	Reverse	8	19	13	40	GUGGACAACGGUCGAGGUAUG
9282667	Forward	13	9	8	30	CUCAAAGCAAUGCAAGCUUGG
9282677	Reverse	10	6	14	30	UAUACACGAUCCAAGCUUGCA
9282777	Forward	12	13	8	33	UUCUUUUGUCUGCCAACAGGC
9283154	Reverse	12	8	10	30	AGAGGAAAAGUCUCACGAUUG
9283160	Reverse	18	10	17	45	ACAAACAGAGGAAAAGUCUCA
9283161	Reverse	25	10	7	42	GACAAACAGAGGAAAAGUCUC
9283587	Forward	15	6	9	30	AUUGAGAUUUUGAUUGAUAGA
9283658	Forward	14	7	16	37	AAACCUAGGUUAGGGGUAGAC
9283682	Forward	7	14	12	33	UCAGAGACAGUGAAAGAGGAC
9283877	Reverse	13	10	15	38	CAGCAGCAGAGCUUCUCUCUC
9283878	Reverse	9	12	14	35	GCAGCAGCAGAGCUUCUCUCU
9283903	Forward	12	10	24	46	UGUCCUCGACUGGCCACGGGA
9284013	Forward	13	13	19	45	CAAAAGAGGAUGUUGAAGAAG
9284016	Forward	9	13	12	34	AAGAGGAUGUUGAAGAAGACG
9284075	Forward	66	38	47	151	CCGUCGAGGUUGAAGUAGUAA
9284336	Forward	13	11	14	38	ACAUCCACCACGGCCACAGUA

Twenty-three nucleotide positions within the unpaired *r* region generate 10 or more detectable small RNAs in an average sequencing run (*i.e.*, 30 or more small RNAs in three sequencing runs combined). The orientation (forward or reverse) as well as the sequences surrounding the first base (underlined) of the small RNAs are shown.

Table S3 Top epsRNA-producing regions (two sequencings pooled)

Rank	Supercontig	Positions	Reads	Small RNA-producing region(s)
1	8	94000–94999	1394461	Between 28S and 18S rRNAs
2	8	97000–97999	643321	Between 28S and 18S rRNAs
3	8	95000–95999	489581	Between 28S and 18S rRNAs
4	8	96000–96999	404418	Between 28S and 18S rRNAs
5	8	93000–93999	213601	Between 28S and 18S rRNAs
6	20	0–999	178111	Region contains no annotated genes
7	8	98000–98999	177829	28S rRNA + right flank
8	9	0–999	72662	28S rRNA
9	8	92000–92999	67128	Between 28S and 18S rRNAs
10	8	189000–189999	58551	18S rRNA
11	2	2454000–2454999	57114	*disiRNA-12*
12	1	8151000–8151999	48117	*disiRNA-9*
13	4	584000–584999	43344	NCU04877 (hypothetical protein)
14	1	5852000–5852999	39019	Between NCU09210 and NCU09211
15	8	99000–99999	38408	Between 28S and 18S rRNAs
16	20	1000–1646	35119	Region contains no annotated genes
17	9	113000–113999	32548	18S rRNA
18	4	5041000–5041999	29773	NCU08845 (hypothetical protein) + right flank
19	2	152000–152999	25193	NCU03580 (hypothetical protein) + right flank
20	5	1797000–1797999	23561	*disiRNA-28*
21	4	3439000–3439999	23061	NCU04417 (hypothetical protein)
22	4	640000–640999	20239	NCU04888 (hypothetical protein)
23	8	88000–88999	19285	28S rRNA
24	3	4643000–4643999	18763	Between NCU08207 and NCU08208
25	8	142000–142999	18689	28S rRNA
26	2	151000–151999	18552	NCU03580 (hypothetical protein)
27	6	1485000–1485999	18540	Between NCU05568 and NCU05567
28	3	4642000–4642999	15818	Between NCU08207 and NCU08208
29	4	494000–494999	15597	Between NCU04857 and NCU04859
30	6	431000–431999	15571	NCU04701 (hypothetical protein)

Small RNAs were isolated from a cross with no artificial unpairings. Supercontigs 1–7 refer to linkage groups I–VII while 8–20 are unmapped.